VNiVERSiDAD
Đ SALAMANCA

MOLECULAR AND CELLULAR MECHANISMS OF NEUROPROTECTION AND PLASTICITY INDUCED BY BRAIN-DERIVED NEUROTROPHIC FACTOR

CARLOS HENRIQUE VIEIRA MELO

Doctoral Thesis in Neurosciences

2013

Blind belief in authority is the greatest enemy of truth

Albert Einstein

CARLOS HENRIQUE VIEIRA MELO

MOLECULAR AND CELLULAR MECHANISMS OF NEUROPROTECTION AND PLASTICITY INDUCED BY BRAIN-DERIVED NEUROTROPHIC FACTOR

Doctoral candidacy thesis, Department of Neurosciences, University of Salamanca

Supervisor – Carlos Bandeira Duarte

Position title – Associate Professor, Department of Life Sciences, University of Coimbra, Portugal

Co-Supervisor – Miguel A. Merchán Cifuentes

Position title – Professor, Department of Neurosciences, University of Salamanca, Spain

CARLOS HENRIQUE VIEIRA MELO

MECANISMOS MOLECULARES Y CELULARES DE LA NEUROPROTECCIÓN E PLASTICIDAD INDUCIDA POR FACTOR NEUROTRÓFICO DERIVADO DEL CEREBRO

Tesis de candidatura doctoral, Departamento de Neurociencias de la Universidad de Salamanca

Director – D. Carlos Bandeira Duarte

Título de la posición – Profesor Asociado del Departamento de Ciencias de la Vida de la Universidad de Coimbra, Portugal

Co-Director – D. Miguel A. Merchán Cifuentes

Título de la posición – Profesor Catedrático del Departamento de Neurociencias de la Universidad de Salamanca, España

Preceptos legales

De conformidad con lo dispuesto en los artículos RD 778/1998, RD 1393/2007 y RD 99/2011, los resultados de los estudios publicados, que fueron utilizados en esta tesis, se indican a continuación. El autor de esta tesis afirma que intervino activamente en el diseño experimental y la ejecución, interpretación de datos y la preparación de los manuscritos publicados, bajo el nombre **MeloC.V.**

Legal Precepts

In accordance with the provisions of Articles RD 778/1998, RD 1393/2007 and RD 99/2011, the results of published studies, which were used in this thesis, are indicated below. The author of this thesis states that heactively intervened in the experimental design and execution, data interpretation and preparation of the published manuscripts, under the name **Melo C.V.**

Baptista M. S.[#], **Melo C. V.**[#@], Armelão M., Herrmann D., Pimentel D. O., Leal G., Caldeira M. V., Bahr B. A., Bengtson M., Ameida R. D., Duarte C. B. (2010) Role of the proteasome in excitotoxicity-induced cleavage of glutamic acid decarboxylase in cultured hippocampal neurons. PloS One 5(4): e10139.

[#]These authors contributed equally.
[@]Corresponding author.

Melo C. V., Okumoto S., Gomes J. R., Baptista M. S., Bahr A., Frommer W. B., Duarte C. B. (2013) Spatiotemporal resolution of BDNF neuroprotection against glutamate excitotoxicity in cultured hippocampal neurons. Neuroscience 237: 66-86. Cover article.

Melo C. V., Mele M., Curcio M., Silva C. G., Duarte C. B. (2013) BDNF regulates the expression of vesicular glutamate transporters in cultured hippocampal neurons. PLoS One 8(1): e53793.

Este estudio fue co-financiado por la Fundación para la Ciencia y la Tecnología (Fundação para a Ciência e Tecnologia - FCT), Portugal, y el Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea, a través de la beca doctoral SFRH/BD/29905/2006, y subvenciones PTDC/SAU-FCF/72283/2006, PTDC/SAL-NEU/104297/2008 y PTDC/SAL-NMC/120144/2010; por la Fundación Calouste Gulbenkian (Fundação Calouste Gulbenkian), a través de una beca de investigación para estudios en el extranjero (Proc. 71949/2005) y la beca para participar en conferencias y / o reuniones científicas internacionales; por la Fundación Luso-Americana (Fundação Luso-Americana - FLAD), a través de una beca para viajes cortos en el extranjero.

This study was co-funded by the Foundation for Science and Technology (Fundação para a Ciência e Tecnologia — FCT), Portugal, and European Regional Development Fund (ERDF), European Union, through the doctoral fellowship SFRH/BD/29905/2006and grants PTDC/SAU-FCF/72283/2006, PTDC/SAL-NEU/104297/2008 and PTDC/SAL-NMC/120144/2010; by the Calouste Gulbenkian Foundation (Fundação Calouste Gulbenkian), through a research fellowship for studies abroad (Proc. 71949/2005) and scholarship for participation in conferences and/ or international scientific meetings; by the Luso-American Foundation(Fundação Luso-Americana — FLAD), through a scholarship for short trips abroad.

Agradecimentos

Em primeiro lugar, gostaria de agradecer ao Professor Doutor Carlos Duarte a sua excelente orientaçãocientifica, empenho no meu sucesso académico e espírito colaborativo demonstradona forma como acolheu as minhas ideias e ajudou a planificar experiencias. O Professor Carlos Duarte tem umaética de trabalho exemplar, nuncadelega as suas aulas teóricas ou práticas em alunos de doutoramento ou pós-doutorados e ajuda verdadeiramente os seus alunos. O Professor Carlos Duarte foi sempre um verdadeiro mentor.

Un agradecimiento muy especial al Profesor Doctor Miguel A. Merchán, por su compromiso en defensa de mi tesis doctoral en la Universidad de Salamanca y orientación durante este proceso de preparación para la defensa de la tesis, siempre con un interés genuino en ayudar.

Agradeço a todos os meus colegas do Centro de Neurociências da Universidade de Coimbra, especialmente, à Margarida, ao João e ao Manadas, com os quais partilhei a bancada laboratorial, e à Elisabete por todo o apoio tecnico e amizade.

También quiero agradecer a todos los amigos de la Universidad de Salamanca, por su apoyo en defensa de mi tesis doctoral.

Agradeço à minha família, em particular aos meus pais e ao meu querido sobrinho Tomás que, passados 6 anos, continua acreditar que fui para a terra do Faísca porque sou piloto de carros de corrida. Agradeço à tia Beca, à tia Stuart e aos meus primos, Belinha, Anita e Rui, pelo seu apoio, bem como aos meus amigos do Porto, que tanto adoro, sobretudo, ao João e à Sofia, à Regina e ao Nelson, Joana e Javi, Lipe, Márcio e à Guida, ao Filipe, ao Kiko e à Marta, mas também ao Bruno e à Ana, aos manos Saraiva, Ana, Teca e Pedro, à Teresa, à Rita, à Inês, ao Quim e à Tânia, entre tantos outros aos quais peço que me desculpem por não mencionar os seus nomes.

Dedico um agradecimento muito especial ao Sr. Carlos e à D. Nina que tem sido uns pais adoptivos para mim, por toda a sua ajuda e carinho. Agradeço a todos os meus amigos da equipa de triatlo do Clube Fluvial Portuense, especialmente ao Nuno e à Marta, à malta do voluntariado e antigos colegas de liceu, Beterraba, Kaká, João, a Sandra e a Rita, entre tantos outros, dos quais tenho muitas saudades.

Acknowledgements

I would like to thank Dr. Wolf Frommer and Dr. Craig C. Garner for welcoming me at Stanford University and my labmates and collaborators Sakiko, Dhananjay, Sergio, Chris, Magali and especially Jackie. Thank you so much Jackie for your genuine friendship! Our 6.30 a.m. lab chats every morning were priceless. Jackie, your cheeky boy, A.K.A. Stanford's bastard son, might just graduate…

I would like to thank all my friends from S. Diego and the Stanford University Triathlon Team. During my MSc., I was extremely fortunate to meet amazing people at the Genomics Institute of the Novartis Research Foundation, thanks to Dr. Claudio Joazeiro, who welcomed mehis laboratory, especially Wei, Mario, Orzala and Jeff, who all made me want to return to California for my PhD, and all others I subsequently met on our backpacking, hiking, skiingand road trips, picnics, potlucks, Monday Movie Nights, Tuesday Fish Tacos and so forth, including Jeff A., Matt, Mimi, Sharon, everyone from the S. Diego Track Club, the Portuguese gang, especially Joana and Joao Dessa, andNuno, Raquel, Marta, Isabel. I will never forget all the laughs, support and the true friendship.

Wei and Danny, you have become more than a family to me, both in S. Diego and in the Bay area. I will never forget that you have literally saved my life when you drove me to Scripps Green Hospital that Sunday morning. I did learn that a full serving of Chechuan spicy chicken number ten upon arrival from a a transcontinental flight and a recently performed quadruple paranasal sinus surgery are definitely not a good combination… Moreover, I learned that the best gifts one can receive in life are friends that will bring you homemade wonton soup to the Hospital and care about you no matter how far you are.

Finally, my friends from the Stanford University Triathlon Team, Katha, thank you for simply being such an amazing woman. Thanks to the Roos brothers, Thomas and Andrew, and Ellen, Ron, Mark, Federico (and his theory on the entropy of corn-flakes halfway through a 3-Km set in the swimming pool), David (300, no stopping!), Nick, John, Lisa, Todd, Bryan, Brian Hass, Brian Hachtmann (you dared me to moon Katie…never dare a Portuguese!), the incredible Sunday morning long-run gang, Natalie, Kendall, Kierann, Major Jason, Martinez, and Tim, Friederike, Steph and Praj (I will never forget those 3-year old kids line-up with their helmets on, following my warm-up instructions before the start of the Saplingathlon. Thanks for visiting me in Porto!).

TABLE OF CONTENTS

RESUMEN

El factor neurotrófico derivado del cerebro (BDNF) es una proteína de pro-supervivencia con alta expresión en el hipocampo y funciones esenciales en las neuronas durante el desarrollo y en el adulto. El BDNF se une preferentemente a los receptores TrkB, activando las vías de señalización Ras-ERK, PI3K/AKT y PLCγ. En condiciones fisiológicas, BDNF regula distintos mecanismos implicados en la plasticidad sináptica, mientras que en condiciones patológicas, se ha comprobado que protege de la toxicidad por sobre-excitación del glutamato. Sin embargo, los mecanismos moleculares implicados en la activación del receptor de BDNF por TrkB con el fin de inducir la protección neuronal no se conocen completamente. Este estudio buscó abordar estas cuestiones y examinar más a fondo la posible relación entre los mecanismos de neuroprotección y/o la recuperación y los mecanismos de plasticidad sináptica inducidos por BDNF.

En la primera parte del estudio se analizaron las alteraciones moleculares inducidas por excitotoxicidad, centrándose en la reducción de la descarboxilasa del ácido glutámico, que es probable que afecte la transmisión sináptica inhibitoria. En este trabajo demostramos que la estimulación excitotóxica de cultivos neuronales del hipocampo con glutamato causa la escisión N-terminal de las isoformas de la descarboxilasas de gluatamato GAD65 y GAD67, tras la ubiquitinación y degradación de una pareja de unión desconocida por el proteosoma. Nosotros observamos en cultivos de neuronas un disminución significativa de aposiciones (puncta) GAD 65 sobre los axones de las neuronas de hipocampo, lo que esta de acuerdo con el análisis bioquímico realizado en extractos de la corteza cerebral y de cerebelo. La relevancia de estos resultados se basa en la demostración de que esta situación podría afectar a la neurotransmisión GABAérgica y el proteosoma actua en la disregulación de GADs en condiciones de excitotoxicidad además de otros sistemas proteolíticos anteriormente implicados en la excitotoxicidad inducida por glutamato (Para mas detalles ver capítulo 1).

En nuestro análisis de la biología celular del proceso de activación de cascadas excitotóxicas prestamos especial interés a la activación diferencial de los tres principales mecanismos proteolíticos (UPS, calpaínas y caspasas) en diferentes compartimentos de la neurona. Además, aportamos datos para la mejor comprensión de las secuencias temporales del efecto protector inducido por BDNF. Estos resultados mostraron una activación temporal diferente de las proteasas aí como la segregación espacial en la neurona de estos mecanismos. De este modo comprobamos que la activación inmediata de calpaína es seguida por una disregulación del proteasoma, en los axones y las dendritas. El proceso de activación de las caspasas se produce más tarde en el cuerpo de la célula y todos los mecanismos proteolíticos disminuyen significativamente por la

pre-incubación con BDNF. Sin embargo, los inhibidores del proteasoma y la calpaína no fueron capaces de imitar el efecto protector de la inhibición de caspasas y BDNF en la prevención de condensación de la cromatina. Por el contrario, la inhibición del proteasoma y la calpaína protegió los marcadores neuronales para dendritas (MAP-2), axones (neurofilamentos H) y los transportadores vesiculares de glutamato (VGLUT1 y VGLUT2), mientras que la inhibición de caspasas no reprodujo el efecto protector de BDNF en las neuritas y marcadores sinápticos. Además, inhibidores de la ruta de PLCγ bloquearon significativamente la acción protectora de BDNF en cultivos neuronales del hipocampo, lo que sugiere un mecanismo neuroprotector dependiente de la actividad sinaptica. En consecuencia, el carácter neuroprotector de BDNF en las sinapsis fue analizado utilizando un nanosensor (FRET glutamato) habiendo comprobado que previene parcialmente la reducción de la actividad sináptica excitatoria, tras medir la liberación de glutamato inducida por KCl. Por lo tanto, nuestra hipótesis es que que la reparación neuronal después de una agresión neuroquímica podría comenzar en el nivel sináptico y BDNF, muy probablemente induciría la recuperación a través de la reactivación de los mecanismos de plasticidad sináptica que involucran la síntesis de novo de proteínas (Por favor, consulte el capítulo 2).

La hipótesis anterior fue evaluada estudiando el efecto del BDNF sobre la expresión de los VGLUT como un paradigma experimental. De este modo comprobamos que la aplicación exógena de BDNF en cultivos de neuronas del hipocampo durante 7 días (DIV7) aumentó rápidamente los niveles de tanto de ARNm como de proteína VGLUT2 en una forma dosis-dependiente, mientras que la expresión de VGLUT1 también aumentó, pero sólo de forma transitoria. Por el contrario a DIV14, la aplicación de BDNF aumentó de manera constante la expresión de VGLUT1, mientras VGLUT2 mantuvo unos niveles bajos de expresion. Por otra parte, experimentos de inhibición de la transcripción y traducción - de VGLUT1 y VGLUT2 - bloquearon completamente el efecto inducido por BDNF en el aumento de la expresión de VGLUTs. Experimentos de microscopía de fluorescencia confocal mostraron un incremento transitorio de tráfico axonal de VGLUT1 y la redistribución de vesículas VGLUT-2-positivas en las neuronas de hipocampo. Estos resultados indican que el BDNF también puede afectar a la distribución subcelular de los VGLUTs durante el desarrollo neuronal.

Además pudimos comprobar que la inhibición de los receptores TrkB y de la señalización vía PLCγ bloquean el efecto inducido por BDNF en el aumento de expresión de VGLUTs, lo que sugiere que este factor y su efecto sobre VGLUT1 podría contribuir a mejorar la liberación de glutamato en la potenciación a largo plazo o LTP (Por favor, consulte el capítulo 3).

En resumen nuestros resultados indican que la neuroprotección mediada por BDNF no se limita al cuerpo celular y a la atenuación de la activación de caspasas. BDNF también protege significativamente las neuronas de los daños excitotoxicos inducidos en axones, dendritas y sinapsis, que predominantemente conllevan la activación de la calpaína y el aumento de la ubiquitinación de proteínas. Por otra parte, el BDNF activa mecanismos coincidentes tanto en condiciones fisiológicas durante el desarrollo neuronal y tras lesiones inducidas por glutamato (excitotoxicidad). BDNF promueve, concomitantemente, la conectividad entre las neuronas y neuroprotección por mecanismos de atenuación de la proteolisis y/o la inducción de la expresión *de novo* de proteínas neuronales, en particular VGLUT1 y VLGUT2. Proponemos en este trabajo de tesis doctoral que la reactivación de los mecanismos de plasticidad mediados por BDNF durante el desarrollo neuronal pueden permitir atenuar daños neuronales. Finalmente, BDNF puede disminuir la activación proteolítica y/o inducir la recuperación de las neuronas del hipocampo en condiciones de neurodegeneración.

ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a pro-survival protein, highly expressed in the hippocampus, with critical functions in both developing and adult neurons. BDNF preferentially binds to TrkB receptors activating, in parallel, the Ras-ERK, PI3K/AKT and PLCγ signaling pathways. Under physiological conditions, BDNF regulates several mechanisms of synaptic plasticiy. Under pathological conditions, BDNF protects hippocampal neurons from glutamate excitotoxicity and ischemia. However, the precise molecular mechanisms BDNF triggers upon TrkB receptor activation to induce neuronal protection and/or recovery are not fully understood. This study sought to address these issues and further examine the possible link between BDNF-induced mechanisms of neuroprotection and/or recovery and mechanisms of synaptic plasticity.

In the first part of this study we examined molecular alterations induced by excitotoxicity, focusing on the downregulation of glutamic acid decarboxylase, which likely affects inhibitory synaptic transmission. We found that excitotoxic stimulation of cultured hippocampal neurons with glutamate leads to a time-dependent N-terminal cleavage of glutamic acid decarboxylase isoforms GAD65 and GAD67, upon ubiquitination and degradation of an unknown binding partner by the proteasome. The characteristic punctate distribution of GAD65 along neurites of differentiated cultured hippocampal neurons and total GAD activity measured in cerebellum or cerebral cortex extracts were significantly reduced. The results showed the deregulation of GADs under excitotoxic conditions, which most likely affects GABAergic neurotransmission, upon UPS activity, in addition to other proteolytic systems previously implicated in glutamate-induced excitotoxicity (Please refer to chapter 1).

We then further evaluated the differential activation of the three main proteolytic mechanisms (UPS, calpains and caspases) implicated in excitotoxicity. We sought to study the protective effect of BDNF in different neuronal compartments and time points towards best understanding the spatiotemporal activation of BDNF-induced mechanisms of neuroprotection. These results showed a time-dependent activation of proteases and spatial segregation of these mechanisms. Calpain activation was followed by proteasome deregulation, in synaptic terminals and neuronal processes. Caspase activation subsequetly occurred in the cell body and all proteolytic mechanisms were significantly decreased by BDNF pre-incubation. Furthermore, proteasome and calpain inhibitors were unable to mimic the protective effect of BDNF and caspase inhibition in preventing chromatin condensation. Conversely, proteasome and calpain inhibition did protect the neuronal markers for dendrites (MAP-2), axons (Neurofilament H) and the vesicular glutamate transporters (VGLUT1 and VGLUT2), whereas caspase inhibition failed to

mimic the protective effect of BDNF on neurites and synaptic markers. BDNF also partly prevented the downregulation of synaptic activity measured by the KCl-evoked glutamate release using a FRET glutamate nanosensor. Moreover, PLCγ chemical inhibitors significantly blocked the protective action of BDNF, suggesting an activity-dependent mechanism of neuroprotection. Thus, we hypothesize that neuronal repair after a degenerative insult may start at the synaptic level and BDNF most likely induces recovery through reactivation of mechanisms of synaptic plasticity involving de novo protein synthesis (Please refer to chapter 2).

The hypothesis above was assessed testing the effect of BDNF on VGLUT expression as an experimental paradigm. Exogenous application of BDNF to cultured hippocampal neurons at DIV7 (days in vitro) rapidly increased VGLUT2 mRNA and protein levels, in a dose-dependent manner, while VGLUT1 expression was only transiently increased. However, at DIV14, BDNF stably increased VGLUT1 expression, whilst VGLUT2 levels remained low. Transcription and translation inhibition fully blocked BDNF-induced VGLUT upregulation. Fluorescence microscopy imaging upon BDNF incubation showed a transient upregulation of VGLUT1 axonal trafficking and redistribution of VGLUT2-positive vesicles. These results suggest that BDNF may also affect VGLUTs subcellular distribution during development. Moreover, inhibition of TrkB receptors and PLCγ signaling precluded BDNF-induced VGLUT upregulation, suggesting that BDNF regulates VGLUT expression during development and its effect on VGLUT1 may contribute to enhance glutamate release in LTP (Please refer to chapter 3).

Overall, these results indicate that BDNF neuroprotection is not restricted to the cell soma and attenuation of caspase activation, also significantly protecting neurons from the excitotoxicity-induced damage to axons, dendrites and synapses, which predominantly results from calpain activation and increased protein ubiquitination. Furthermore, BDNF activates overlapping mechanisms under physiological and pathological conditions. BDNF concomitantly promotes connectivity between neurons and neuroprotection, by attenuating proteolytic mechanisms and/or inducing de novo expression of key neuronal markers, namely VGLUT1 and VGLUT2. Thus, we propose that reactivating BDNF-mediated developmental mechanisms of neuronal plasticity may enable to attenuate neuronal damages. BDNF may decrease proteolytic activation and/or induce recovery of hippocampal neurons under conditions of neurodegeneration.

ABBREVIATIONS

AD: Alzheimer's Disease
AIF: Apoptosis-Inducing Factor
AMPA: 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
aPKC-ι: atypical protein kinase C-ι
Arc: Activity-regulated cytoskeleton-associated protein
bFGF: basic Fibroblast Growth Factor
ARAP: Rap-dependent RhoGAP 3
BDNF: Brain-Derived Neurotrophic Factor
BHLHB2: Basic Helix-Loop-helix B2
CaMKIγ: Ca^{2+}/calmodulin-dependent protein kinase CLICK-III (CL3)
CARD: Caspase Recruitment Domain
CaRE1: Ca^{2+}-response element 1
CaRTF: Calcium-Responsive Transcription Factor
CRE: cAMP/Ca^{2+}-Response Element
CREB: cAMP Response Element-Binding Protein
CRMP2: Collapsin Response Mediator Protein-2
CNTF: Ciliary Neurotrophic Factor
DAG: Diacylglycerol
DIV: Days in vitro
ECD: Extracellular Domain
EPSP: Excitatory Postsynaptic Potentials
eIF4E: eukaryotic Initiation Factor 4E
4E-BP1: 4E-Binding Protein 1
ER: Endoplasmic Reticulum
ERK: Extracellular signal-Regulated Kinase
GAD: Glutamate Decarboxylase
GEF: Guanine nucleotide Exchange Factor
GDNF: Glial cell line-Derived Neurotrophic Factor
GSK3β: Gycogen Synthase Kinase 3 β
HD: Huntington's Disease
ICD: Intracellular Domain
IGF: Insulin-like Growth Factor
IKK-β: IκB kinase-β
ILK: Integrin-Linked Kinase
IP3: Inositol 1,4,5-triphosphate

IRAK: Interleukin-1 Receptor-Associated Kinase

IRS-1: Insulin Receptor Substrate 1

JNK: c-Jun N-terminal kinase

LINGO: Leucine rich repeat and Ig domain containing 1

LTD: Long-Term Depression

LTP: Long-Term Potentiation

MAG: Myelin-Associated Glycoportein

MAGE: Melanoma-Associated Antigen

MAP: Mitogen-Activated Protein

MBGI: Myelin-BasedGrowth Inhibitor

MCAO: Medial Cerebral Artery Occlusion

MeCP2: Methyl-CpG-binding Protein 2

MEF2D: Myocyte Enhancer Factor 2D

miR: microRNA

MKK: MAP kinase kinase

MKP-1: MAP kinase phosphatase-1

mTORC1: mTOR (mammalian target of rapamycin) complex 1

NF-H: Neurofilament H

NgR: Nogo-66 Receptor

NMDA: *N*-Methyl-D-aspartic acid

NPAS4: Neuronal PAS domain protein 4

NRAGE: Neurotrophin Receptor p75 interacting MAGE homologue

NRIF: Neurotrophin Receptor-Interacting Factor

NT3: Neurotrophin 3

NT4/5: Neurotrophin 4/5

NGF: Nerve Growth Factor

NF-kB: Nuclear Factor-kB

OAG: one-oleoy1–1 acetyl-sn glycerol

OMgp: Oligodendrocyte Myelin Glycoprotein

$p75^{NTR}$: p75 Neurotrophin Receptor

PARP-1: Poly (ADP-ribose) Polymerase-1

PIP2: Phosphatidylinositol 4,5-bisphosphate

PLC-γ: Phospholipase Cγ

PKAcβ: Protein Kinase A catalytic β

RanBPM: Ran-Binding Protein in Microtubule-organizing center

RasGEF: Ras guanine nucleotide exchange factor

RhoA: Ras homologue member A

RIP-2: Receptor-Interacting Protein 2

ROCK: Rho-activated kinase

ROS: Reactive Oxygen Species

SC1: Schwann Cell Factor 1

SorCS2: Sortilin-related VPS10 domain-containing receptor 2

SNP: Single-Nucleotide Polymorphism

SVZ: Subventricular zone

TACE: Tumor necrosis factor-α-converting enzyme

TGF-β: Transforming Growth Factor-β

TGN: Trans-Golgi Network

TrkB: Tropomyosin-related kinase B

TRAF: TNF Receptor Associated Factor

TRPC: Transient Receptor Potential Canonical

UPS: Ubiquitin-Proteasome System

VGLUT: Vesicular Glutamate Transporter

WAVE: Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) (Barde et al., 1982) is a pro-survival protein highly expressed in the hippocampus, amygdala, cerebral cortex and hypothalamus (Katoh-Semba et al., 1997; Conner et al., 1997; Ernfors et al., 1990). This member of the neurotrophin family is encoded by the homonymous gene *Bdnf*, with multiple transcript variants, bidirectional transcription, complex splicing and several functional promoters, specifically used in different tissues and brain regions (Pruunsild et al., 2007). BDNFis expressed as a 32-kDa precursor protein, proBDNF (Lee et al., 2001), which is processed by constitutive or activity-dependent regulated mechanisms (Goodman et al., 1996; Farhadi et al., 2000; Lessman and Brigadski, 2009), and through numerous post-translational modifications, including N-glycosylation and cleavage into the mature protein (Mowla et al., 2001). The non-covalently linked stable BDNF homodimer binds tropomyosin-related kinase B (TrkB) receptors activating, in parallel, the Ras-ERK, PI3K/Akt and PLCγ signaling pathways (Huang & Reichardt, 2003; Reichardt, 2006).

BDNF has critical functions in both developing and adult neurons, under physiological and pathological conditions, either implicated in genetic and multifactorial neurological diseases or contributing for neuroprotection against glutamate excitotoxicity and ischemia (Bramham and Nessaoudi, 2005; Almeida et al., 2005; Cunha et al., 2010; Murray and Holmes, 2011; Nagahara and Tuszynski, 2011; Gomes et al., 2012 Hartmann et al., 2012; Park & Poo, 2013).

During development, BDNF regulates neuronal differentiation by stimulating the formation of appropriate synaptic connections, concomitantly controlling the direction and rate of axon growth (Wang and Poo, 2005; Li et al., 2005) as well as the shape of dendritic arbors and spines (Ji et al, 2005; An et al., 2008; Kwon et al., 2011), and promoting the survival of selected neuronal populations of the peripheral (Oppenheim et al., 1992; Song et al., 2008) and central nervous systems (Lindsay et al., 1985; Phillips et al., 1990;Klöcker et al., 2000;Gupta et al., 2009; Gomes et al., 2012).

In many regions of the adult CNS, BDNF not only enhances excitatory transmission (Lohof et al., 1993; LeVine et al., 1995; Kang and Schuman, 1995, Tyler and Pozzo-Miller, 2001; Carvalho et al., 2008) but also favors inhibitory synaptic activity in order to negatively control the homeostatic scaling of intrinsic neuronal excitability (Desai et al., 1999; Swanwick et al., 2006; Pozo and Goda, 2010). In addition, BDNF regulates several forms of synaptic plasticity (Vicario-abejon et al., 1998; Rutherford et al., 1998; Huang et al., 1999; Minichiello, 2009). In the hippocampus, this neurotrophin is involved in both learning (Linnarsson et al., 1997; Minichiello et al., 1999;Minichiello, 2009) and memory (Liu et al., 2004; Beckinschtein et al., 2008a; Beckinschtein et al., 2008b)

formation. BDNF is also essential for a variety of adaptive neuronal responses dependent on short-term plasticity (Cirulli et al., 2004; Ninan et al., 2010), while facilitating long-term potentiation (LTP) (Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996) and attenuating long-term depression (LTD) (Akaneya et al. 1996; Huber et al. 1998; Kinoshita et al. 1999), whereas pro-BDNF has the opposite effect, facilitating hippocampal LTD (Woo et al., 2005).

BDNF is also implicated in the most prevalent late-onset neurodegenerative conditions (Connor et al., 1997; Canals et al., 2004; Yanpallewar et al., 2012). Decreased BDNF levels in the hippocampus of Alzheimer's disease (AD) patients (Connor et al., 1997) and axonal transport deficits of BDNF-containing vesicles in mice with presenilin-1 mutations and hyperphosphorylated tau (Peethumnongsin et al., 2010) indicate that modulating neurotrophin signaling is a potential therapeutic approach to this pathology. Increasing BDNF expression prevents neuronal loss, corrects motor dysfunction and improves hippocampal learning and synaptic plasticity in transgenic murine (Arancibia et al., 2008; Blurton-Jones et al., 2009) and non-human primate (Nagahara et al., 2009) models of Alzheimer's disease. Aerobic exercise, which increases BDNF levels (Neeper et al., 1995), also prevents the decline in spatial learning and memory through hippocampal LTP improvement in an AD mouse model (Liu et al., 2011). Similarly, decreased BDNF levels in the striatum of Huntington’s disease (HD) patients (Ferrer et al., 2000) expressing mutant huntingtin, which impairs BDNF gene expression (Zuccato et al., 2001) and anterograde vesicular transport from cerebrocortical afferents (Gauthier et al., 2004), can be rescued by BDNF overexpression (Xie et al., 2010; Giralt et al., 2011) and pharmacological agents that increase BDNF expression (Rigamonti et al., 2007; Simmons et al., 2009). BDNF signaling also correlates with the etiology of Parkinson’s disease (Mogi et al, 1999; Parain et al., 1999; Collier et al., 2005), Multiple Sclerosis (Stadelmann et al., 2002) and Amyotrophic Lateral Sclerosis (Yanpallewar et al., 2012).

BDNFis additionally linked to stress response (Vollmayr et al., 2001) and the biology of mood disorders (Hall et al., 2003), especially anxiety and depression (Eisch et al., 2003; Martinowich et al., 2007), mediating resilience to chronic stress (Taliaz et al., 2011). BDNF variants increase the susceptibility to numerous psychiatric disorders, for example, schizophrenia (Krebs et al., 2000) and aberrant eating behaviors, including anorexia nervosa restrictive type (Ribases et al., 2003), bulimia nervosa 2 (Ribases et al., 2003) and severe obesity (Liao et al., 2012). BDNF-deficient mice show aggressiveness (Lyons et al., 1999), locomotor hyperactivity (Rios et al., 2001), hyperphagia (Gray et al., 2006; Unger et al., 2007) and type-2 diabetes (Sha et al., 2007). Furthermore, BDNF is a critical regulator of energy balance (Xu et al., 2003), pain (Pezet and McMahon, 2006) and drug addiction, particularly opioid and cocaine dependence (Vargas-Perez et al.,

2009; Mao et al., 2009; Lu et al., 2010). Conversely, exogenous BDNF application rescues different neural tissues from excitotoxic, ischemic, traumatic and toxic injuries, in vitro and in vivo, upon acute or long-term neurotrophic treatment, both before and after the insult (Coughlan et al., 2009;Pereira et al., 2009; Murray and Holmes, 2011; Noble et al., 2011). In vitro, BDNF protects cultured cortical (Hetman et al., 1999; Sun et al., 2008), cerebellar granule (Bazán-Peregrino et al., 2007; Wang et al., 2010) and hippocampal (Almeida et al., 2005; Johnson-Farley et al., 2007; Gomes et al., 2012) neurons from apoptotic cell death through activation of the ERK and PI3K signaling pathways. BDNF also rescues cortical neurons from oxygen-glucose deprivation (Ferenz et al., 2012) and prevents N-Methyl-D-aspartic acid (NMDA)-induced protein kinase C (PKC) inactivation, equally providing maximal protection from cell death when pre-incubated, either continuously for 8 hours, or transiently between 8 and 4 hours, prior to NMDA excitotoxicity (Tremblay et al., 1999). In vivo, long-term (7-day) BDNF intraventricular infusion beginning 24 hours (Schabitz et al., 1997) or immediately before (Beck et al., 1994) the injury reduces infarct size and protects hippocampal CA1 neurons in a rat model of transient forebrain ischemia, respectively. Infusion (Yamashita et al., 1997) and vehicle-mediated intravenous (Wu and Pardridge, 1999; Schabitz et al., 2000) administration of BDNF shortly (up to 30 minutes) after medial cerebral artery occlusion (MCAO) produce similar neuroprotective results, mimicked even with delayed (up to 2 hours) intravenous application of BDNF conjugated to a blood-brain barrier drug targeting system (Zhang and Pardridge, 2001).

BDNF neuroprotection in vivo extends to other insults, namely hypoxic-ischemic (Han and Holtzman, 2000), traumatic and spinal cord (Oppenheim et al., 1992; Ikeda et al., 2002) injuries, kainate excitotoxicity (Gratacòset al., 2001) and neonatal hypoxia (Galvin and Oorschot, 2003), and includes the most recent therapeutic strategies, namely ex vivo gene therapy (Yasuhara et al., 2006; Shi et al., 2009; Takeshima et al., 2011) and transplantation of BDNF-overexpressing human neuronal stem cells (NSCs) grafted into the brain region overlying the lesion (Lee et al., 2010).

Unsurprisingly, BDNF is often called the “wonder molecule” (Monk, 2009) or “miracle-gro for the brain” (Ratey, 2008). BDNF does play a panoply of roles in neuronal function, which makes its study just as fascinating as complex, frequently hindering the distinction between different developmental and neuroprotective effects triggered by endogenous and exogenous BDNF, respectively. Furthermore, the use of BDNF in clinical applications is limited by unfavorable pharmacokinetics, specifically its short plasma half-life (less than 1 minute in rats) and the low rate of transport across the blood-brain barrier (BBB) (Pardridge et al, 1994; Poduslo and Curran, 1996), poor intraparenchymal penetration (Morse et al., 1993) and adverse side-effects, mainly resulting from the low-

affinity BDNF-p75 neurotrophin receptor ($p75^{NTR}$) interaction, which can induce pain (Zhang et al., 2008), among other factors (Kingwell, 2010).

However, recent advances through the development of alternative BDNF delivery methods, for example, pegylation (Pardridge et al., 1998), chimeric peptide approaches (Zhang and Pardridge, 2001; Wu, 2005), adeno-associated viral (AAV) vector-mediated gene delivery (Baumgartner and Shine, 1997; Martin et al., 2003; Kells et al., 2004; Shi et al., 2009), genetically engineered bone marrow mesenchymal stem cells (Kurozumi et al., 2004; Sasaki et al., 2009; Harper et al., 2009; Makar et al., 2009; Park et al., 2012) and the use of poly(ethylene vinyl acetate) (EVAc) polymers (Sirianni et al., 2010) as vehiclesforlong-term in vivo delivery of BDNF, in addition to partial (Schmid et al., 2012) or selective (Jang et al., 2010; Bai et al., 2010; Chen et al., 2011) TrkB agonists and BDNF peptidomimetics (O'Leary and Hughes, 2003; Fletcher et al., 2008; Massa et al., 2010), have enabled to overcome such drawbacks and successfully promote neurotrophic activities and neuroprotection aimed at designing new therapeutic strategies.

Notwithstanding, in vitro studies using primary cultures of dissociated neurons enable to follow individual neurons and dynamic changes of their morphology over time, facilitating the access to the surface of cells and high-resolution imaging, allowing a high number of experimental conditions to be tested simultaneously, among several other advantages (Dotti et al., 1988; Potter and DeMarse, 2001; Kaech and Banker, 2006). Moreover, neuronal cultures normally reproduce in vivo aspects of neuronal development and neurodegeneration, despite lacking some three-dimensional features, and results initially found in such preparations are usually confirmed in animal models afterwards (Kaech et al., 2012a, b, c).

Excitotoxicity has been recently proposed by an increasing number of researchers as the converging mechanism of neurodegeneration in several neuropathological conditions (Dong et al., 2009; Lau and Tymianski, 2010; Ehrnhoefer et al., 2011). In particular, glutamate-induced excitotoxicity has been found to trigger the critical events of in vivo neurodegeneration in cultured hippocampal neurons (Luetjens et al., 2000; Aarts and Tymianski, 2004; Almeida et al., 2005; Hilton et al., 2006; Gomes et al., 2011; Gomes et al., 2012). Hippocampal neurons are further susceptible to excitotoxic damage (Hollmann and Heinemann, 1994) because they lack the p50 subunit of Nuclear Factor-kB (NF-kB) (Yu et al., 1999) and show enhanced expression of corticosteroid receptors, calcium channel activity and synaptic plasticity with consequent vulnerability to hyperexcitability (reviewed in Murray and Holmes, 2011).

In the following sections, we will address the state-of-the-art on the role of BDNF in mechanisms of synaptic plasticity and neuroprotection,in further detail, focusing on mechanisms of plasticity and neuroprotection against glutamate excitotoxicity induced by

BDNF in cultured hippocampal neurons. The aim is to best understand the therapeutic potential of BDNF in the treatment of excitotoxicity-based neurodegenerative conditions, whether through reactivation of BDNF-induced developmental gene expression to trigger functional recovery, or attenuation of cellular damage by downregulation of proteases and pathogenic mechanisms triggered on programmed cell death. Therefore, the identification of the key molecular targets of physiological BDNF signaling is critical to develop new approaches for BDNF-induced neuroprotection against different pathological conditions.

1. Neurotrophins

Neurotrophins are agroup of secreted growth factors critical for the development and maintenance of the vertebrate nervous system(Allen and Dawbarn, 2006).Nerve growth factor (NGF) was the first neurotrophin identified, more than half a century ago, during a search for survival factors that could explain the death of developing sensory and motor neurons in the absence of peripheral targets, as a result of a degenerative process rather than a failure of differentiation (Hamburguer and Levi-Montalcini,1949; reviewed in Thoenen and Edgar, 1985). Subsequently, brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin 3 (NT3) (Ernfors et al., 1990; Jones and Reichardt, 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin 4/5 (NT4/5) (Berkemeirer et al., 1991; Hallböök et al., 1991; Ip et al., 1992) were identified in different tissues and cloned through homology screens, establishing this family of functionally and structurally related proteins.

In addition to neurotrophins, several other polypeptide factors have shown neurotrophic activities, including the ciliary neurotrophic factor (CNTF) (Ip et al., 1996), insulin-like growth factor (IGF) (Doré et al., 1997), basic fibroblast growth factor (bFGF) (Walicke et al., 1986), transforming growth factor-β (TGF-β) (Farkas et al., 2003), glial cell line-derived neurotrophic factor (GDNF) (Lindsay and Yancopoulos, 1996) and Sonic hedgehog (Miao et al., 1996), among others, which promote the survival of specific neuron populations and protect such cells from toxic insults.

1.1- The neurotrophin hypothesis

The neurotrophin hypothesis was set on three principles: i) specific target tissues produce limiting amounts of a neurotrophin; ii) responsive neurons projecting to these targets compete for the limiting amounts of neurotrophin; iii) the neurotrophin binds to selective receptors on afferent terminals and is internalized and retrogradely transported to the neuronal cell body, where it provides signals affecting neuronal survival and differentiation (Oppenheim, 1991; Lucidi-Phillipi and Gage, 1993; Snider, 1994; Burek and Oppenheim, 1996; Wright et al., 1997; Pettmann and Henderson, 1998).However, neurotrophins are not just retrogradely transported target-derived growth factors. Recent studies demonstrating the anterograde transport of neurotrophins, which can also act in autocrine and paracrine modes, upon constitutive and activity-dependent release, has lead to the reassessment of this hypothesis, whereby neurotrophin access rather than quantity is the limiting factor determining cellular fate (Conner et al., 1998).

1.2 - Neurotrophin structure

All neurotrophins are post-transcriptionally processed from precursor forms (pre-pro-neurotrophins) into mature polypeptides with similar molecular size, roughly 13 kDa. Secreted proteins typically have basic isoelectric points (pI = 10.5 for BDNF) (Mowla et al., 2001; Kanato et al., 2008), which limits their diffusion (Yan et al., 1994; Dittrich et al., 1996; Lodovichi et al., 2000) and range of action (Bibel and Barde, 2000).

Neurotrophins exist as non-covalently linked and stable homodimers in solution (Sofroniew et al., 2001), sharing a highly homologous protomer structure, with approximately 50% amino acid identity and overlapping three-dimensional conformations, encompassing three pairs of anti-parallel β-strands and cysteine residues, arranged in a cysteine "knot" motif, formed by three intramolecular disulfide bonds between the polypeptide chains(McDonald et al., 1991; McDonald and Hendrickson, 1993). The β-strands maintain the conformation of the dimer interface and delineate a flat surface with a hydrophobic core that includes highly conserved residues. The highly conserved β-strands are interconnected by four highly variable β-loops, which significantly contribute for the different receptor specificities (Jungbluth et al., 1994; Lindsay et al., 1996; Kullander et al., 1997).The highly variable β-loops enable different structures of receptor-ligand complexes and receptor binding kinetics, determining the variability of interactions between the intracellular domains of the receptors and adaptor proteins, their phosphorylation, and the association with membrane microdomains on the cell surface, specialized for signaling and trafficking(Robinson et al., 1999).Such issues are addressed in further detail, in section 4, with emphasis on BDNF and its mechanism of action.

1.3- Neurotrophin homology

Evolutionary studies have shown that a single ancestor gene underwent two independent duplication events at an early stage of vertebrate evolution, leading to the formation of the current neurotrophins (Götz et al., 1994). According to sequence comparisons and isolation of neurotrophin genes from various vertebrates, NGF/NT3 and BDNF/NT4/5 evolved from those separate duplication events (Hallböök, 1999).

No homologs of neurotrophins or their receptors have been identified, thus far, in invertebrate phyla (Chao, 2000), despitethe complete genome sequencing of invertebrate organisms, in recent years, particularly those most used by geneticists, including *Caenorhabditis elegans* (Ruvkun and Hobert, 1998) and *Drosophila melanogaster* (Myers et al., 2000). However, growth factors other than neurotrophins, including FGF (Klämbt et al., 1992; Roubin et al., 1999) and TGF-β (Krishna et al., 1999; Raftery and Sutherland,

1999), have been identified in invertebrates. Therefore, a nervous system with precise wiring, chemical neurotransmission and developmentally regulated elimination of neurons can be assembled in the absence of neurotrophins, suggesting that neurotrophins may be involved in adaptive responses of long-lived higher vertebrates to environmental stimuli (Bibel and Barde, 2000).

In vertebrates, two novel neurotrophins from the platyfish and carp have been cloned and named neurotrophin-6 (Götz et al., 1994) and neurotrophin-7 (Nilsson et al., 1998), respectively. Although they appear to interact with the same receptors as the mammalian proteins, no orthologs of neurotrophin-6 and neurotrophin-7 have been found in mammals or birds. Moreover, molecular phylogeny studies on the evolution of the neurotrophins,in general (Kullander et al., 1997), and BDNF, in particular(Tettamanti et al., 2010),have shown that neurotrophins are a paradigmatic example of how natural selection acts on mammals, separating them from other classes. Conversely, this also suggests that neurotrophins are key determinants of the extremely high complexity of nervous systems found in such organisms (Jaaro et al., 2001).Furthermore, BDNF was considerably more conserved in structure and function than nerve growth factor during vertebrate evolution (Götz et al., 1992), and is the most abundantly expressed neurotrophin in the brain,despite the high homology shared by neurotrophins, further indicating the key role of BDNF in neuronal function.

1.4- Neurotrophin receptors

All four known members of the neurotrophin family activate two classes of cell surface receptors, the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors (TrkA, TrkB and TrkC), and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor superfamily, through which neurotrophins trigger their biological functions (Figure 1).

NGF is the preferred ligand for TrkA (Cordon-Cardo et al., 1991; Kaplan et al., 1991; Klein et al., 1991a), TrkC preferentially binds NT3 (Hohn et al., 1990; Lamballe et al., 1991; Soppet et al., 1991; Ip et al., 1993), and TrkB can bind either BDNF or NT4 depending on the cell type (Glass et al., 1991; Soppet et al., 1991; Ip et al., 1992, 1993). TrkA and TrkB also display low-affinity binding for NT3 (Cordon-Cardo et al., 1991; Glass et al., 1991; Soppet et al., 1991; Ip et al., 1993), which demonstrates the redundancy of this family. Furthermore, NGF, BDNF, NT3 and NT4 also bind to $p75^{NTR}$ with a similar, low affinity of $1x10^{-9}$M (Radeke et al., 1987; Meakin and Shooter, 1992; Barbacid, 1993).

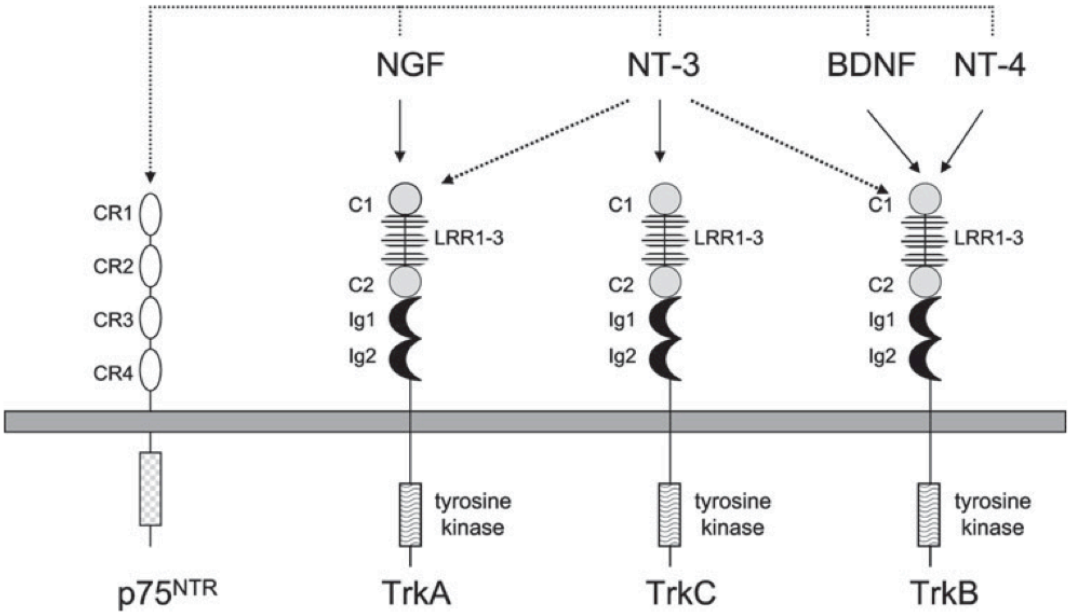


Figure 1. Neurotrophins and their receptors (*in* Skaper, 2012). Neurotrophins interact with three Trk receptors: NGF binds TrkA, BDNF and NT-4 bindTrkB, and NT-3 binds TrkC. In some cellular contexts, NT-3 can also activate TrkA and TrkB, albeit with less affinity. All neurotrophins bind to and activate the $p75^{NTR}$. CR1–CR4 cysteine-rich motifs, C1/C2 cysteine-rich clusters, LRR1–3 leucine-rich repeats, Ig1/Ig2 immunoglobulin-like domains.

1.4.1- Trk receptors

1.4.1.1- Trk receptor structure and binding specificity to neurotrophins

The Trk receptors are modular, single-pass membrane proteins consisting of an N-terminal signal peptide followed by two cysteine-rich domains separated by three leucine-rich motifs, two immunoglobulin-like domains and a transmembrane, a juxtamembrane and a kinase domain preceding the C-terminus (reviewed in Barbacid, 1995). The C-terminal immunoglobulin-like domain (Urfer et al., 1995) and the second leucine-rich motif (Windisch et al., 1995), may both mediate the direct interaction with neurotrophins, in the extracellular surface of neurotrophin receptors, but recent evidence suggests that only the former (Ig-C2) domain actually contacts with NGF in the TrkA receptor-NGF complex structure (Wehrman et al., 2007). In turn, neurotrophins bind to Trk receptors through an analogous region on the surface, formed by conserved amino acids, while the variable residues on the periphery of this common region confer Trk receptor specificity (Ultsch et al., 1999; Wiesmann et al., 1999; Wiesmann et al., 2000). Discontinuous stretches of amino acid residues along the primary sequence group together on one side of the

neurotrophin dimer three-dimensional structure, forming thiscommon interface responsible for binding to and activating Trk receptors. This interface includes the N-terminus, some of the variable loop regions and a beta-strand (Robinson et al., 1999; Banfield et al., 2001).

The N-terminus of neurotrophins only becomes ordered upon complex formationwith the Trk receptor and this ordering ismainly directed by the complementary receptor surface, which additionally determines receptor specificity (Stanzione et al., 2010). These features further explain the limited crossreactivity between neurotrophins and Trk receptors and the high-affinity binding associated with the respective ligand-receptor pairs (Banfield et al., 2001). The two symmetrical surfaces formed along the two-fold axis of the neurotrophin dimer provide a model for ligand-mediated receptor dimerization (Ibáñez et al., 1993). However, the magnitude of binding constants is insufficient, by approximately threeorders of magnitude, to promote receptor dimerization at physiologically active concentrations (Philo et al., 1994). In dispute of the classical model of Trk receptor dimerization upon neurotrophin binding, TrkB receptors have been quite recently shown to exist as homodimers formed in the endoplasmic reticulum (ER), before ligand binding, and the isoforms lacking the intracellular domain cannot form such dimeric structure (Shen and Maruyama, 2012).

1.4.1.2- Trk receptor isoforms

All three Trk receptors show different isoforms with either deletions in the extracellular region or intracellular truncations, including the tyrosine kinase domain (Shelton et al., 1995; Palko et al., 1999; Kryl et al., 1999), resulting from alternative splicing in the case of TrkB and TrkC loci (Klein et al., 1990; Middlemas et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993; Garner and Large, 1994),and post-transcriptional ectodomain cleavage in the case of TrkA receptors (Cabrera et al., 1996; Díaz-Rodríguez et al., 1999).

Extracellular-domain Trk variants have different ligand specificities, namely the TrkB splice variant lacking exon 9, which shows decreased interaction with NT3 and NT4/5 (Strohmaier et al., 1996), and the TrkA variant with increased specificity for NGF and decreased specificity for NT3 (Clary and Reichardt, 1994).

Trk splice variants lacking the canonical intracellular tyrosine kinase domain are the most highly expressed isoforms in the adult brainand show multiple functions in mammalian development (Klein et al., 1990; Allendoerfer et al., 1994; Escandon et al., 1994; Fryer et al., 1996). Truncated TrkB receptor isoforms, TrkB.T1 and TrkB.T2, contain short intracellular domains with 23 and 21 amino acid residues, respectively (Klein et al., 1990; Middlemas et al., 1991). Both truncated forms of TrkB receptors are upregulated in

early postnatal development and predominate over the full-length receptor in the adult brain (Fryer et al., 1996).

Truncated TrkB receptors internalize BDNF, which may restrict neurotrophin availability (Biffo et al., 1995) or, conversely, enable BDNF recycling (von Bartheld et al., 2001), although TrkB.T1 receptors predominantly recycle back to the cell surface by a default mechanism, whilst endocytosed TrkB-FL receptors recyclein a hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)-dependent manner, which relies on its tyrosine kinase activity (Huang et al., 2009). Truncated TrkB receptors act as dominant-negative modulators of Trk signaling (Eide et al., 1996; Ninkina et al., 1996), especially in full-length TrkB-mediated regulation of cell survival (Dorsey et al., 2006), but they also induce their own signaling cascades, independently of full-length TrkB (Takai et al., 2001; Ohira et al., 2006; Manadas et al., 2007). TrkB.T1 deletion in mice causes increased anxiety and morphological abnormalities in basolateral amygdala neurons, consistent with an independent signaling function for this receptor (Carim-Todd et al., 2009). In astrocytes, TrkB.T1 receptors mediate inhibition of RhoA (Ohira et al., 2006),a GTPase responsible for Ca^{2+}-dependent activation of p38 MAPK, coupled to neuronal death under excitotoxic conditions (Semenova et al., 2007).

The physiological levels of truncated TrkB receptors are significantly altered under pathological conditions (Manadas et al., 2007). The frontal cortex of patients with AD has decreased full-length TrkB expression and increased truncated TrkB expression (Ferrer et al., 1999). TrkB signaling is impaired in ALS patients, who show increased total TrkB mRNA in the spinal cord but decreased TrkB receptor phosphorylation, which suggests that alterations in the TrkB response to BDNF, rather than insufficient neurotrophin supply, may be the underlying cause of disease in ALS, probably involving the expression of truncated TrkB isoforms (Mutoh et al., 2000; Küst et al. 2002). Accordingly, TrkB.T1 deletion in a mouse model of ALS delays disease onset (Yanpallewar et al., 2012).

Under excitotoxic conditions, the expression of truncated TrkB receptors is increased in hippocampal neurons (Rudge et al., 1998; Gomes et al., 2012), while BDNF and TrkB.FL immunoreactivity decreases preceding neuronal loss, in damaged areas (Goutan et al., 1998; Ferrer et al., 2001). Calpains mediate the initial step of TrkB.FL degradation, while the TrkB.T receptors expressed de novo may have a dominant negative effect on the signaling activity of the remaining TrkB.FL receptors. However, this may be compensated by the neuroprotective signaling activity of the truncated receptors (Gomes et al., 2012). In addition, the physiological levels of TrkB.T1 receptors are key regulators of neuronal complexity and full-length TrkB signaling in vivo because loss of TrkB.T1 can partially rescue BDNF haploinsufficiency (Carim-Todd et al., 2009).

1.4.2- p75^{NTR}

1.4.2.1- p75^{NTR} expression

The p75^{NTR} is a 75-kDa protein encoded by the human *NGFR* gene located on chromosome 17q21.The rat, mouse and human p75^{NTR} promoter sequences are highly homologous (Schor, 2005). p75^{NTR} receptor expression can be detected early on in the nervous system development, both in neural stem cells (Yan et al., 1988) and on the surface of glial cells (Chen et al., 2009). During the CNS development, p75^{NTR} is highly expressed in the neocortex, hippocampus and midbrain, while in the adult brain p75^{NTR} is mainly restricted to cholinergic neurons in the forebrain, motor neurons and Purkinje neurons in the cerebellum. In the peripheral nervous system, p75^{NTR} is found in sympathetic neurons and dorsal nerves (Chen et al., 1996).

There are two isoforms of the receptor p75^{NTR}, a full-length receptor and a truncated variant form, which results from alternative splicing of exon III of the NGFR locus and lacks the neurotrophin-binding site, both of which are expressed in nerve cells (von Schack et al., 2001).

1.4.2.2- p75^{NTR}molecular structure and ligand-binding affinity

p75^{NTR} is a transmembrane protein with 399 amino acid residues, upon cleavage of the signal peptide, containing an extracellular domain (ECD), a transmembrane portion and an intracellular domain (ICD) (Johnson et al., 1986). The ECD consists of a stalk region connecting the transmembrane domain and four negatively charged cysteine-rich repeats (CR1-4, Fig. 1). The third and fourth cysteine-richrepeats are the neurotrophin-binding site (Chapman et al., 1995; Shamovsky et al., 1999; Dechant and Barde, 2002; Skeldal et al., 2011). The ICD of p75^{NTR} is a global-like domain, known as the death domain, consisting of two sets of perpendicular helices arranged in trios. The death domain connects to the transmembrane portion through a flexible linker region, which may be involved in signal transduction (Liepinsh et al., 1997).

Unlike the Trk receptors, the cytoplasmic region of p75^{NTR} shows no intrinsic ligand-inducible enzymatic function (Locksley et et al., 2001). Furthermore, in contrast with other TNF receptors, the p75^{NTR} has a type-2 death domain, rather than a type-1, and the p75^{NTR} does not self-associate in solution (Hempstead, 2002).

All neurotrophins in their mature form bind to p75^{NTR} with low affinity (Radeke et al., 1987; Meakin and Shooter, 1992; Barbacid, 1993a). Contrary to the mature form, the pro-neurotrophins pro-NGF and pro-BDNF can bind to p75^{NTR} with high affinity (Figure 3),

approximately 1000 fold higher than mature neurotrophins (Lee et al., 2001; Teng et al., 2005). Although pro-neurotrophins do not directly bind or activate Trk receptors, endocytosis and cleavage of pro-neurotrophins generate mature forms of neurotrophins that are capable of inducing Trk activation (Boutilier et al., 2008).

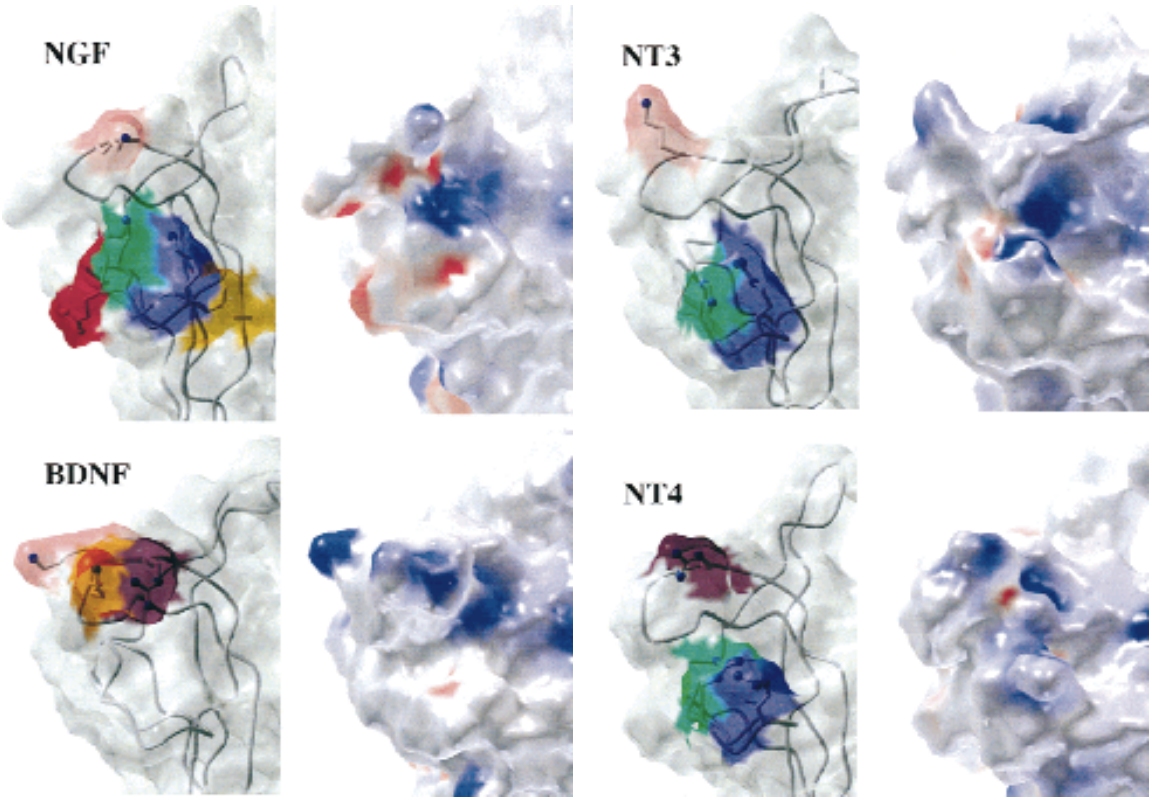


Figure 2. Surface representations of a region of the neurotrophin homodimers critical for $p75^{NTR}$ binding (*in* Robinson et al., 1999). The first and third panels from the left show a transparent surface on top of a wire frame (gray) of each neurotrophin. Residues known to bind to $p75^{NTR}$ are displayed and their surface contribution colored: Ile31 (NGF), gold; Lys32 (NGF), Arg31 (NT3), Arg34 (NT4), blue; Lys34 (NGF), His33 (NT3), Arg36 (NT4), green; Lys95 (BDNF), orange; Lys95 (NGF), Lys96 (BDNF), Lys97 (NT3), pink; Arg97 (BDNF), Arg107 (NT4), purple, and Glu35 (NGF), red. The right panels show an opaque surface charge representation of each neurotrophin dimer in the same orientation as the corresponding left panel. Blue regions represent positive and the red ones denote negatively charged regions.

Whether neurotrophins promote or not receptor homodimerization upon binding to $p75^{NTR}$ is still under debate. The initial model proposed the binding of a single ectodomain of deglycosylated $p75^{NTR}$ toan NGF dimmer, in an asymmetric structure, thereby enabling $p75^{NTR}$to form heterocomplexes with other co-receptors, including Trk receptors, given the

similarity of $p75^{NTR}$ extracellular domains withthose of TNF receptors bound to a trimer of ligands (He and Garcia, 2004). However, O-glycosylation of the $p75^{NTR}$ stalk domain is required for receptor sorting (Yeaman et al., 1997; Bronfman and Fainzilber, 2004). Recent results have shown that NT-3 and NGF bind to $p75^{NTR}$ in a 2:2 ligand-receptor stoichiometry, forming a central homodimer around which two glycosylated $p75^{NTR}$ molecules bind symmetrically (Gong et al., 2008; Feng et al., 2010). The $p75^{NTR}$ forms dimers even in the absence of neurotrophins through cysteinyl residues within the transmembrane domain, and it was proposed that the interaction of the neurotrophins with the receptor complex induces a conformational chance in the $p75^{NTR}$ intracellular domain which may allow the recruitment of the effector molecules (Vilar et al., 2009b; Vilar et al., 2009a). Furthermore, no evidence for a direct TrkA/$p75^{NTR}$ interaction has been found (Wehrman et al., 2007), suggesting that $p75^{NTR}$ and Trk receptors most likely communicate through convergence of downstream signaling pathways and/or shared adaptor molecules, rather than direct extracellular interactions (Chen et al., 2009).

1.4.2.3- $p75^{NTR}$ post-translational modifications and regulation of activity

$p75^{NTR}$ is palmitoylated by thioester formation at cysteine 279 (Barker et al., 1994), which facilitates translocation to cholesterol- and sphingolipid-rich bilayer membranes (Huang et al. 1999; Wahrle et al., 2002). Although the singleN-glycosylation site is not required for receptor sorting, the juxtamembrane region of the extracellular domainis rich in O-glycosylated serine/threonine residues contains an apical targeting signal for $p75^{NTR}$ sorting in polarized cells (Yeaman et al., 1997).

$p75^{NTR}$ undergoes regulated intramembrane proteolysis (RIP) by the tumor necrosis factor-α-converting enzyme (TACE), also known as disintegrin and metalloprotease domain17 (ADAM17), a phorbol 12-myristate 13-acetate (PMA)-inducible membrane protein α-secretase, generating a soluble extracellular domain (p75ECD). This cleavage may be followed by a presenilin 1 (PS1)-dependent γ–secretase cleavage of the resulting 25 KDa fragment, releasing the soluble 20 KDa fragment of the intracellular domain (p75ICD) into the cytoplasm (Urdiales et al., 1998; Kanning et al., 2003; Srinivasan et al., 2007; Ceni et al., 2010). This γ-secretase cleavage occurs in the middle of the transmembrane domain (Jung et al., 2003) and requires prior α-secretase cleavage in a 15-amino-acid strech of the $p75^{NTR}$ stalk domain (Zampieri et al., 2005).

Palmitoylation of the $p75^{NTR}$ C-terminal fragment regulates apoptotic signaling and is required for subsequent cleavage by γ-secretase (Underwood et al., 2008).

The sequential α- and γ-secretase-mediated cleavage is required for $p75^{NTR}$-triggered intracellular signaling, which leads to either apoptotic cell death, upon JNK3 activation (Kenchappa et al., 2006; Srinivasan et al., 2007), or cell survival, dependent on activation of the PI3K/Akt signaling pathway (Ceni et al., 2010).

1.4.2.4- $p75^{NTR}$-mediated signaling pathways and cellular effects

Several signaling pathways are activated upon neurotrophin binding to $p75^{NTR}$through recruitment of adaptor proteins, including TRAF6, neurotrophin receptor-interacting factor (NRIF), melanoma-associated antigen (MAGE), neurotrophin receptor p75 interacting MAGE homologue (NRAGE), Schwann cell factor 1 (SC1), RhoGDI and other proteins (Yamashita et al., 2005; Schecterson and Bothwell, 2010).

The activation of $p75^{NTR}$ triggers multiple downstream proapoptotic signals, including the stress-activated c-Jun N-terminal kinase (JNK), ceramides and the small GTP binding protein Rac. In contrast, $p75^{NTR}$ also promotes cell survival signaling through the transcription factor NF-κB and acts as a cell cycle regulator of neuronal precursor cells.

1.4.2.4.1- $p75^{NTR}$-inducedNF-κB activation and cell survival

Neurotrophin binding to $p75^{NTR}$ induces NF-κB activation, thereby promoting NF-κB-dependent neuronal survival (Hamanoue et al. 1999; Middleton et al. 2000) through TRAF6 interaction with the cytoplasmic domain of $p75^{NTR}$ (Khursigara et al. 1999). Interleukin-1 receptor-associated kinase (IRAK) is recruited to this complex, resulting in formation of a complex of TRAF6 and IRAK with atypical protein kinase C-ζ (aPKC-ζ) and the aPKC-interacting protein p62 (Wooten et al. 2001; Vandenplas et al. 2002). IκB kinase-β (IKK-β), which is a substrate of aPKC, is recruited to and activated in this complex. IKK-mediated phosphorylation of IκB releases the transcription factor NF-κB, which then translocates to the nucleus activating the transcription of survival genes. Both the p62 and the kinase activity of IRAK are required for NF-κB activation. The Ran-binding protein in the microtubule-organizing center (RanBPM) negatively regulates the $p75^{NTR}$-induced activation of the NF-κB pathway by interacting with TRAF6 and affecting its ubiquitination (Wang et al., 2012).

NF-κB can also be activated by the interaction between the caspase recruitment domain (CARD) of receptor-interacting protein 2 (RIP-2) and the death domain of $p75^{NTR}$, demonstrating how adaptor proteins account for the ability of neurotrophins to trigger a bifunctional switch for cell survival or cell death through the same $p75^{NTR}$ (Khursigara et

al., 2001). Alternatively, upon neurotrophin binding to $p75^{NTR}$, the mitogen-activated protein (MAP) kinase p38beta2, activated by MAP kinase kinase (MKK) 6, specifically interacts with the 5^{th} and 6^{th} helices of the $p75^{NTR}$ intracytoplasmic region and induces its phosphorylation, thereby increasing the activation of NF-κB and decreasing that of AP-1 (Wang et al., 2000). Upon excision from the $p75^{NTR}$, p75ICD can translocate to the nucleus, through an unknown mechanism (Peng et al., 2004; Srinivasan et al., 2007), and activate NF-κB (Kanning et al., 2003) in concert with the TNF receptor associated factor 6 (TRAF6). The latter requires the NGF-induced association between PS1 and TRAF6 for TRAF6 E3 ligase-mediated ubiquitination of $p75^{NTR}$ and the regulated intramembrane proteolysis of $p75^{NTR}$ (Powell et al., 2009). TrkA and TrkB but not TrkC receptors mediate this cleavage (Kanning et al., 2003). TrkA and TrkB induce MEK-dependent phosphorylation of ADAM17 at the intracellular residue threonine 735, activating this transmembrane cysteine protease and triggering cleavage of $p75^{NTR}$ in primary cerebellar granule neurons (Ceni et al., 2010). In turn, Trk-induced ADAM17 phosphorylation and generation of the p75ICD is required for neurotrophin-induced Erk and Akt activation and survival signaling in a feedback mechanism sustaining Trk-dependent cell survival (Kommaddi et al., 2011). However, this contrasts with the $p75^{NTR}$-induced NF-κB activation, which promotes apoptosis in neonatal rat oligodendrocytes (Yoon et al. 1998) and prion-infected neurons (Bai et al., 2008).

Overall, $p75^{NTR}$-induced NF-κB activation may be ligand-dependent, through NGF binding (Carter et al., 1996) or ligand-independent, through signaling inducedby disulfide-crosslinked $p75^{NTR}$ dimmers (Vilar et al., 2009a).

1.4.2.4.2- $p75^{NTR}$-inducedJNK activation and apoptosis

$p75^{NTR}$ signaling promotes apoptosis through two regions of the receptor, the cytoplasmic juxtamembrane “chopper” domain (Coulson et al., 2000) and the intracellular death domain (Wang et al., 2001), and an array of adaptor proteins, including the melanoma-associated antigen (MAGE), neurotrophin receptor p75 interacting MAGE homologue (NRAGE) and Schwann cell factor 1 (SC1), triggering the Jun kinase cascade (Yamashita et al., 2005; Niewiadomska et al., 2011). Palmitoylation of $p75^{NTR}$ is necessary for the induction of apoptosis through the “Chopper” domain (Diarra et al., 2009).

Key intermediates of $p75^{NTR}$-induced activation of the Jun kinase cascade also include the neurotrophin receptor interacting factor (NRIF) and the E3 ubiquitin ligase TRAF6 (Yeiser et al. 2004; Linggi et al. 2005), which form a complex whereby TRAF6-mediated K63 ubiquitination of NRIF enables NRIF translocation into the nucleus (Geetha et al. 2005b). Nuclear transport of NRIF complexed to p75ICD requires ligand-dependent

γ-secretase-mediated cleavage of $p75^{NTR}$ (Kenchappa et al. 2006) and activates the expression of pro-apoptotic genes, including *BAX*, *BAD* and *BAK*, and caspases-9, -6 and -3. (Coulson et al., 2000; Huang and Reichardt, 2003; De Felice et al., 2008; Diolaiti et al., 2007; Khwaja et al., 2006). Upon neurotrophin binding, $p75^{NTR}$ stimulation may also activate p53 and the expression of pro-apoptotic genes, including *BAX*, concomitantly inducing the expression of Fas in neuronal cells and promoting apoptosis through Fas receptor activation (Reichardt, 2006).

In sympathetic neurons, activation of the Jun kinase cascade by $p75^{NTR}$ involves Cdc42 (Bazenet et al. 1998) and the downstream MAP kinase kinase kinase apoptosis signal-regulated kinase 1 (ASK1), which inhibits cell death induced by constitutively active Cdc42 (Kanamoto et al. 2000). The Jun kinase kinase MKK7 likely provides a link between ASK1 and Jun kinase (Kanamoto et al. 2000). In oligodendrocytes, Rac activation is required for $p75^{NTR}$-induced apoptosis, demonstrating that different cells may use different intermediates in the pathway that leads to activation of the Jun-kinase signaling-pathway (Harrington et al. 2002).

Activation of $p75^{NTR}$ is not always related with neuronal death and, in fact, during development of the sympathetic nervous system, the $p75^{NTR}$ has a dual function: promoting survival together with TrkA in response to NGF and inducing cell death upon binding pro- or mature BDNF.BDNF-binding to $p75^{NTR}$ activates JNK3, inducing TACE/ADAM17 mRNA and protein up-regulation, which promotes receptor proteolysis, leading to prolonged activation of JNK3 and subsequent apoptosis in sympathetic neurons (Kenchappa et al., 2010). Conversely, TrkA may form a heteromeric complex with either the full-length $p75^{NTR}$ or the 25 KDa membrane-tethered C-terminal fragment to promote neuronal survival, but not with the p75ICD. This indicates thatγ-secretase-mediated removal of the $p75^{NTR}$ transmembrane domain may disturb the interaction with TrkA, thereby affecting the formation/disassembly of the $p75^{NTR}$-TrkA receptor complex, by regulating the availability of the transmembrane domain required for this interaction (Jung et al., 2003). However, results subsequently found have contradicted the formation of the full-length $p75^{NTR}$-TrkA receptor complex (Wehrman et al., 2007).

1.4.2.4.3- $p75^{NTR}$-inducedceramide production and downstream signaling

Neurotrophin binding to $p75^{NTR}$ activates acidic sphingomyelinase, resulting in the production of ceramides, lipid components of cell membranes, which can also act as second messengers (Dobrowsky et al., 1994). Ceramides may induce both apoptotic and prosurvival effects downstream of $p75^{NTR}$ activation, according to their levels within cells (DeFreitas et al., 2001; Song and Posse de Chaves, 2003). They trigger a number of

signaling pathways, including the Erk, Jun kinase and NF-κB signalling pathways, in addition to TrkA receptor activity, which is mediated through phosphorylation of serine residues (MacPhee and Barker, 1997; Muller et al., 1998). Ceramides bind Raf, thereby inhibiting ERK signaling (Muller et al., 1998), and may also inhibit or promote PI3-Kinase signaling through different mechanisms, depending on the neural populations (MacPhee and Barker, 1997; Zundel et al., 2000) and signal intensity (Blöchl and Blöchl, 2007).

1.4.2.4.4- $p75^{NTR}$-induced RhoA activation and neurite outgrowth

CNS neurons are mostly unable to regrow naturally after neural injury for several reasons, including scar formation, myelin inhibition and decreased intrinsic regrowth potential after development (Chen et al., 2009). Myelin-based growth inhibitors (MBGIs), including Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp), all bind to the Nogo-66 receptor (NgR), negatively regulating neurite outgrowth (Wang et al., 2002) by modulating Ras homologue member A (RhoA) activity (Gehler et al., 2004). The NgR lacks an intracellular domain and, thus, requires the formation of the NgR-Leucine rich repeat and Ig domain containing 1 (LINGO-1)-$p75^{NTR}$ complex to trigger its cellular effects (Wong et al., 2002; Mi et al., 2004). RhoA inhibits regeneration, as demonstrated by NgR, $p75^{NTR}$ and RhoA siRNA silencing, which significantly blocks myelin inhibition (Ahmed et al., 2005), but RhoA does not bind directly to the NgR-LINGO-1-$p75^{NTR}$ complex. RhoA-GDP is usually bound to the Rho guanine dissociation inhibitorα (Rho-GDIα), which prevents RhoA activation by guanine exchange factors (GEFs), whilst the GTPase activating proteins (GAPs) accelerate the intrinsic GTPase activity of Rho proteins (Luo, 2000).

The binding of MBGIs to the NgR-LINGO-1-$p75^{NTR}$ complex enables $p75^{NTR}$ to bind to Rho-GDIα through the fifth helix of its death domain, releasing RhoA from Rho-GDI. After being activated by GEFs, RhoA stimulates Rho-activated kinase (ROCK), depolymerizing actin filaments and leading to growth cone collapse (Yamashita and Tohyama, 2003). Conversely, the binding of mature NGF (Yamashita et al., 1999), NT-3 (McQuillen et al., 2002) and BDNF (Gehler et al., 2004) to $p75^{NTR}$ abolishes the interaction of $p75^{NTR}$ with Rho-GDIα, which leads to the inactivation of RhoA, likely through p190RhoGAP and Rap-dependent RhoGAP (ARAP3), and promotes neurite outgrowth (Jeon et al., 2010). $p75^{NTR}$ additionally facilitates internalization of Trk receptors, through recruitment of an E2–E3 ubiquitin ligase complex that ubiquitinates Trk receptors, and subsequent signaling events that promote axon growth, retrograde transport and nuclear signaling at the cell soma (Geetha et al., 2005). Likewise, $p75^{NTR}$ promotes retrograde

transport of neurotrophins *in vivo*, which may enhance axon growth through the same mechanism (Curtis et al., 1995; von Bartheld et al., 1996; Butowt et al., 2009).

However, the precursor forms of NGF, BDNF and NT-3 apparently have the opposite effect, mimicking MBGI-induced inhibition of neurite outgrowth. In hippocampal neurons, the Rac GEF Trio dissociates from the $p75^{NTR}$–sortilin-related VPS10 domain-containing receptor 2 (SorCS2) upon pro-NGF binding, decreasing Rac activity and impairing filopodial formation. In parallel, PKC is activated, phosphorylating and inactivating the actin-bundling protein fascin, which leads to destabilization and collapse of actin filaments and, consequently, acute growth cone retraction (Deinhardt et al., 2011). In cortical and dorsal root ganglion neurons, pro-BDNF promotes a dose- and time-dependent activation of RhoA via $p75^{NTR}$ and significant neurite collapse, mediated by ROCK and the collapsin response mediator protein-2 (CRMP-2) (Sun et al., 2012).

Therefore, the specificity of mature neurotrophins vs. proneurotrophins actions partly depends on the formation of distinct co-receptor complexes (Teng et al., 2011).

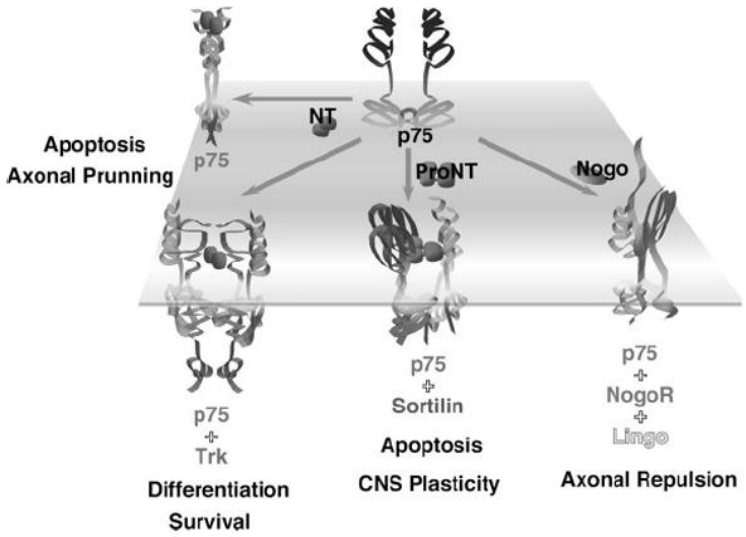


Figure 3. Schematic representation of mature neurotrophin (NT) and proneurotrophin (proNT) actions, and the diversity of coreceptor interactions (*in* Teng et al., 2010).

Neurite growth and connectivity are severely affected upon full knockdown of $p75^{NTR}$, which demonstrated that $p75^{NTR}$ has a key role in the growth cone outgrowth (Bentley et al., 2000). However, contradicting results on regulated proteolysis of $p75^{NTR}$and neurite outgrowth and regeneration have been reported. On the one hand, MAG binding to $p75^{NTR}$ cerebellar neurons induces sequential α- and γ-secretase proteolytic cleavage of $p75^{NTR}$, which is required for the activation of Rho and inhibition of neurite outgrowth (Domeniconi et al., 2005). On the other hand, TACE-induced regulated

intramembranous proteolysis of $p75^{NTR}$ abrogates axon growth inhibitory signaling, thereby enabling CNS axon/neurite growth (Ahmed et al., 2006).

Additional neurite growth inhibitory factors, including semaphorin3A (Tang et al., 2004), repulsive guidance molecule b (Liu et al., 2009) and ephrin-B3 (Benson et al., 2005), preclude the regeneration of CNS neurons through $p75^{NTR}$-mediated signaling (Naska et al., 2010).

1.4.2.4.5- $p75^{NTR}$-induced Ras/ERK signaling

Similarly to Trk receptors, $p75^{NTR}$ can also activate the Ras/ERK pathway because the death domain has two tyrosine residues, Y337 and Y336, which can be phosphorylated upon $p75^{NTR}$ activation. In particular, Y366 phosphorylation stimulates Ras, a small GTPase involved in neuron growth regulation, through the same adaptor proteins as the Trk receptors (Blöchl et al., 2004). Furthermore, ceramide (Song et al., 2003) and Ras (Xue et al., 2000) promote robust neuronal survival by a mechanism dependent on the juxtamembrane sequence of the cytoplasmic region of $p75^{NTR}$ and involving the activation of PI3K and the downstream kinase Akt (Costantini et al., 2005; Roux et al., 2001). Akt is responsible for the balance between pro- and anti-apoptotic proteins, including Bcl-2, promoting neuronal survival (Frade et al., 1996; Wehrman et al., 2007).

However, while $p75^{NTR}$ activation of Ras is relatively brief, lasting only a few minutes, rapidly returning to basal levels (Sahai et al., 2001; Peng et al., 2004), activation of Ras by Trk receptors, which is also fast, is sustained for much longer periods of time (Shamovsky et al., 1999; Schor et al., 2005; Diarra et al., 2009). Therefore, $p75^{NTR}$-induced short-term activation of Ras signaling mediates neuronal proliferation, whilst Trk receptor-induced long-term activation may also trigger the differentiation process, inducing ERK-mediated long-lasting effects, through up-regulation of genes encoding proteins involved in both differentiation and proliferation (Cargnello and Roux, 2011).

In summary, neurotrophin stimulation of differentiating neurons shifts the initially trophic role of $p75^{NTR}$ signaling during development into a negative growth regulator and an apoptosis inductor upon overstimulation (Blöchl and Blöchl, 2007).

1.5- Neurotrophin heterodimers

Neurotrophins form stable, non-covalently linked homodimers at physiologically active concentrations but may also form non-covalent heterodimers (Radziejewski and Robinson, 1993). BDNF and NT-3 establish highly stable heterodimers, although10-fold less active in neuronal survival assays than a 50:50 mixture of BDNF and NT3 homodimers (Jungbluth et al., 1994). In contrast, the formation of heterodimers involving NGF is extremely unfavorable (Arakawa et al., 1994) and heterodimers containing NGF subunits undergo gradual rearrangement to the homodimers (Radziejewski and Robinson, 1993). Nevertheless, the NGF-NT4 heterodimer has been shown to produce neuronal differentiation in PC12 cells as effectively as NGF (Treanor et al., 1995).

The crystal structures of NGF (McDonald et al., 1991) and NT4 (Robinson et al., 1999) homodimers, in addition to BDNF/NT3 (Robinson et al., 1995) and BDNF/NT4 (Robinson et al., 1999) heterodimers have been reported, enabling the comparison of such highly homologous structures (Figure 4).

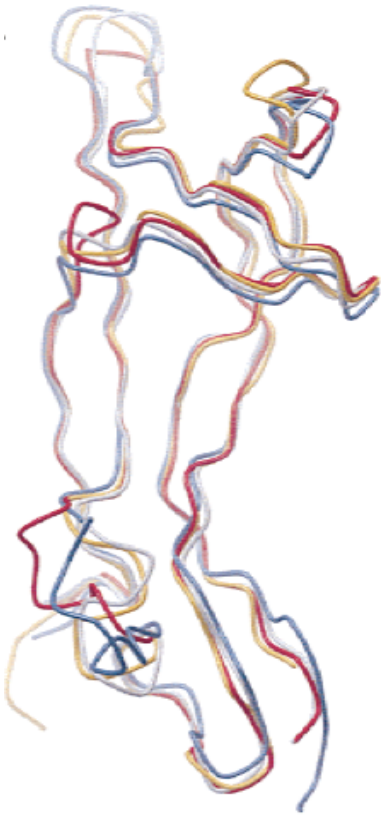


Figure 4. Superpositions of the four neurotrophins (*in* Robinson et al., 1999) with mouse NGF in green, human BDNF from the BDNF/NT3 heterodimer in red, NT3 from the BDNF/NT3 heterodimer in yellow and NT4 from the BDNF/NT4 heterodimer in blue.

2. *BDNF* gene structure and expression

In humans, the *Bdnf* gene spans approximately 70Kb in the short arm of chromosome 11 at position 13,between the loci FSHB and HVBS1 (Jones and Reichardt, 1990), has a complex structure consisting of 11 exons and 9 functional promoters (Pruunsild et al., 2007), and each transcript contains multiple 5' upstream translation initiation codons regulating translational efficiency (Kidane et al, 2009). In mice and rats, 11 different 5′ untranslated regions (UTRs) generate 22 different transcripts, encoded by 9 exons (Figure 5). As in humans, each 5′ exon is alternatively spliced to downstream exon IX (the coding region of BDNF) and a 3′UTR containing two potential polyadenylation signals (Aid et al., 2007).

The human and rodent (mice and rat) *Bdnf* genes have similar structures and splicing patterns. However, the *Bdnf* gene has two human-specific exons, Vh and VIIIh, unused as 5' exons but always spliced with exon V, a longer and more complexly spliced exon IX 5'UTR and a non-coding antisense *Bdnfos* gene (Figure 6), absent in rodents (Liu et al., 2005). In addition, the splice donor site in human exon VII contains nucleotides GG instead of the conventional GU sequence characteristic of eukaryotes (Black, 2003). Brain *Bdnf* and *Bdnfos* transcripts form dsRNA duplexes, in vivo, potentially acting as cis-antisense RNAs, targeting one of the initial transcripts (Borsani et al., 2005), or directly inhibiting *Bdnf*transcription and/or regulating pre-mRNA splicing, although the mRNA levels are not specifically reduced in tissues with high expression of *Bdnfos* transcripts (Pruunsild et al., 2007).

Multiple *BDNF* promoters enable a developmentally, tissue specific and activity-dependent regulated bidirectional transcription (Pruunsild et al., 2007).

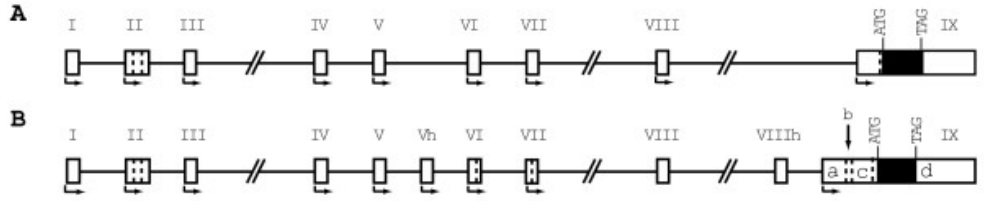


Figure 5. Schematic representation of rodent (A) and human (B) BDNF gene structures (adapted from Koppel et al., 2009). *BDNF* genes consist of multiple untranslated 5' exons spliced together with a common 3' protein-coding sequence in exon IX (transcriptional start sites are indicated with arrows). BDNF transcription can also start from exon IX introducing a unique 5' UTR sequence. Filled boxes indicate BDNF coding sequences whilst unfilled boxes correspond to untranslated sequences. Hatched lines indicate sites of alternative splicing. *Bdnf* exon IX, which encodes the BDNF protein and 3'UTR,

undergoes internal splicing, and transcription may also start from this exon, divided into regions “a”, “b”, “c” and “d” as indicated in the box marking the position of exon IX.

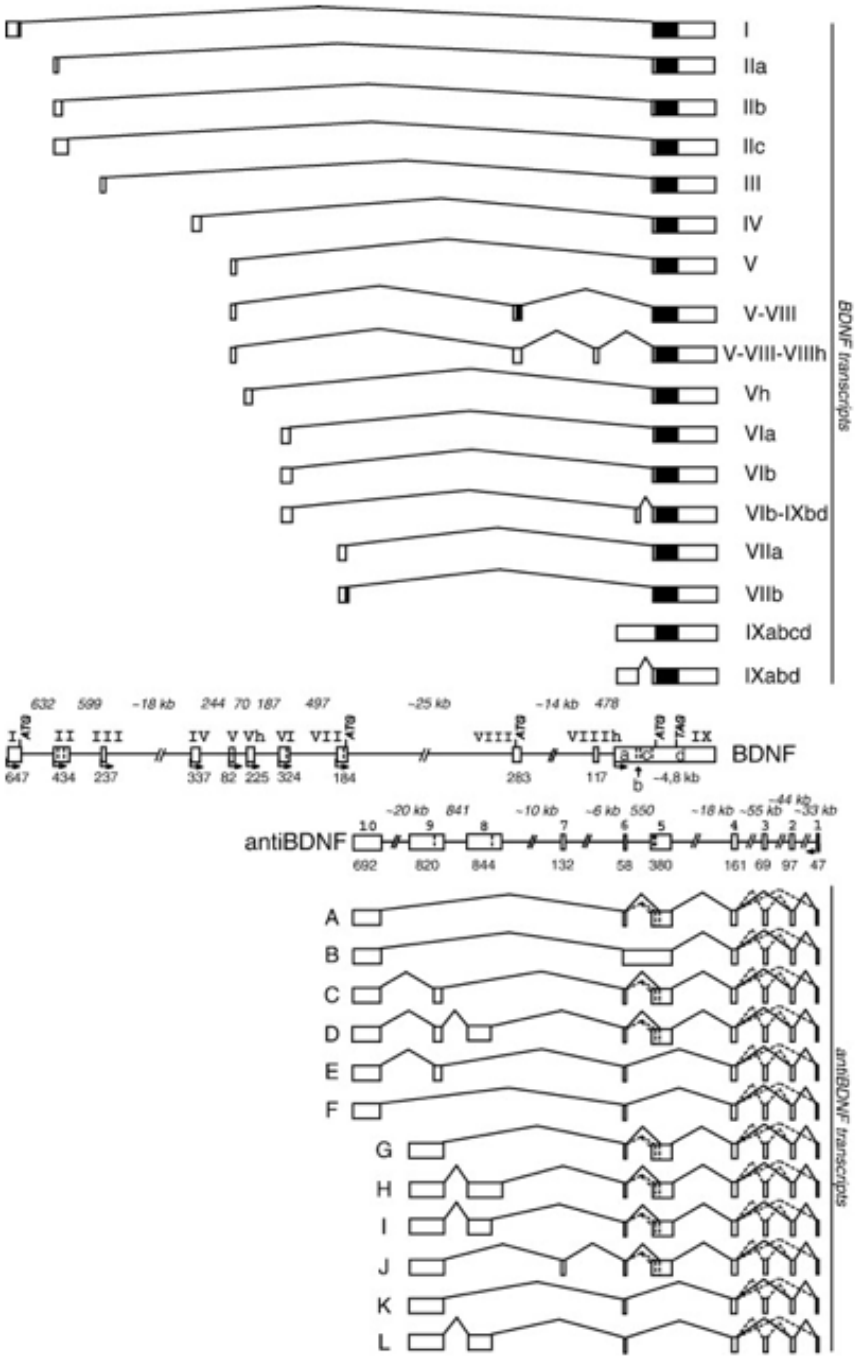


Figure 6. Alternative transcripts of the human *Bdnf* (top) and *antiBdnf* (bottom) genes (adapted from Pruunsild et al., 2007). The numbers below exons (boxes) and above introns (lines) indicate their sizes in base pairs, if not shown otherwise. ATG and TAG mark the positions of translational star and stop codons, respectively. Vertical dashed lines indicate alternative splicing sites for the respective axons.

2.1- Developmental regulation of *Bdnf* expression

During embryonic development, BDNF expression is more abundant in the nervous system than other tissues. However, the highest levels are reached during post-natal development, when BDNF mRNA and protein expression significantly increase in the brain, peaking at P10-14 and decreasing thereafter (Katoh-Semba et al., 1997). While BDNF exons I- and II-containing transcripts are only found in adults, exons III and IV transcripts are mainly expressed during the postnatal period (Pattabiraman et al., 2005).

2.2- Subcellular and tissue-specific *BDNF* expression

In terms of tissue-specific expression, the hippocampus shows the highest levels of *Bdnf*expression in the nervous system, followed by the amygdala, cerebral cortex and hypothalamus (Ernfors et al., 1994; Timmusk et al., 1994; Kawamoto et al., 1996; Conner et al., 1997). In general, *Bdnf*is found in almost all brain tissues, but the dentate nucleus, white matter of the cerebellum, substatia nigra and epiphysis show very low BDNF expression (Pruunsild et al., 2007). In the peripheral tissues BDNF is found in very low concentrations (Katoh-Semba et al., 1997). Although BDNF is more expressed in the developing brain than in other organs, this neurotrophin is also found in non-neural tissues of adults, especially the thymus, liver, spleen, heart, and lung (Maisonpierre et. al., 1990; Hohn et al., 1990; Ernfors et al., 1990; Maisonpierre et. al., 1991; Katoh-Semba et al., 1997). *Bdnf* transcripts containing exons I, II and III are expressed throughout the brain, whereas exon IV transcripts are mainly expressed outside the brain (Tardito et al., 2006).

In neurons, *Bdnf* exons I- and II-containing transcripts are typically excluded from the soma whilst exon IV is strongly expressed in the soma and proximal dendrites (Aliaga et al., 2009) and exon III is restricted to the soma (Pattabiraman et al., 2005). In hippocampal neurons, silencing individual endogenous transcripts or overexpressing *Bdnf-Gfp* transcripts has demonstrated that exons I- and IV-containing transcripts selectively affect proximal dendrites, whilst II and VI affect distal dendrites, reflecting a segregation of BDNF transcripts, which results in a highly selective activation of TrkB receptors, thereby enabling BDNF to differentially shape distinct dendritic compartments (Baj et al., 2011). Exons I- and II-containing transcripts are absent from cultured hypothalamic neurons (Aliaga et al., 2009) and only expressed in adult cerebrocortical neurons (Pattabiraman et al., 2005).

2.3- Activity-dependent *Bdnf* expression

Neuronal activity regulates *Bdnf* transcription through calcium-mediated pathways (West et al., 2001; Flavell and Greenberg, 2008) acting on promoters I and IV (Tao et al., 2002; Rattiner et al., 2004; Kidane et al., 2009; Koppel et al., 2010) and distinct 3'UTRs (Lau et al., 2010). Upregulation of BDNF transcription requires increased intracellular calcium concentrations ($[Ca^{2+}]_i$) (Zafra et al., 1992; Ghosh et al., 1994; Sano et al., 1996), likely dependent on Ca^{2+}influx through L-type voltage-gated calcium channels (VGCCs) or NMDA receptors (Shieh et al., 1998; Tao et al., 1998; Tabuchi et al., 2000). In fact, numerous studies have shown that *Bdnf* mRNA expression is increased upon glutamate receptor activation (Zafra et al., 1990; Zafra et al., 1991; Lindefors et al., 1992; Lauterborn et al., 2000), but halted upon γ-aminobutyric acid type A ($GABA_A$) receptor activation (Lindholm et al., 1994; Berninger et al., 1995).

Rodent *Bdnf* (r*Bdnf*) genes have several effectors of calcium-dependent upregulation on promotersI and IV. Depolarization-induced Ca^{2+}influx upon neuronal activity leads to CaM Kinase IV-dependent phosphorylation of cAMP response element-binding protein (CREB) (alias CREB1) on Ser-133 (Shieh et al., 1998; Tao et al., 1998). The duration of CREB phosphorylation is longer when calcium influx occurs through L-type VGCCs rather than through NMDA receptor, showing that distinct stimuli trigger different gene expression readouts (Hardingham et al., 1999).

CREB1 binding to cAMP/Ca^{2+}-response element (CRE) upregulates the *Bdnf* promoter IV (Shieh et al., 1998; Tao et al., 1998), which is modulated by upstream stimulatory factors (USFs) and calcium-responsive transcription factor (CaRF) binding to an E-box element and CaRE1 (Ca^{2+}-response element 1) element, respectively (Tao et al., 2002; Chen et al., 2003a). MeCP2 (methyl-CpG-binding protein 2), BHLHB2 (basic helix-loop-helix B2) and NFκB (nuclear factor κB) also regulate promoter IV (Chen et al., 2003b; Jiang et al., 2008) but CRE has a key role because CRE knock-in mutations block mouse *Bdnf* (m*Bdnf*) promoter IV activity-responsiveness *in vivo* (Hong et al., 2008).

Neuronal activity also induces r*Bdnf* exon I (Timmusk et al., 1993) through CREB, USFs, MEF2D (myocyte enhancer factor 2D), and NFκB (Tabuchi et al., 2002; Lubin et al., 2007; Flavell et al., 2008). NPAS4 (neuronal PAS domain protein 4) binds unspecified sites of m*Bdnf* promoters I and IV (Liu et al., 2006). Similarly, human *Bdnf* transcripts containing exons I and IV and 5'-extended exon IXa show strong activity-dependent induction in vivo, while exon II and III transcripts show only moderate induction (Koppel et al, 2009). The absence of induction of exon VI-containing transcripts is also consistent the results found for *Bdnf* mRNAs in rodents (Timmusk et al., 1993; Metsis et al., 1993; Aid et al., 2007).

Taken together, the evidences available indicate that a cooperation between CREB/CRE and CaRF/CaRE1 mediate activity-dependent *Bdnf* transcription.

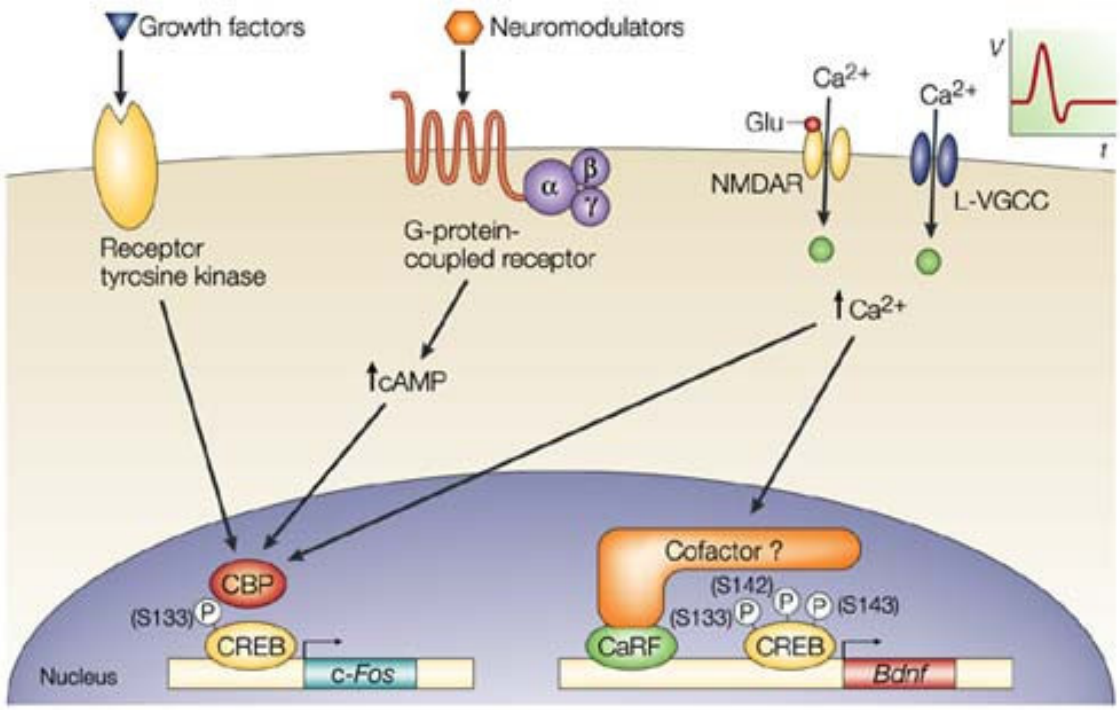


Figure 7. Activity-dependent *Bdnf* transcription model (in West et al., 2002). The activation of plasma-membrane channels, including NMDA receptors and L-type VGCCs, which allow the influx of calcium, lead to the transcriptional activation of *c-Fos* and *BDNF*. The calcium influx induces phosphorylation of CREB at Ser133, recruitment of the transcriptional coactivator CBP (CREB-binding protein) and activation of the transcription factor CaRF (calcium-response factor). The phosphorylation of CREB at Ser142 and 143 inhibits the association of phosphorylated Ser133 with CBP, suggesting that an alternative cofactor might be recruited to CREB in response to calcium signals. CaRF cooperates with CREB to promote *Bdnf* transcription.

Recently, the direct interaction between scaffolding proteins RACK1 and 14-3-3ζ has been show to regulate *Bdnf*transcription by triggering cAMP-dependent RACK1 nuclear translocation (Neasta et al., 2012). However, the effectors of RACK1-regulated BDNF transcription remain completely unknown.

*Bdnf*may be considered an immediate-early gene but its transcription is rather slow (2-4h), when compared to prototype *c-fos*, supporting two critical findings. Firstly, activity-dependent *Bdnf* expression is further associated to long-lasting alterations with synaptic physiology and morphology (Lu, 2003). *Bdnf* mRNA expression in the hippocampus

significantly increases 2-4h after tetanic stimulation (Patterson et al., 1992; Morimoto et al., 1998), which correlates with late long-term potentiation (L-LTP) and long-term memory formation (Abel et al., 1997; Miller et al., 2002; Villareal et al., 2002). Secondly, synapse-specific modulation by BDNF expression relies on local protein translation of previously targeted *Bdnf* transcripts (Steward and Schuman, 2001). *Bdnf* transcripts result from regulated polyadenylation at the two alternative sites in the *Bdnf* gene, generating two mRNA pools with either a long or a short 3'UTR (Timmusk et al., 1993). The long *Bdnf* 3′UTR is a cis-acting translation suppressor at rest but promotes fast and strong activation of translation upon neuronal activity (Lau et al., 2010). *Bdnf* transcripts with a long 3′UTR are targeted to dendrites (Tongiorgi et al., 1997; An et al., 2008) for local protein translation (Kaneko et al., 2012) while those with a short 3′UTR remain in the soma, maintaining basal levels of *Bdnf* expression. Exons II and IV transcripts localize to distal dendrites upon activity, whilst I and IV remain in the soma, even after strong neuronal activation (Pattabiraman et al., 2005; Chiaruttini et al., 2008). As previously mentioned, exons I- and IV-containing transcripts selectively affect proximal dendrites, whilst II and VI affect distal dendrites (Baj et al., 2011). Dendritic trafficking of *Bdnf* mRNA can be mediated by translin-dependent (Chiaruttini et al., 2009) or -independent (Wu et al., 2011) mechanisms but the activity-dependent targeting, induced by exogenous application of recombinant BDNF, is not blocked by translin knockdown, in cultured hippocampal neurons (Wu et al., 2011). The G196A (Val66Met) mutation blocks the trafficking of *Bdnf* transcripts to dendrites (Chiaruttini et al., 2009), which plays a key role in mediating synaptic plasticity (An et al., 2008; Lu et al., 2008; Tongiorgi, 2008).

Thus far, no evidence for intra-axonal protein synthesis of BDNF has been found, although local translation also occurs in both growing and mature axons (Jung et al., 2012). In hippocampal neurons, dendritic BDNF synthesis drives retrograde homeostatic plasticity of presynaptic function (Jakawich et al., 2010) but the lack of postsynaptic BDNF localization in adult neurons suggests otherwise (Dieni et al., 2012). Nevertheless, BDNF activity-dependent recycling (Santi et al., 2006) and self-amplifying autocrine actions (Cheng et al., 2011) indicates that axons may not require local BDNF synthesis, but instead rely on fast anterograde axonal transport of TrkB- and BDNF-containing vesicles (Adachi et al., 2005, Huang et al., 2011).

Bdnf expression is also regulated at a post-transcriptional level by microRNAs, small non-coding RNAs of approximately 22 nucleotides, which target mRNA sequences by 3'UTR base-pairing, triggering mRNA cleavage or translation repression (Filipowicz et al., 2008). The more complex a gene is, the higher the probability of harbouring miRNA targets will be (Cui et al., 2007). Predictably, considering the high complexity of *Bdnf* cis-regulation, numerous miRNA targets have been validated for human *Bdnf* 3'UTR

sequences, including miR-1/206 (Lewis et al., 2003), miR-30a, miR-30a-5p and miR-195 (Mellios et al., 2009), miR-124 and let-7d (Chandrasekar and Dreyer, 2009), miR-15a (Friedman et al., 2009), miR-210 (Fasanaro et al., 2009) and allele-specific miR-26a and miR26b (Caputo et al., 2011). Although no activity-dependent mechanism for regulation of BDNF expression directly through microRNA has been found, thus far, BDNF increases miR-212 (Im et al., 2010; Remenyi et al., 2010) and miR-132 (Kawashima et al., 2010) expression, through the MAPK/ERK signaling pathway, in cultured cortical neurons, specifically up-regulating postsynaptic, but not presynaptic proteins, namely the glutamate receptor subunitsGluN2A, GluN2B, and GluA1 (Kawashima et al., 2010). In turn, miR-212 and miR-132 have recognition elements in the extended 3'UTR of brain MeCP2, down-regulating MeCP2 translation and, consequently, BDNF levels in vivo, through a CREB-induced homeostatic mechanism of feedback regulation (Klein et al., 2007). Additionally, miR-206 represses BDNF expression during myogenic differentiation, specifically in skeletal muscle cells (Miura et al., 2012), but the mechanisms involved remain incompletely elucidated.

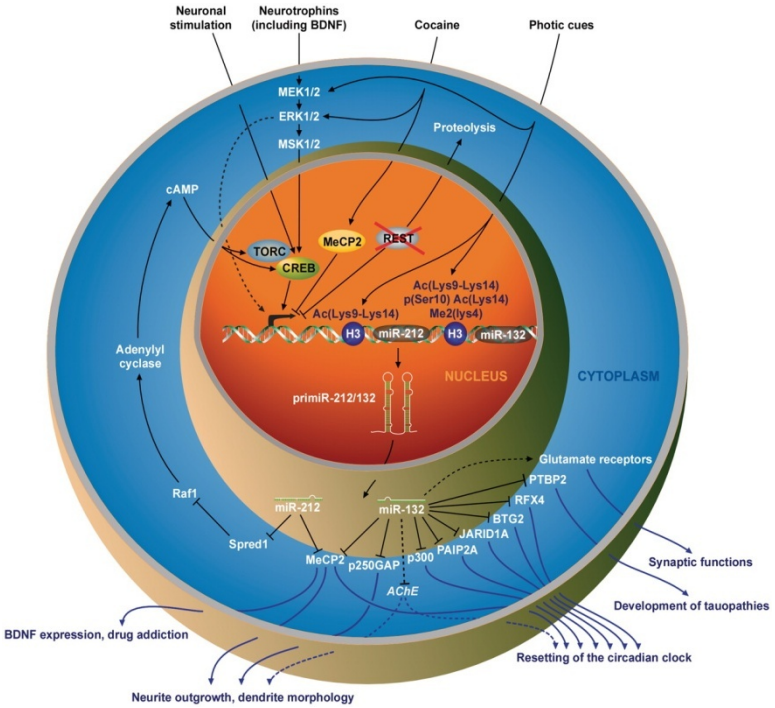


Figure 8. Inducers and targets of the miR-212/132 locus (*in* Wanet et al., 2012). In neurons, various stimuli (neurotrophins, including BDNF, photic cues or an extended access to cocaine) lead to transcription of the miR-212/132 locus through CREB activation, although an unidentified ERK1/2-dependent, MSK1/2- and CREB-independent mechanism may also contribute to miR-212/132 expression in BDNF-stimulated neurons (dashed arrow). miR-212/132 are involved in neurite outgrowth, dendrite morphology and

resetting of the circadian clock, in addition toparticipating in synaptic functions by up-regulating the expression of the glutamate receptors GluN2A, GluN2B and GluA1.

Various studies in animal models have shown that BDNF expression is also regulated by visual stimuli (Castren et al., 1992; Schoups et al., 1995; Pollock et al., 2001). Similarly to darkness (Karpova et al., 2010), monocular activity blockade causes a marked decrease in *Bdnf* mRNA and protein in the visual cortex corresponding to the deprived eye (Bozzi et al., 1995; Rossi et al, 1999; Lein and Shatz, 2000). Conversely, sensory stimulation of whiskers up-regulates BDNF in the barrel cortex (Rocamora et al., 1996; Nanda and Mack, 2000).

Aerobic exercise, namely running (Neeper et al., 1996; Oliff et al., 1998; Russo-Neustadt et al., 1999; Berchtold et al., 2001; Chen et al., 2009; Zajac et al., 2010; Gomez-Pinilla et al., 2011), sleep and circadian rhythm (Bova et al., 1998; Liang et al., 1998; Berchtold et al., 1999; Cirelli and Tononi, 2000; Fujihara et al., 2003), environmental enrichment (Thiriet et al., 2008; Zajac et al., 2010; Kuzamaki et al., 2011) and dietary restriction (Lee et al., 2000) are found among some of the factors that differentially affect BDNF gene expression under physiological conditions.

Under pathological conditions, decreased BDNF levels have been found in the hippocampus of Alzheimer's disease (AD) patients (Connor et al., 1997) and in the striatum of Huntington's disease (HD) patients (Ferrer et al., 2000). Stress (Smith et al., 1995; Ueyama et al., 1997; Taliaz et al., 2011; Yu et al., 2012), depression (Kokaia et al., 1993; Kawahara et al., 1997) and ischemia (Lindvall et al., 1992; Miyake et al., 2002) also influence BDNF expression. *Bdnf* II-IX mRNA is significantly reduced in the dorsolateral prefrontal cortex (DLPFC) of patients with schizophrenia (Wong et al., 2010).

In summary, miRNA-dependent regulation (Klein et al., 2007), spatial segregation of different BDNF transcripts (Tongiorgi et al., 2006; Chiaruttini et al., 2008; Baj et al., 2011) and local-protein translation at proximal and distal dendrites (Baj et al., 2011) modulate BDNF availability and function. This tight control of *BDNF* expression and high homology among species, from fish to mammals, with nearly identical primary sequences and conserved tissue distribution (Heinrich and Pagtakhan, 2004; Aid et al., 2007; Pruunsild et al., 2007; Tettamanti et al., 2010), imply a strong functional significance (Cohen-Cory et al., 2010).

3. BDNF post-translational modifications, transport and release

All *Bdnf* transcripts encode the same 32-kDa precursor protein, proBDNF, regardless of the promoter used and polyadenylation site (Lee et al., 2001). BDNF is expressed as a preproprotein, which consists of the mature BDNF domain, corresponding to the C-terminal portion of polypeptide precursor, a prosequence of 112 amino acids and a N-terminal signal sequence of 18 amino acid residues (Pruunsild et al., 2007). Exons I, VII and VIII contain in-frame ATG codons that could be used at alternative translation sites originating prepro-BDNF proteins with longer N-termini (Figure 9).

(A)

(B)

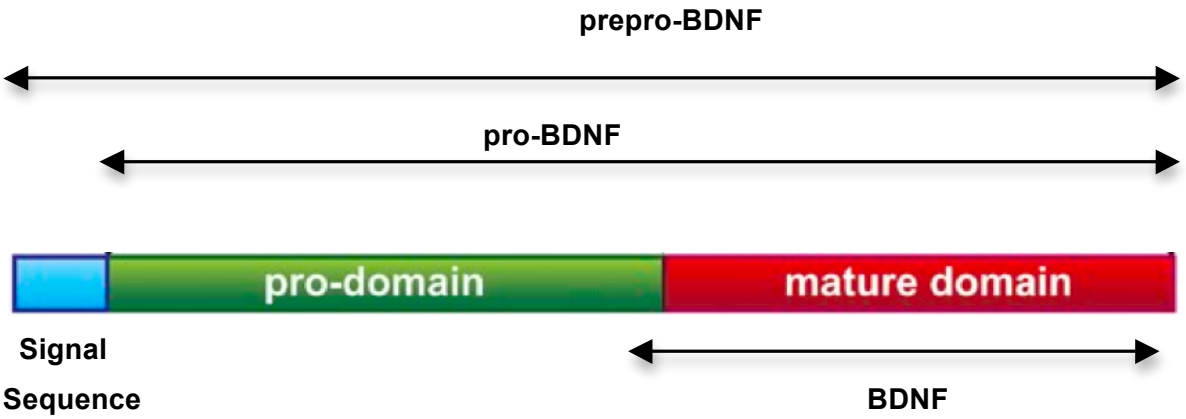


Figure 9. Amino acid sequence of potential prepro-BDNF N-termini (adapted from Pruunsild et al., 2007). (A) Sequences encoded by exon IX are in black and sequences encoded by alternative 5′ exons in blue. The transcripts encoding the respective N-termini of BDNF are listed adjacent to the N-terminal sequences. (B) Schematic representation of prepro-BDNF, consisting of N-terminal signal sequence (in blue) and pro-BDNF (formed by the pro-domain (in green) and mature domain (in red), the latter encompassing the BDNF monomer released upon endoproteolytic cleavage.

3.1-BDNF proteolytic processing and secretion

The signal sequence immediately following the initiation codon is cleaved off upon translation into the Endoplasmic Reticulum (ER), yielding the precursor form of the neurotrophin, pro-BDNF, which undergoes further posttranslational modifications through the Golgi apparatus and the trans-Golgi network (TGN). proBDNF may be cleaved intracellularly by endoproteases, including furin (Mowla et al., 2001) and proprotein convertases (PCs) (Seidah et al., 1996; Mowla et al., 1999), and secreted as the 14 kDa mature BDNF upon excision of the COOH-terminal and/or NH_2-terminal basic residues by exoproteases, carboxypeptidases and aminopeptidases, respectively (Figure 10) (Thomas and Davies, 2005; Leßmann and Brigadski, 2009). The cleaved BDNF peptide may also undergo N-acetylation of amino-terminal glycine, alanine, serine or threonine residues (GAST substrates) adjacent to aspartate, glutamate or asparagine residues (DEN group), catalyzed by N-acetyltransferase (NAT) (Bradshaw et al., 1998), or C-terminal amidation, catalyzed by peptidylglycine alpha-amidating monooxygenase (PAM) to prevent ionization of the COOH-terminus (Eipper et al., 1992; Mulcahy and Nillni, 2007), before release into the extracellular matrix. Alternatively, proBDNF may be secreted first and then cleaved by extracellular endoproteases, including the serine protease plasmin, generated from tPA cleavage of plasminogen (Lee et al., 2001; Pang et al., 2004), and matrix metalloproteinases MMP-3, MMP-7 or MMP-9 (Lee et al., 2001; Hwang et al., 2005; Mizoguchi et al., 2009), into the pro-domain and mature BDNF.

The mechanisms regulating intracellular or extracellular cleavage of pro-BDNF are not fully understood, possibly depending on the optimal pH of proteases and sequence information in the pro-domain C-terminal region (Nomoto et al., 2007), but the extracellular processing of pro-BDNF is more efficient and pro-BDNF secretion prevails over the release of mature BDNF (Goodman et al., 1996; Farhadi et al., 2000; Mowla et al., 2001; Nagappan et al., 2009).

Furthermore, both pro-BDNF and mature BDNF may be sorted and packaged either through the default constitutive secretion pathway or through the regulated pathway. Although the relative role of each pathway in the release of the neurotrophin in vivo has not been clearly established, the regulated pathway (Chen et al., 2005; Lou et al., 2005) is particularly relevant in the release of pro-BDNF in distal neuronal processes (Brigadski et al., 2005). Thus far, no single prodomain consensus sequence regulating this process of differential sorting has been found (Leßmann and Brigadski, 2009). However, several sequence motifs and protein domains contribute to cargo sorting into the regulated secretion pathway, through electrostatic interactions between the negatively charged amino acids in the pro-domain and the positively charged residues in the mature

domain (Ma et al., 2008), or molecular interactions between the pro-domain and TGN membrane-resident sorting receptors of secretory granules, including select convertases, namely subtilisin/kexin (Seidah et al., 1999), carboxypeptidase E (Lou et al., 2005; Park et al., 2008), sortilin (Chen et al., 2005) and chromogranins (Li et al., 2005).

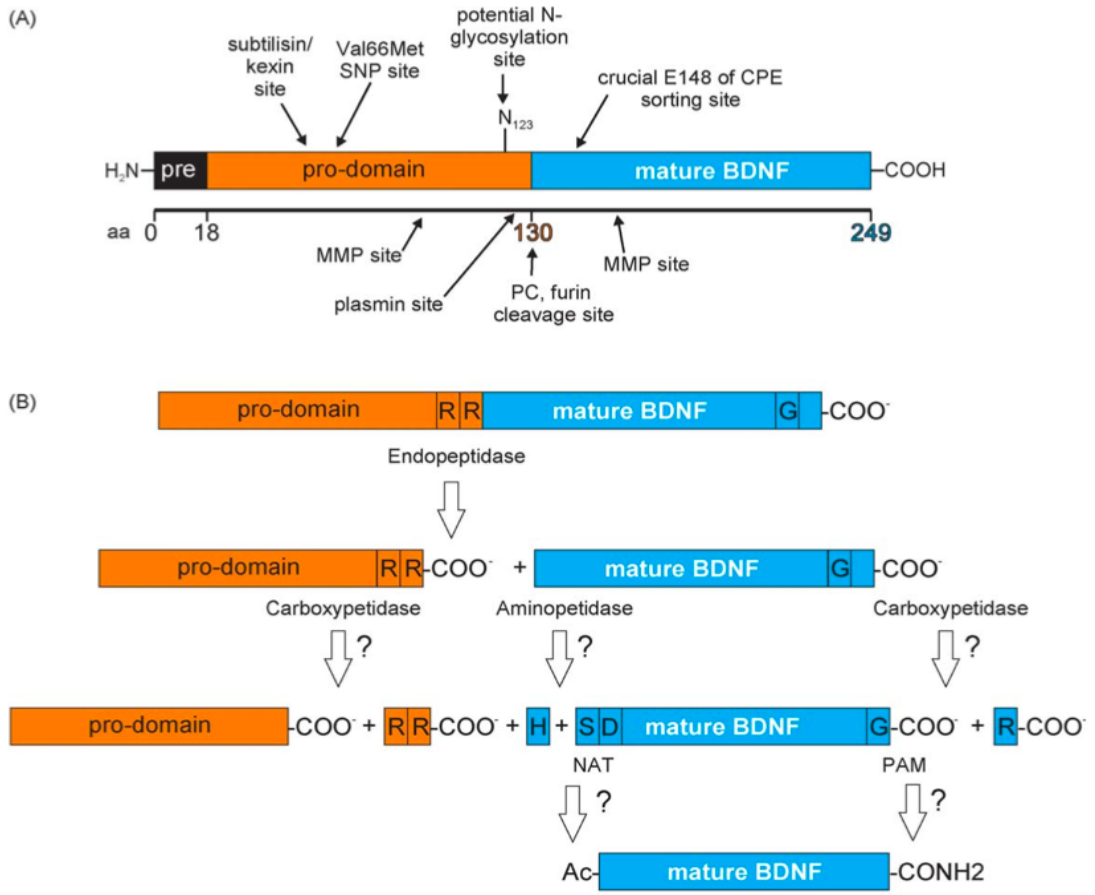


Figure 10. Schematic representation of the BDNF precursor pro-BDNF and the two cleavage products, pro-peptide and BDNF (in Leßmann and Brigadski, 2009). (A) The different domains of BDNF are drawn to scale along the length of its amino acid sequence. Different endoproteases, including protein convertases (PCs), furin, and plasmin can cleave at position 130. The position of the val66met single nucleotide polymorphism in the prodomain, the carboxypeptidase recognition site, two putative matrix-metalloproteinase (MMP) and subtilisin/kexin cleavage sites and a putative N-glycosylation site are also indicated. (B) proBDNF is first cleaved endoproteolytically by an intracellular endopeptidase (furin or PC) or by extracellular plasmin. Intracellular cleavage can be followed by the removal of carboxy- and/or amino-terminal (preferentially basic) residues by exoproteases (carboxypeptidases and aminopeptidase, respectively). The cleaved peptides can undergo further modification: a peptidylglycine alpha-amidating monooxygenase (PAM) catalyzes the C-terminal peptide amidation to prevent ionization of the COOH-terminus. The N-acetyltransferase (NAT) catalyzes the acetylation of amino-terminal.

In cultured hippocampal neurons, high-frequency neuronal activity controls the ratio of extracellular pro-BDNF/mBDNF by regulating the secretion of extracellular proteases. Low-frequency stimulation predominantly induces pro-BDNF secretion, whereas high-frequency stimulation preferentially induces the secretion of tissue plasminogen activator and increases extracellular mBDNF. Furthermore, inhibition of extracellular, but not intracellular cleavage of pro-BDNF greatly reduces high-frequency stimulation-induced extracellular mBDNF (Nagappan et al., 2009; Greenber et al., 2009).

3.2- Activity-dependent BDNF sorting and release

3.2.1- Sorting mechanisms and sub-cellular sites of BDNF release

According to the consensus model, proBDNF binding to sortilin in the Golgi enables the correct folding of the mature domain, which then binds carboxypeptidase E, thereby sorting the neurotrophin to the regulated secretory pathway (Chen et al., 2005; Lou et al., 2005). Syt-IV was recently shown to mediate activity-dependent sorting of BDNF-containing vesicles into distinct vesicle pools targeted to axons or dendrites, in hippocampal neurons (Dean et al., 2012). The neurotrophin is either transported along dendrites via secretory granules (Kohara et al., 2001; Brigadski et al., 2005) or within large dense core vesicles in axons (Fawcett et al., 1998; Adachi et al., 2005). Ultimately, synaptotagmin-IV mediates neurotrophin release (Dean et al., 2009) through different activity-dependent exocytic mechanisms in axons and dendrites (Matsuda et al., 2009).

Upon calcium influx due to spiked synaptic activity, CAPS2 promotes synaptic and extrasynaptic secretion specifically in axons (Sadakata et al., 2004; Sadakata et al., 2007; Shinoda et al., 2011), while BDNF is secreted by dendrites through “full-collapse” vesicle fusion and complete content extrusion with only a brief increase in activity, which merely generates “kiss-and-run” exocytosis in axons (Matsuda et al., 2009).

In addition to calcium influx-dependent release from presynaptic (Balkowiec and Katz, 2002) and postsynaptic (Hartmann et al., 2001) sites through VGCCs, a third, calcium influx-independent mechanism regulates BDNF release upon calcium release from intracellular stores (Griesbeck et al., 1999).

The methods used to measure BDNF secretion have relied mainly on recombinant overexpression of BDNF tagged with Green Fluorescent Protein (GFP) (Kojima et al., 2001; Matsuda et al., 2009) or indirect assays measuring endogenous BDNF release to the extracellular medium through scavengers (Gubellini et al., 2005; Walz et al., 2006;

Magby et al., 2006; Mohajerani et al., 2007; Crozier et al., 2008). Consequently, neither spatiotemporal data on the secretion of endogenous BDNF, nor direct evidences of site-specific endogenous BDNF release have been found thus far (Cohen-Corey et al., 2010).

Most studies indicate a predominantly postsynaptic release of endogenous BDNF and a retrograde mode of action (Kuczewski et al., 2009; Lessmann and Brigadski, 2009). In cultured hippocampal neurons, postsynaptic depolarization elicits calcium-dependent release of BDNF that diffuses retrogradely and enhances presynaptic transmitter release (Magby et al., 2006 Crozier et al., 2008).

Similarly, postsynaptic BDNF release and retrograde action can be spontaneously induced by giant depolarization potentials during a period of the postnatal development (Mohajerani et al., 2007; Kuczewski et al., 2008b). Concurrently, homeostatic modulation of presynaptic function, in response to synaptic inactivity caused by 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA) receptor blockade, requires postsynaptic BDNF release as a retrograde messenger, locally synthesized in dendrites (Lindskog et al., 2010; Jakawich et al., 2010). However, recent in vivo findings (Dieni et al., 2012) have challenged the activity-dependent dendritic synthesis and release of BDNF in adult neurons, supporting previous evidence in favor of an anterograde mode of action in striatal (Altar et al., 1997) and hippocampal (Zakharenko et al., 2003) neurons. Thus, BDNF released form axon terminals partly accounts for spontaneous, rapid calcium synaptic transients on CA3 dendrites (Lang et al., 2007). In addition, the θ-burst-induced release of endogenous BDNF from hippocampal mossy fibers suffices to trigger intracellular changes in postsynaptic CA3 neurons (Li et al., 2010) and presynaptic pools of BDNF support the formation of a postsynaptic form of LTP in the dorsal striatum (Jia et al., 2010).

Ultrastructural resolution of endogenous BDNF subcellular location unequivocally shows no localization of this neurotrophin in hippocampal dendritic spines, in contrast with a strong labeling associated with secretory vesicles, in presynaptic terminals (Figure 11) (Dieni et al., 2012).

Several other studies have concluded that BDNF is stored and released in both axons and dendrites, albeit strictly in cultured hippocampal neurons (Haubensak et al., 1998; Hartmann et al., 2001; Wu et al., 2004; Adachi et al., 2005; Brigadski et al., 2005; An et al., 2008; Dean et al., 2009; Matsuda et al., 2009).

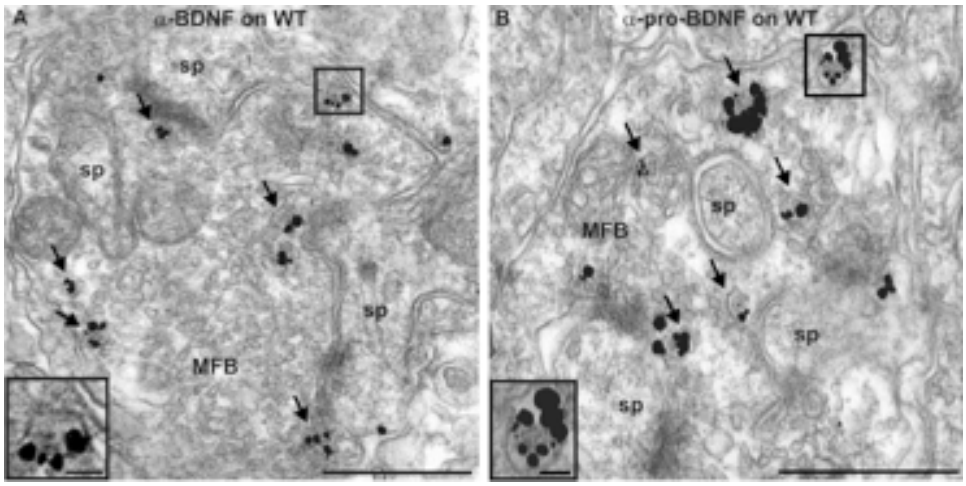


Figure 11. Ultrastructural localization of BDNF and pro-BDNF in mossy fiber boutons (MFB) (adapted from Dieni et al., 2012). (A) Electron micrograph of an ultrathin section of wild-type (WT) stratum lucidum (SL) prelabeled with anti-BDNF immunogold. Gold clusters, indicated with arrows, are associated with large secretory vesicles within the bouton (sp, dendritic spine). (B) Anti-pro-BDNF-labeled WT MFB containing cluster-labeled secretory vesicles are indicated with arrows. The inset image shows a large labeled dense-core vesicle (DCV).

Whether delivered by axonal anterograde transport, in dense core vesicles, to presynaptic terminals (Fawcett et al., 1998; Kohara et al., 2001; Adachi et al., 2005; Ng et al., 2007), or postsynaptic terminals, via secretory granules (Goodman et al., 1996, Haubensak et al., 1998; Hartmann et al., 2001; Kohara et al., 2001; Adachi et al., 2005; Brigadski et al., 2005), the differential secretion and release of pro-BDNF and BDNF are essential for numerous neuronal functions, contributing for the specificity of their effects.

3.2.2- Val66Met BDNF variant

A single-nucleotide polymorphism in the human *Bdnf* gene, expressed by 40% of humans, of whom 5% are homozygous, results in a valine to methionine substitution (Val66Met) in the pro-domain, leading to reduced trafficking and activity-dependent regulated secretion of BDNF, abnormal hippocampal-specific short-term plasticity and impaired episodic and working memory (Egan et al., 2003; Hariri et al., 2003; Dempster et al., 2005; Tan et al., 2005; Ho et al., 2006). The Met allele also increases susceptibility to neurodegenerative pathologies, namely Alzheimer's disease (Kunugi et al., 2001; Riemenschneider et al., 2002; Ventriglia et al., 2002), levodopa-induced dyskenesias in

Parkinson's disease (Foltynie et al., 2009) and psychiatric conditions, including depression (Kaufman et al., 2006; Kim et al., 2007), anxiety (Sen et al., 2003; Jiang et al., 2005; Lang et al., 2005), aberrant eating behavior (Rosas-Vargas et al., 2011; Skledar et al., 2012; Monteleone and Maj, 2013) and bipolar disorder (Neves-Pereira et al., 2002; Sklar et al., 2002; Chen et al., 2004).

Even though the Met allele shows a functional advantage regarding cognitive control, when response inhibition is required (Beste et al., 2010), a common clinical symptom of these disorders may be the variable impairment of cognitive functions (Gratacòs et al., 2007; Frustaci et al., 2008). Furthermore, the Met allele has been linked to lower levels of hippocampal N-acetylaspartate (Egan et al., 2003) and significant alterations in brain anatomy, including decreased gray matter volume throughout the prefrontal cortex and middle temporal lobes, in addition to limbic structures such as the amygdala (Pezawas et al., 2004; Ho et al., 2006, 2007; Montag et al., 2009).

The knock-in *Bdnf* (*Bdnf*$^{Met/Met}$) mouse, which expresses the BDNFMet variant under the regulation of endogenous BDNF promoters, shows an approximately 30% decrease in activity-dependent secretion of endogenous BDNF and hippocampal volume, impairment in hippocampal contextual but not cue-dependent fear conditioning, and increased anxiety-related behaviors, fully mimicking the phenotypic hallmarks in humans with this single-nucleotide polymorphism (SNP) of *Bdnf* (Chen et al 2006; Chen et al., 2008) and, thus, validating a vertically integrated approach to studying human genetic variants (Dincheva et al., 2012).

3.3- Physiological roles of BDNF processing andrelease

Endogenous BDNF release triggers postsynaptic calcium currents, which can be spontaneous, fast and frequent upon activation of voltage-gated sodium and calcium channels (Lang et al., 2007), or slow and sustained when mediated by transient receptor potential canonical subfamily 3 (TRPC3) channels (Amaral and Pozzo-Miller, 2007a,b), along dendrites of CA1 hippocampal neurons. These calcium signals may account for the BDNF-dependent unsilencing of presynaptically non-functional synapses by actin cytoskeleton remodelling (Shen et al., 2006), and the "unmasking" of postsynaptically silent synapses in developing neurons, through insertion of AMPA receptors into NMDA receptor-only membranes, thereby increasing responsiveness to glutamate (Itami et al., 2003; Caldeira et al., 2007b; Nakata and Nakamura, 2007). Endogenously released BDNF was also shown to mediate increases in dendritic spine volume (Tanaka et al., 2008), inducing growth of PSD95 positive postsynaptic specializations in glutamatergic

synapses (Yoshii and Constantine-Paton, 2007), and presynaptic bouton enlargement (Li et al., 2011).

Selective presynaptic knockdown of syt-IV increases spontaneous quantal release, whilst postsynaptic loss of syt-IV increases quantal amplitude, revealing a mechanism whereby syt-IV-mediated regulation of BDNF secretion modulates synaptic strength in a useful range during LTP (Dean et al., 2009). Accordingly, extracellular cleavage of proBDNF into the mature form, the non-covalent, stable homodimers, by serine protease plasmin, is critical for protein synthesis-dependent late-phase long-term potentiation (L-LTP) in hippocampal neurons (Pang et al., 2004) and pro-survival effects of BDNF (Lee et al., 2001). In contrast, constitutively secreted proBDNF facilitates long-term depression (LTD) (Woo et al., 2005) and pro-apoptotic effects (Teng et al., 2005) by high-affinity binding to $p75^{NTR}$ (Fayard et al., 2005).

Mature BDNF may also bind to $p75^{NTR}$, albeit with low affinity (Rodriguez-Tébar et al., 1990), or truncated TrkB receptor isoforms lacking the tyrosine kinase domain required for downstream signaling (Klein et al., 1990). The latter receptors negatively modulate BDNF signaling through heterodimerization with full-lenght TrkB receptors and clearance of BDNF from the extracellular space upon receptor internalization (Haapasalo et al., 2002), although this possibility remains highly controversial, as previously discussed in section 1.4.2.4.

Nevertheless, extracellular BDNF preferentially binds to full-length tropomyosin-related kinase B (TrkB) receptors, setting off the trans-autophosphorylation of tyrosine residues in the intracellular domain, Y490 and Y816, which recruit SH2 proteins activating, in parallel, the Ras-ERK, PI3K/AKT and PLCγ signaling pathways (Huang & Reichardt, 2003; Reichardt, 2006).

In summary, numerous factors contribute to the extremely complex regulation of BDNF expression, secretion and release through the constitutive or activity-dependent regulated pathways. In addition, the intracellular or extracellular proteolytic processing of pro-BDNF, the highly regulated release of BDNF in response to different stimuli, and both pro-BDNF and BDNF differential affinity for and activation of TrkB receptors and $p75^{NTR}$, enable the panoply of effects triggered by this neurotrophin, mediated by the numerous signaling pathways it activates upon receptor binding (Figure 12).

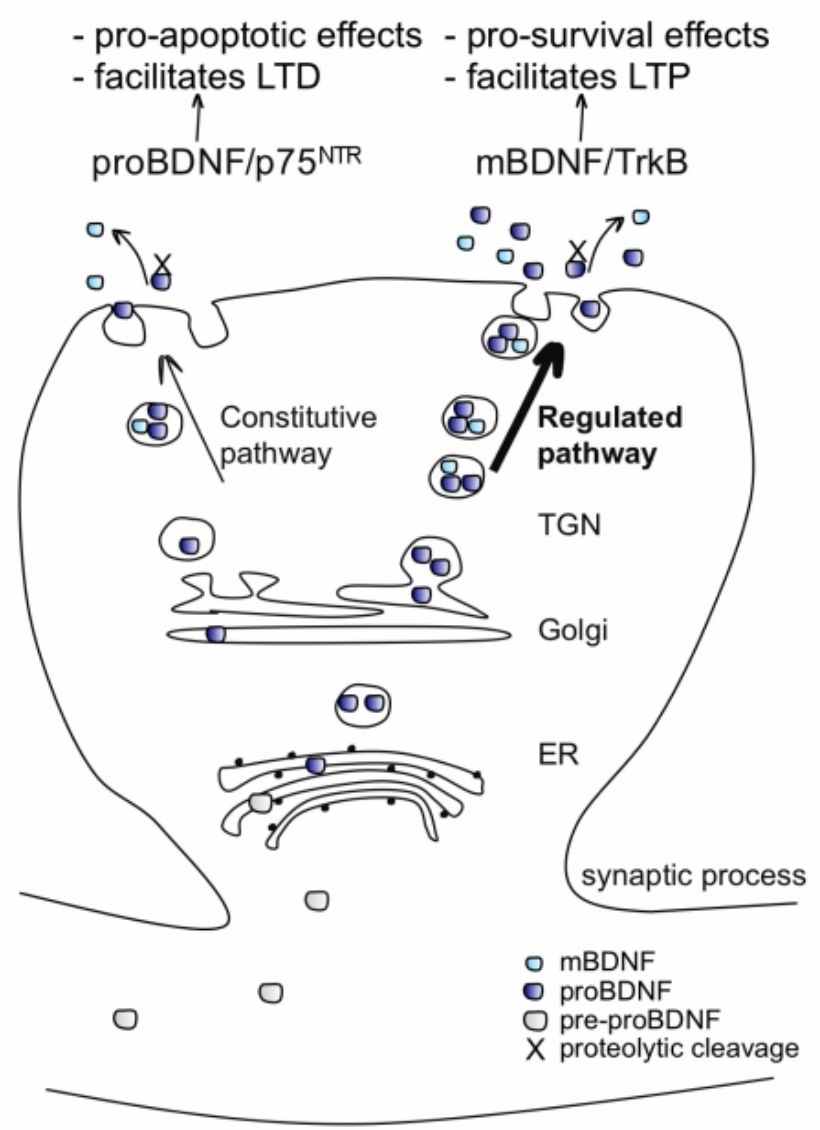


Figure 12. BDNF processing, packaging and secretion in neurons (*in* Cunha et al., 2010). BDNF is synthesized as a pre-proBDNF protein, which has its pre-sequence cleaved off in the endoplasmic reticulum (ER) and the resulting 32-kDa proBDNF moves, via the Golgi apparatus, into the trans-Golgi network (TGN) where it is sorted into the constitutive and activity-dependent regulated secretory pathways. ProBDNF is either proteolytically cleaved and secreted as 14-kDa mature BDNF (mBDNF) or secreted as proBDNF and cleaved by extracellular proteases. Secretion of the proBDNF predominates and both proBDNF and mBDNF are preferentially packaged into vesicles of the regulated secretory pathway. Once released, proBDNF binds preferentially to pan-neurotrophin receptor $p75^{NTR}$while mBDNF binds preferentially to bothpre-and post-synaptic TrkB receptors, triggering different intracellular secondary messenger cascades and affecting distinct cellular responses.

4. BDNF: mechanism of action

The mature form of BDNF is a 27.0 kDa homodimer of two polypeptides with 119 amino acid residues, each binding to two distinct classes of receptors, one with low affinity, the $p75^{NTR}$ (Rodriguez-Tébar et al., 1990) and one with high affinity, the TrkB receptor (NTRK2) (Soppet et al., 1991; Squinto et al., 1991; Klein et al., 1991; Haniu et al., 1997; Naylor et al., 2002). proBDNF also interacts with sortilin (SORT1) controlling the sorting of BDNF at the Golgi apparatus to the regulated secretory pathway (Chen et al., 2005). The pro-BDNF-sortilin interaction is also involved in the formation of a trimeric complex withthe $p75^{NTR}$, which promotes apoptosis (Teng et al., 2005; Skeldal et al., 2012). Additional interactions with other receptors, which would determine the mechanism of action of this neurotrophin, have not been identified thus far (Figure 13).

.

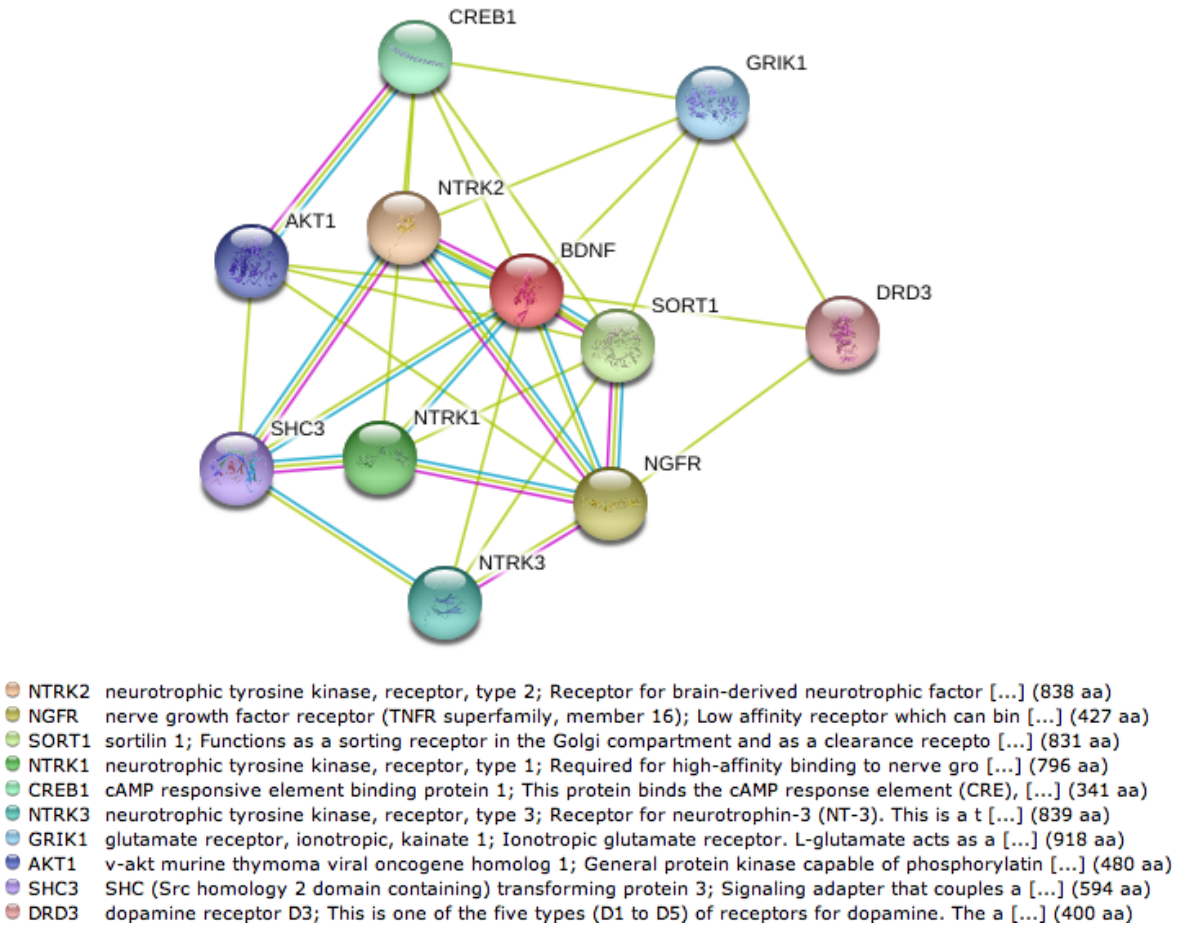


Figure 13. Network display based on evidence of BDNF interactions. Nodes are either colored (if directly linked to BDNF as in the table below) or white (nodes of a higher iteration or depth). Edges represent predicted functional links and consist of up to eight lines, one color for each type of evidence. Source: STRING 9.0 software.

BDNF binding to TrkB receptors activates, in parallel, the Ras-ERK, PI3K/AKT and PLCγ signaling pathways (Figure 14) (Huang & Reichardt, 2003).

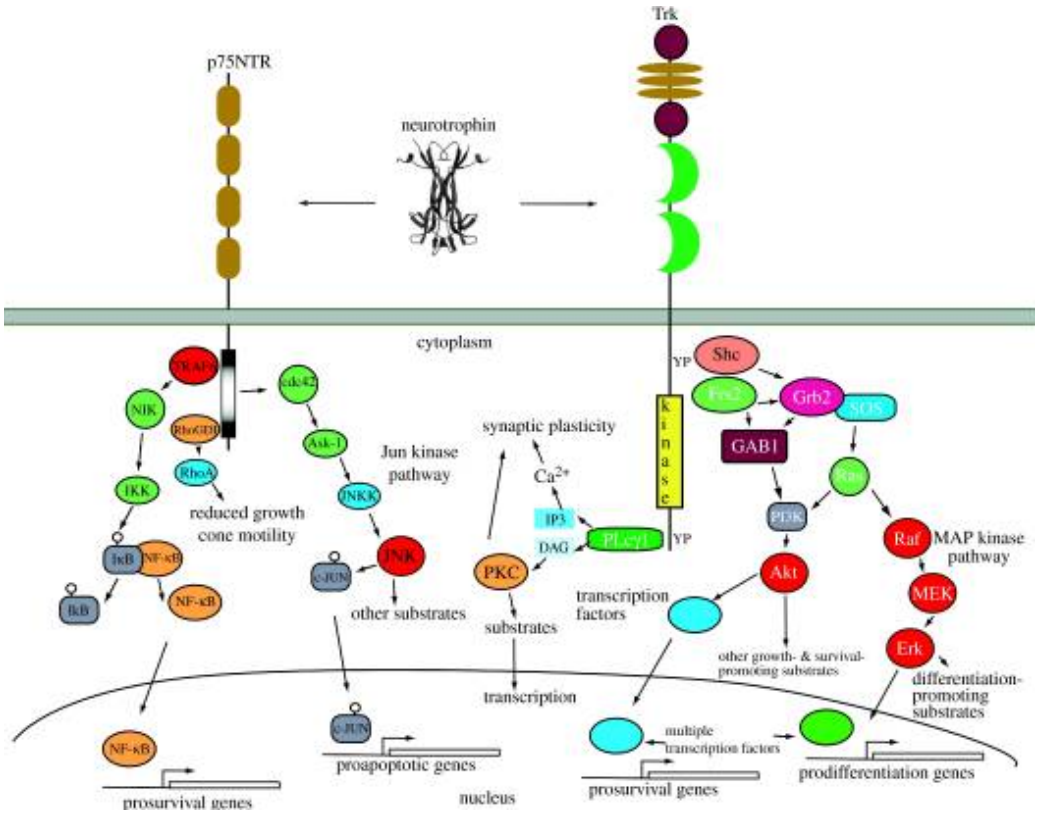


Figure 14.BDNF signaling (*in* Reichardt, 2006). Interactions of the neurotrophin BDNF with $p75^{NTR}$and Trk receptors and the main intracellular signaling pathways activated by each receptor. $p75^{NTR}$ regulates NF-κB activation, which results in transcription of multiple genes, including several that promote neuronal survival. Activation of the Jun kinase pathway similarly induces the expression of several genes, some of which promote neuronal apoptosis. $p75^{NTR}$ also regulates the activity of Rho, which controls growth cone motility. Each Trk receptor also controls three main signaling pathways. Activation of Ras results in activation of the MAP kinase-signaling cascade, which promotes neuronal differentiation including neurite outgrowth. Activation of PI3K through Ras or Gab1 promotes survival and growth of neurons. Activation of PLC-γ1 results in activation of Ca^{2+}- and protein kinase C-regulated pathways that promote synaptic plasticity. Each of these signaling pathways also regulates gene transcription. Several other adaptors for $p75^{NTR}$ and Trk receptors have been omitted for simplicity.

Upon binding to TrkB receptor, BDNF sets off the trans-autophosphorylation of tyrosine residues Y490 and Y816, in the intracellular domain. Trans-autophosphorylation of Y816 recruits and activates cytoplasmic PLCγ, which hydrolyzes PIP2 into IP3 and DAG. IP3 promotes Ca^{2+} release from internal stores activating $[Ca^{2+}]_i$-regulated enzymes, including CAMKs and PKC isoforms (Ouyang et al., 1997). Concomitantly, DAG stimulates DAG-regulated PKC isoforms, for example, PKCδ (Huang and Reichardt, 2003). The PLCγ pathway is central in LTP (Minichiello et al., 2002; Gärtner et al., 2006; Gruart et al., 2007), neurotrophin-mediated neurotrophin release (Canossa et al., 1997) and growth cone guidance (Li et al., 2005), retrograde synaptic modification (Du and Poo, 2004) and dendritic spine morphology (Amaral and Pozzo-Miller, 2007) regulated by activation of TRPC channels.

Trans-autophosphorylation of Y490 enables recruitment of Shc, IRS1 and IRS2 linker proteins activating the Ras-ERK and PI3K/Akt cascades. Following docking to TrkB receptor, Shc binds to adaptor protein Grb2 and guanine nucleotide exchange factor SOS, initiating the GTP loading of Ras followed by sequential activation of Raf, MEK and ERK (Reichardt et al., 2006). ERK translocates to the nucleus upon phosphorylation, regulating gene expression through isoform-specific activation of transcription factors, including CREB (ERK1/2/5), MEF2 (ERK5) or Elk1 (ERK1/2) (Grewal et al., 1999). The Ras-ERK signaling pathway is crucial for neurogenesis (Barnabé-Heider and Miller, 2003), inhibition of pro-apoptotic proteins (Datta et al., 1997), stimulation of pro-survival gene expression (Bonni et al., 1999) and protein synthesis-dependent plasticity (Kelleher et al., 2004).

The PI3K/Akt cascade can be triggered by Grb2 recruitment of intermediary binding protein Gab1 (Holgado-Madruga et al., 1997) or direct interaction between PI3K and Ras or IRS1/IRS2 (Yamada et al., 1997). The PI3K/Akt pathway has a pivotal role in cell survival (Brunet et al., 2001), neuroprotection (Almeida et al., 2005), trafficking of synaptic proteins (Yoshii and Constantine-Patton, 2007) and can also directly control protein synthesis through mTOR activation and 4EBP phosphorylation (Takei et al., 2004).

Nonetheless, there is significant interplay between the signaling pathways activated by TrkB receptors. DAG regulates PKCδ-dependent mitogenic activation of MAPK/ERK (Corbit et al., 1999) and is implicated in MAPK/ERK-mediated neurite outgrowth (Williams et al., 1994; Dimitropoulou and Bixby, 2000). Furthermore, neuroprotection by BDNF against apoptotic cell death involves crosstalk between the ERK and PI3K pathways (Almeida et al., 2005).

4.1- PLCγ signaling pathway and the modulation of TRPC channels

BDNF induces the activation of docked PLC-γ1 through TrkB-mediated phosphorylation, hydrolyzing PtdIns(4,5)P2 into IP3 and diacylglycerol (DAG) (Figure 15). IP3 triggers the release of Ca^{2+} from endoplasmic reticulum stores through IP3 receptors (IP3Rs) (Berridge, 1998), which causes Ca^{2+} elevations in embryonic cultures of cerebrocortical (Zirrgiebel et al., 1995; Behar et al., 1997; Matsumoto et al., 2001) and hippocampal (Berninger et al., 1993; Marsh and Palfrey, 1996; Canossa et al., 1997; Finkbeiner et al., 1997; Li et al., 1998) neurons. This pathway converges on the same IP3R-containing Ca^{2+} stores targeted by other metabotropic receptors in hippocampal pyramidal neurons, including group-I metabotropic glutamate receptors (mGluR) (Conn and Pin, 1997). Concomitantly, DAG stimulates DAG-regulated protein kinases, including almost all PKC isoforms (Reichardt, 2006). The activation of PKC-δ may also trigger MEK1 and Erk1/2 activation, promoting crosstalk between the signaling pathways (Corbit et al., 1999).

As a result of PLCγ signaling, several Ca^{2+}-calmodulin-dependent protein kinases and other Ca^{2+}-calmodulin-regulated targets are activated, which regulate the expression and activity of several proteins, including ion channels and transcription factors critical for BDNF-induced mechanisms of synaptic plasticity (Toledo-Aral et al., 1995; Minichiello et al., 2002; Klein et al., 2005). BDNF-triggered Ca^{2+} transients induce the translation and synaptic incorporation of the AMPA receptor subunit GluA1 upon IP3 receptor-mediated and TRPC-dependent Ca^{2+} release from cytoplasmic intracellular stores in cultured cerebrocortical (Nakata and Nakamura, 2007) and hippocampal (Fortin et al., 2012) neurons. The rise in the intracellular calcium concentration also up-regulates Ca^{2+}-sensitive adenyl cyclase (AC) activity, which is required for CREB-dependent gene transcription (Nguyen et al., 1994; Shaywitz and Greenberg, 1999) and the synthesis (Ji et al., 2005), transport (Yoshii and Constantine-Paton, 2007) and synaptic delivery of PSD-95-TrkB complexes, upon PKMζ-dependent and ZDHHC8-mediated palmitoylation of PSD-95 (Yoshii et al., 2011). Mutant mice with a deficiency in the coupling of TrkB receptors to PLCγ show impaired hippocampal LTP and associative learning (Minichiello et al., 2002; Gartner et al., 2006; Gruart et al., 2007).

Focal and brief applications of BDNF to dendritic spines of dentate granule cells induce Ca^{2+} transients (rapid and short-lived Ca^{2+} elevations) and fast membrane depolarization, mediated by TTX-insensitive Nav1.9 channels (Kafitz et al., 1999; Blum et al., 2002) and sensitive to VGCC blockers (Kovalchuk et al., 2002). Therefore, such BDNF-induced Ca^{2+} signals translate a voltage-gated Ca^{2+} influx, secondary to membrane depolarization and independent of PLCγ/IP3R-mediated Ca^{2+} mobilization from

intracellular stores (Amaral et al., 2007). However, in the absence of extracellular Ca^{2+}, BDNF-induced Ca^{2+}transients are significantly reduced, though not completely blocked, suggesting that both Ca^{2+} influx and its mobilization from intracellular stores contribute to the effects of BDNF (Canossa et al., 1997; Finkbeiner et al., 1997; Marsh and Palfrey, 1996; Li et al., 1998) and, consequently, the involvement of non-selective cationic currents mediated by plasma membrane TRPC channels (Montell et al., 2002; Clapham, 2003). Accordingly, TrkB receptor signaling through PLC-γ1-mediated release of intracellular Ca^{2+} and DAG also activates several TRPC channels, including TRPC3, which co-distribute and associate with TrkB receptors (Li et al., 1999; Li et al., 2005) and TRPC6 (Li et al., 2005).

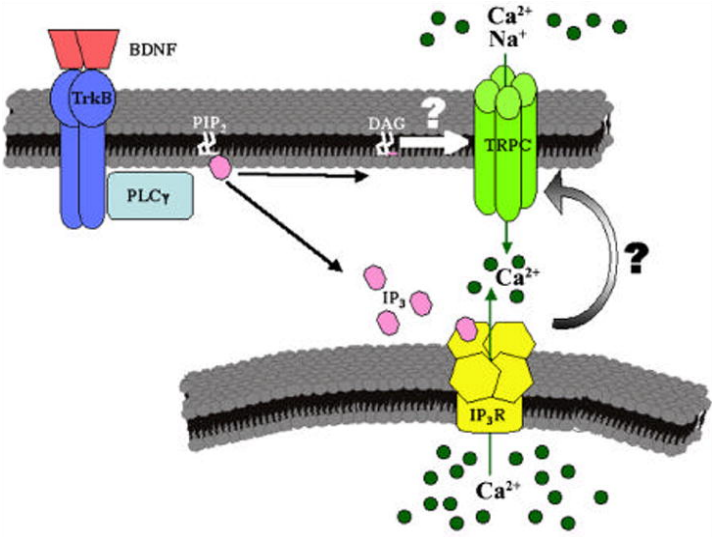


Figure 15. TrkB-induced stimulation of PLCγ causes PIP2 hydrolysis and formation of DAG and IP3 (*in* Amaral et al., 2007). Activation of IP3Rs leads to Ca^{2+} mobilization from intracellular stores. TRPC channels, most likely heteromultimeric, may be gated by different mechanisms: a diffusible factor, by Ca^{2+} ions released from IP3-sensitive Ca^{2+} stores, a physical interaction with activated IP3Rs or by DAG itself. TRPC channels are known to mediate a non-selective cationic current that requires intact IP3R signaling, full intracellular Ca^{2+} stores and extracellular Ca^{2+} ions.

TRP channels are a group of structurally related and membrane-localized ion channels, originally described in *Drosophila* photoreceptors (Montell et al., 1985). The mammalian TRP homologues are classified into TRPC (canonical, or short form), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), and TRPML (mucolipin) subfamilies (Clapham, 2003). Mammalian TRP channels are involved in capacitative Ca^{2+} entry (Birnbaumer et al., 1996) and sensory transduction, including temperature, touch, pain, osmolarity, pheromone and taste, in addition to modulation of the cell cycle (Clapham, 2003; Montell et al., 2002).

All mammalian TRPC proteins (TRPC1 through TRPC7) are widely expressed in the brain (Mizuno et al., 1999), including the hippocampus (Philipp et al., 1998; Li et al., 1999; Strubing et al., 2001), and function as receptor-operated channels (Ma et al., 2000). TRPC channels can be activated in neurons by stimulation of Gq/G11-type G protein-coupled receptors and tyrosine kinase receptors. IP3Rs (Kiselyov et al., 1998), DAG (Hofmann et al., 1999) and a soluble Ca^{2+}influx factor produced in response to Ca^{2+}store depletion (Clapham et al., 2001) have all been implicated in TRPC channel gating, as a result of the distinct activation properties between functional homomeric and heteromeric channels, but the different models of activation and modulation of native TRPC channels remain highly controversial (Clapham, 1996; Birnbaumer et al., 1996; Boulay et al., 1997; Zhu et al., 1998; Clapham, 2003; Montell et al., 2002; Putney, 2004; Putney, 2007; Shen et al., 2011).

Independently of the gating mechanism, TRPC channels are implicated in BDNF-induced Ca^{2+} transients at growth cones and synapses (Li et al., 2005; Amaral and Pozzo-Miller, 2007). I_{BDNF}, a slow and sustained nonselective cationic current mediated by TRPC channels (Amaral and Pozzo-Miller 2007; Li et al. 1999), is consistently associated with slow and sustained Ca^{2+} elevations in voltage-clamped CA1 pyramidal neurons, in the presence of the Na^{+} channel blocker tetrodotoxin. The rapid increase of quantal vesicular transmitter release induced by BDNF in CA1 pyramidal neurons depends on such slow and sustained nonselective cationic current mediated by TRPC channels or I_{BDNF} (Amaral and Pozzo-Miller, 2012). In addition, TRPC channels are also required for BDNF-induced changes in dendritic spine density because both siRNA-mediated TRPC3 knockdown and TRPC inhibitors effectively prevent the increase in spine density induced by BDNF (Amaral and Pozzo-Miller, 2007b). Similarly, local application of BDNF to intact *Xenopus laevis* optic tectum induces rapid retrograde synaptic modification by persistent potentiation of retinal ganglion cell synapses, dependent on TrkB receptor activation, phospholipase Cγ activity and TRPC-mediated intracellular Ca^{2+} elevation (Du and Poo, 2004).

Consistent with the model of TRPC activation by BDNF-induced TrkB signaling, TRPC3/6 channels mediate BDNF-evoked Ca^{2+} signals in growth cones of cultured cerebellar granule cells (Li et al., 2005). Furthermore, the xTRPC1 channel, a *Xenopus* homolog of TRPC1, plays a similar role in BDNF-induced growth cone turning *in vitro* (Wang and Poo, 2005) and similar non-selective cationic currents have been previously described in cortical and hippocampal neurons (Alzheimer, 1994; Haj-Dahmane and Andrade, 1996; Congar et al., 1997).

TRPC channels are additionally involved in BDNF-mediated neuroprotection. In particular, TRPC3 and 6 protect cerebellar granule neurons from serum deprivation-

induced cell death (Jia et al., 2007). Suppression of NMDA receptor-dependent calpain degradation of the TRPC6 N-terminal domain, with a fusion peptide derived from the calpain cleavage site in TRPC6, prevents ischemic neuronal brain damage and preserves neuronal survival, in a rat model of stroke, improving behavioral performance through the CREB signaling pathway (Du et al., 2010). Furthermore, the TRPC6 agonist one-oleoy1–1 acetyl-sn glycerol (OAG) significantly increases retinal ganglion cell survival in a rat model of retinal ischemia/reperfusion-induced cell death, through a mechanism mediated by BDNF (Wang et al., 2010).

In summary, focal application of BDNF triggers fast calcium transients at postsynaptic sites in developing hippocampal neurons (Lang et al., 2007) and the PLCγ pathway is central in LTP (Minichiello et al., 2002; Gartner et al., 2006; Gruart et al., 2007), neurotrophin-mediated neurotrophin release (Canossa et al., 1997) and growth cone guidance (Li et al., 2005), retrograde synaptic modification (Du and Poo, 2004) and dendritic spine morphology (Amaral and Pozzo-Miller, 2007a) regulated by activation of TRPC channels.

4.2- MAPK/ERK signaling pathway, neuronal differentiation and plasticity

The MAPK/ERK signaling pathway is activated downstream of TrkB upon Shc recruitment and phosphorylation, which promotes Shc interaction with the adaptor protein Grb2. Grb2 recruits and activates the GEF SOS, which promotes the removal of GDP from Ras. This enables Ras activation by GTP binding, leading to the sequential phosphorylation of Raf, Mek1 and/or Mek2, which in turn phosphorylate and activate Erk1 and Erk2 (English et al., 1999; Reichard, 2006). MAPK/ERK signaling regulates CREB-dependent transcription as phosphorylated Erk translocates to the nucleus, directly phosphorylating and activating the transcription factor CREB (Shaywitz and Greenberg, 1999). The MAPK/ERK signaling pathway also regulates protein synthesis-dependent plasticity by increasing the phosphorylation of eukaryotic initiation factor 4E (eIF4E), 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (Kelleher et al., 2004; Klann and Dever, 2004).

In hippocampal neurons, targeted inhibition of ERK signaling significantly impairs the induction of LTP, implicating this signaling pathway in hippocampal-dependent behavior, while mice lacking the neuronal-specific Ras guanine-releasing factor (Ras-GR) have severely impaired LTP in the amygdala and a corresponding deficit in long-term memory for aversive events (Orban et al., 1999; Sweatt, 2004).

ERK1 knockdown results in a stimulus-dependent increase of ERK2 signaling, likely due to its enhanced interaction with the upstream kinase MEK, which enhances

synaptic plasticity in striatal neurons and facilitates long-term adaptive changes underlying learning, memory and drug addiction (Mazzucchelli et al., 2002). ERK2 knockdown causes a reduction in cerebrocortical thickness due to impaired proliferation of neural progenitors during the neurogenic period and the generation of fewer neurons (Samuels et al., 2008), as previously found by inhibiting MAPK signaling (Vaccarino et al., 1999; Ménard et al., 2002; Barnabe-Heider and Miller, 2003; Dono, 2003; Ohkubo et al., 2004; Zheng et al., 2004; Paquin et al., 2005; Thomson et al., 2007). ERK2 also regulates the timing of oligodendrocyte differenciation (Fyffe-Maricich et al., 2011), while MEK1 inhibition causes the retention of neuronal precursor cells in the subventricular zone (SVZ)/VZ in an undifferentiated state, blocking neurogenesis (Ménard et al., 2002; Barnabe-Heider and Miller, 2003; Paquin et al., 2005). Therefore, MAPK/ERK signal transduction directs neurogenesis and concomitantly suppresses gliogenesis (Miller and Gauthier, 2007).

Although the various signaling pathways activated by TrkB receptors are often analyzed separately, there is a significant interplay between them. PLCγ-dependent production of DAG regulates PKCδ-dependent mitogenic activation of MAPK/ERK, upon BDNF binding to TrkB receptors (Corbit et al., 1999), contributing for MAPK/ERK-mediated neurite outgrowth (Williams et al., 1994; Dimitropoulou et al., 2000). Neuroprotection mediated by BDNF against apoptotic cell death involves crosstalk between the ERK and PI3K pathways in cultured hippocampal neurons (Almeida et al., 2005), and both ERK (Thomas and Huganir, 2004) and PI3K (Lin et al., 2001) pathways have been implicated in LTP, in addition to PLCγ. PI3K-activated Akt regulates downstream effector Cdc42, involved in growth cone guidance (Menna et al., 2009), synaptic vesicle docking (Shen et al., 2006) and cytoskeletal dynamics (Chen et al., 2012; Rosário et al., 2012), which respond to PLCγ-modulated secondary messengers $[Ca^{2+}]_i$ and cAMP (Mai et al., 2009).

4.3- PI3K/Akt signaling pathway, apoptosis regulation and neuroprotection

The PI3K/Akt signaling pathway can be activated downstream of TrkB either by the Shc/Grb2/SOS complex or by IRS1/2. Recruitment of Gab1 by phosphorylated Grb2 enables the subsequent binding and activation of PI3Kinase (Holgado-Madruga et al. 1997). In some neurons, Trk receptor activation results in phosphorylation of IRS1, which also enables the recruitment and activation of PI3Kinase (Yamada et al. 1997).

Activated PI3K generates P_3-phosphorylated phosphoinosides, which, in combination with otherphosphoinositide kinases, change the plasma membrane composition, in the cytoplasmic side, enabling the membrane translocation and activation

of protein kinase Akt, with multiple effects on neuronal survival and development (Reichardt, 2006). Activated Akt controls, through phosphorylation, the activation of key regulators of thecaspase cascade, including BAD, a Bcl2-family member. BAD promotes apoptosis upon binding to Bcl-xL, thereby preventing Bcl-xL from inhibiting the pro-apoptotic activity of Bax. 14-3-3 proteins sequester phosphorylated BAD in the cytoplasm, preventing its pro-apoptotic actions (Yuan & Yankner 2000; Brunet et al. 2001; Yuan et al. 2003a). On the one hand, Akt regulates the activity of transcription factors, including the forkhead transcription factor FKHRL1, promoting theirinteraction with 14-3-3 proteins, thereby sequestering them in the cytoplasm and precluding thetranscription of a number of genes encoding pro-apoptotic proteins (Brunet et al. 2001).

Akt-mediated phosphorylation of IκB causes its degradation, releasing NF-κB, which promotesthe transcription of genesinvolved in sensory neuron survival (Hamanoue et al. 1999). Additionally, PI3K-Akt-mediated signaling downstream of TrkB receptor activation triggers integrin-linked kinase (ILK) activation (Li et al., 2012). ILK inactivates glycogen synthase kinase 3-β (GSK3-β) by phosphorylation (Zhou et al. 2004) and can also function upstream of Akt, affecting Akt activity (Mills et al. 2003). The convergence of BDNF signaling in the ILK-mediated inactivation of GSK3-β has been implicated in neuroprotection (Guo et al., 2008) and the control of apoptosis (Jantas et al., 2009; Ortega et al., 2010).

PI3Kinase activation promotes axon growth and pathfinding, in addition to neuronal differentiation. The 3-phosphoinositides formed by PI3Kinase activity recruit several signaling proteins to the membrane, including GEFs for Cdc42, Rac and Rho (Yuan et al. 2003b), whichregulate F-actin cytoskeleton organization, enabling BDNF gradients to steer filopodial dynamics (Luikart et al., 2008). PI3K activity localizes to the tip of developing hippocampal axons (Shi et al., 2003), enhancing BDNF secretion and membrane insertion of TrkB receptors. PI3K activity simultaneously promotes the anterograde transport of TrkB receptors, further enhancing local BDNF/TrkB signaling, in a dual self-amplifying autocrine loop (Cheng et al., 2011).

PI3Kinase activation downstream BDNF-binding to TrkB receptors also facilitates local protein translation in dendrites by activation of mammalian target of rapamycin (mTOR) (Schratt et al., 2004; Takei et al., 2004; Smart et al., 2004), one of the main regulators of protein synthesis (Sarbassov et al., 2005). Akt phosphorylates TSC2 directly on multiple sites, mitigating the inhibitory effects of the TSC1-TSC2 complex on Rheb and mTORC1 [mTOR (mammalian target of rapamycin) complex 1] (Huang and Manning, 2009). PI3K-Akt-mTOR signaling mediates the BDNF-induced phosphorylation of eukaryotic initiation factor 4E binding protein (4EBP1) and p70 ribosomal S6 kinase (p70S6K), and the dephosphorylation of eukaryotic elongation factor 2 (eEF2) (Chen et

al., 2009). 4EBP1 phosphorylation promotes the assembly of the eIF4F complex and enhances the translation initiation of subsets of mRNAs at active synapses (Santos et al., 2010; Yoshii and Constantine-Paton, 2010).

Overall, these TrkB-activated signaling pathways account for nearly all BDNF synaptic effects but their biological responses likely reflect BDNF or TrkB receptor levels and the spatiotemporal pattern of BDNF stimulation, especially when activated pre- and/or postsynaptically (Cunha et al., 2010).

4.4- BDNF and lipid rafts:
Signaling regulation through the compartmentalization of TrkB receptors

Upon binding, BDNF may induce a rapid translocation of full-length TrkB receptors into cholesterol-rich lipid rafts. Accordingly, the depletion of cholesterol from the cell surface prevents the strengthening effect of BDNF in glutamate release and long-term regulation of dendritic growth (Suzuki et al., 2004), in addition to chemotropic guidance of axonal growth cones (Guirland et al., 2004).

In dendrites, sequential prenylation and kinase-activity-dependent palmitoylation of the Ca^{2+}/calmodulin-dependent protein kinase CLICK-III (CL3)/CaMKIγ C-terminal region is essential for CL3 membrane anchoring and targeting into dendritic lipid rafts, significantly contributing to BDNF-stimulated dendritic growth in cortical neurons. CL3 acts upstream of the RacGEF STEF and Rac, in lipid rafts, thereby linking Ca^{2+}-dependent modifications in lipid raftsto extrinsic activity-regulated dendrite formation (Takemoto-Kimura et al., 2007). In axons, TrkB receptor localization within the membrane or graded receptor activation by BDNF along discrete axon sites polarizes growth, by triggering gradients of cytoplasmic secondary messengers, including intracellular Ca^{2+} and cyclic adenosine monophosphate (cAMP) (Mai et al., 2009). Therefore, BDNF-induced polarized signaling differentially affects cytoskeletal rearrangementsinvolved in the initiation of dendritic branchingand axon growth as BDNF application to cultured neurons rapidly affects filopodia and lamellipodia dynamics through localized changes in the actin cytoskeleton (Gibney and Zheng, 2003; Menna et al., 2009).

Different mechanisms participate in the regulation of TrkB recruitment to lipid rafts. Neuronal activity promotes BDNF-induced recruitment of TrkB receptors from extrasynaptic sites into lipid rafts and TrkB receptor endocytosis, which is a key signaling event for many long-term BDNF functions (Nagappan and Lu, 2005). BDNF-induced translocation of TrkB receptors into lipid rafts is mediated *in vivo* by Fyn and is required for full activation of TrkB receptors and of downstream PLCγ signaling (Pereira and Chao,

2007). The localization of $p75^{NTR}$ to lipid rafts after its phosphorylation at Ser304 by protein kinase A catalytic βsubunit (PKAcβ) inhibits the BDNF-dependent translocation of TrkB receptors into lipid rafts and causes the inactivation of Rac1 (a small guanosine triphosphatase [GTPase] regulator of actin cytoskeleton) and the suppression of neurite outgrowth (Higuchi et al., 2003).

In cultured cortical and hippocampal neurons, but not in glial cells, BDNF elicits *de novo* cholesterol biosynthesis, rather than the mere incorporation of extracellular cholesterol, increasing cholesterol, the lipid raft marker protein caveolin-2 and presynaptic proteins, exclusively in lipid rafts, which indicates that BDNF promotes the development of neuronal lipid rafts with a key role in the readily releasable pool of synaptic vesicles (Suzuki et al., 2007). The integrity of lipid rafts is not required for BDNF regulation of neuronal survival (Suzuki et al., 2004), suggesting a direct role of these lipid microdomains in the spatial organization of signalling mechanisms mediated by TrkB receptors (Ibáñez, 2004).

5. The role of BDNF in neuronal connectivity

During development, BDNF regulates neuronal differentiation by stimulating the formation of appropriate synaptic connections, concomitantly controlling the direction and rate of axon growth (Wang and Poo, 2005; Li et al., 2005), in addition to the shape of dendritic arbors and spines (Ji et al, 2005; An et al., 2008; Kwon et al., 2011).

5.1- BDNF and the development of dendritic arbors and spines

BDNF affects apical and basal dendritic branching in opposite ways (McAllister et al., 1995; McAllister et al., 1997), either promoting dendritic arbor branching (McAllister et al., 1995; Horch and Katz, 2002; Wirth et al., 2003) or limiting its size (McAllister et al., 1997; Lom et al., 2002). In cultured hippocampal neurons, BDNF increases the number of primary dendrites and dendritic spines (Ji et al., 2005) although conditional knockout of TrkB receptors in adult mice shows no deficits in dendritic number, branching or length, affecting only synapse formation (Luikart et al., 2005). In cortical pyramidal neurons, the presence of BDNF in dendritic arbors can be rapidly detected upon exposure to the neurotrophin, in a spatially restricted and activity-dependent manner (McAllister et al., 1996; Horch and Katz, 2002). In cerebellar Purkinje neurons, BDNF affects the shape and number of dendritic spines but not the complexity of dendritic arbors, both *in vitro* (Shimada et al., 1998) and *in vivo* (Bosman et al., 2006). Therefore, BDNF differentially affects synaptic connectivity of developing neurons, modulating the development of dendritic arbors and spines, depending on the neuronal population (Cohen-Cory et al., 2010).

Evidence suggests a direct postsynaptic action of BDNF on dendritic branching and spine formation. Firstly, TrkB receptors can be found in the dendrites of cerebrocortical (Gomes et al., 2006) and hippocampal neurons (Drake et al., 1999). Secondly, GFP-tagged BDNFendogenously released from presynaptic, excitatory neurons, and anterogradely transferred to post-synaptic, inhibitory neurons, regulates dendritic development through this transsynaptic route (Kohara et al., 2003).

The effect of BDNF on dendritic arbors likely translates local changes in signaling regulating dendritic filopodial density, motility and connectivity (Cohen-Cory et al., 2010). Focal application of BDNF in dendrites induces localized calcium transients at nascent synapses along dendrites (Lang et al., 2007). In addition, BDNF controls the branching and laminar refinement of dendritic arbors in different subtypes of retinal ganglion cells (Liu et al., 2007; Liu et al., 2009).

5.1.1 – Dendritic branching and MAP2

Microtubule-associated protein 2 (MAP2) is a long filamentous protein that belongs to the MAP family and plays a key role in dendritic branching (Audesirk et al., 1997). All MAP family proteins are natively unfolded, adopt specific conformation upon binding to their targets (Uversky, 2002), contain several microtubule-binding repeats near the carboxyl terminus (Lewis et al., 1998) and include a conserved KXGS motif that can be phosphorylated (Dehmelt and Halpain, 2005) (Figure 16). MAP2, in particular, contains several PEST sequences, susceptible to calpain-mediated breakdown (Fischer et al., 1991; Johnson et al., 1993). In combination, these factors enable MAP2 to bind along the length of microtubules altering their dynamic behavior by crosslinking microtubules with intermediate filaments and other microtubules (Al-Bassam et al., 2002).

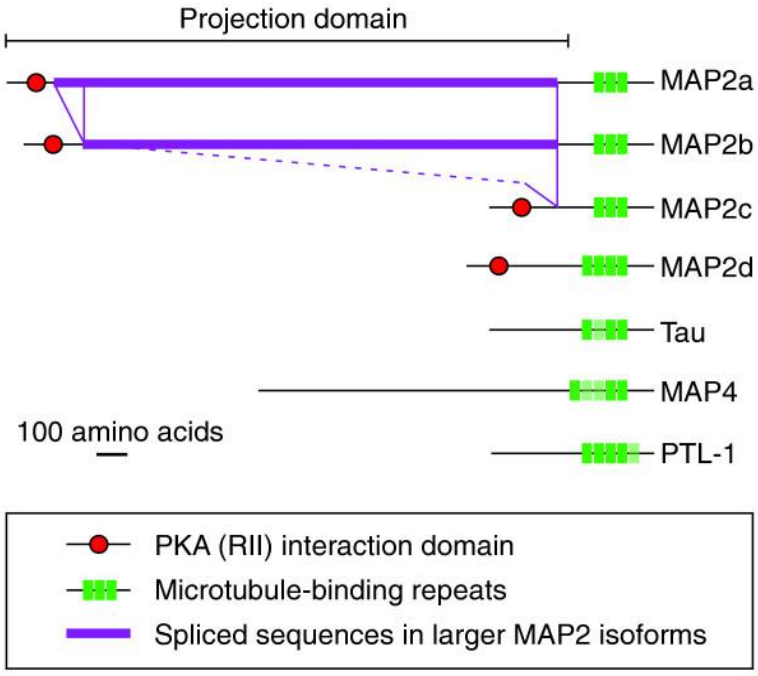


Figure 16. The domain organization of MAP family proteins (*in* Dehmelt and Halpain, 2005). Selected isoforms of the human homologs are shown, in addition to the nematode homolog PTL-1. All family members have alternative splice forms with varying numbers of carboxy-terminal microtubule-binding repeats and amino-terminal projection domains of varying lengths. PKA (RII) indicates a domain interacting with the RII subunit of protein kinase A.

MAP2 can be regulated by calpain-mediated cleavage (Friedrich and Aszódi, 1991) and by phosphorylation (Brugg and Matus 1991), which contribute for its role in dendritic remodeling (Fadis et al., 1997; Huang et al., 2007). Phosphorylation mediated by several kinases, including PKA, the microtubule affinity regulating kinase (MARK) (Drewes et al., 1995) and JNK1 (Chang et al., 2003), decreases MAP2 affinity for microtubules (Ozer and Halpain, 2000). MAP2 dephosphorylation promotes microtubule polymerization and bundling, whereas MAP2 phosphorylation and the consequent spacing of microtubules enhance branching (Hely et al., 2001).

In hippocampal neurons, the extracellular matrix protein Agrin (Mantych and Ferreira, 2001), the tyrosine kinase Abl (Jones et al., 2004), the brain-specific Ras guanine nucleotide exchange factor (RasGEF) very-KIND (Huang et al., 2007; Huang et al., 2011) and cholesterol (Fan et al., 2002) have all been implicated in MAP2 regulation of microtubule stability in a phosphorylation-dependent manner, through different mechanisms.

5.2 – BDNF and axon branching

BDNF significantly affects axonal arborization and addition of the neurotrophin in the optic tectum of *Xenopus laevis* tadpoles elicited axon branching within two hours of treatment (Cohen-Cory and Fraser, 1995). Furthermore, BDNF-mediated TrkB signaling enhances axon arborization and synaptic connectivity of hippocampal axons (Danzer et al., 2002) and, accordingly, mice lacking TrkB show reduced axon branching in the hippocampus (Martinez et al., 1998). The effects of BDNF on axon extension and branching are mediated through different mechanisms, involving gene and protein expression, in addition to posttranslational modifications of key regulatory proteins, especially phosphorylation, depending on the neuronal population (Bilimoria and Bonni, 2013).

BDNF-dependent activation of ERK1/2 increases Pol1-mediated nucleolar transcription, which regulates axon outgrowth in rat forebrain neurons (Gomes et al., 2011). BDNF also targets microRNA 9 (miR-9), which represses the translation of Map1b, a key protein inmicrotubule stability, thereby mediating BDNF-dependent axon branching and linking regulatory signaling processes with dynamic translation mechanisms (Dajas-Bailador et al., 2012).

In hippocampal neurons, TrkB interaction with ephrinA5 via its second cysteine-rich domain (CC2), which is necessary and sufficient for binding to ephrinA5, enhances PI-3 kinase/Ak signaling, axon branching and synapse formation (Marler et al., 2008). In parallel, BDNF-induced activation of TrkB receptors results in phosphorylation of beta-

catenin at residue Y654, which colocalizes with the cytoskeleton at growth cones and increase axon growth and branching (David et al., 2008).

In spinal motor and sensory neurons, Sprouty 3 (Spry3) is expressed in a BDNF-dependent manner and inhibits the ability of BDNF to induce filopodia by repressing calcium release downstream of BDNF signaling, thereby regulating BDNF-induced axonal branching of motoneurons (Panagiotaki et al., 2010).

BDNF signaling downstream TrkB receptors further promotes axon outgrowth and branching by stimulating two Dock3-dependent pathways: actin polymerization and microtubule assembly. On the one hand, Dock3 forms a complex with Fyn and Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous (WAVE) proteins at the plasma membrane, triggering Rac1 activation and promoting actin polymerization (Namekata et al., 2010). On the other hand, Dock3 binds to and inactivates GSK-3β at the plasma membrane, thereby increasing the nonphosphorylated active form of collapsin response mediator protein-2 (CRMP-2), which promotes microtubule assembly (Namekata et al, 2012). Furthermore, exogenous application of BDNF induces GSK-3β phosphorylation and CRMP-2 dephosphorylation, in hippocampal neurons. GSK-3β phosphorylation is associated with regeneration of axons in transgenic mice overexpressing Dock3 upon optic nerve injury (Namekata et al, 2012).

BDNF-induced expression of MAP kinase phosphatase-1 (MKP-1) also controls axon branching by spatiotemporal deactivation of JNK, which controls the formation and the maintenance of axons (Oliva et al., 2006). JNK dephosphorylationnegatively regulates the phosphorylation of stathmins (Tararuk et al., 2006), including SCG10 (Suh et al., 2004) and SCLIP (Poulain and Sobel, 2007), and neurofilament H (De Girolamo and Billett, 2006; Yamasaki et al., 2011), precluding microtubule destabilization (Jeanneteau et al., 2011).

Taken together, the available evidences indicate that BDNF regulates axon branching through different mechanisms depending on the cell type and possibly the signaling machinery available.

5.2.1- Neurofilament H and regulation of axon morphogenesis

The main constituents of the neuronal cytoskeleton are microtubules (MTs), microfilaments (MFs) and the intermediate filaments (IFs).

In axons, IFs are 10 times more abundant than MFs and MTs. IFs are classified into five types, according to gene structure and amino acid sequence (Sihag et al., 2007). Type IV IF proteins are expressed in CNS mature neurons and include the neurofilaments, NF light (NF-L; 68 kDa), NF medium (NF-M; 150 kDa) and NF heavy (NF-H; 200 kDa) (Figure 17), in addition to α-internexin (66 kDa) and peripherin (57 kDa).

Neurofilaments are heteropolymers formed by the assembly of subunits that differ by C-terminal phosphorylation, bundling, and axonal transport rate in growing axons (Yabe et al., 2001). They are normally synthesized and assembled in the cell body, and subsequently transported along the axon where they form an array responsible for maintaining axonal caliber and optimal conduction (Ohara et al., 1993; Shah et al., 2000; Motil et al., 2006).

Neurofilament proteins are closely linked to axon development and neuronal homeostasis, as demonstrated by the tight regulation of post-transcriptional changes in Neurofilament mRNA transport, translation and stability, in addition to Neurofilament gene transcription during axon regeneration (Szaro and Strong, 2010). In particular, the balance of kinase and phosphatase activities regionally and temporally regulates C-terminal phosphorylation of neurofilaments (Shea and Chan, 2010).

The proper formation of a neurofilament axonal network is essential for the establishment and maintenance of axonal calibre and consequently for the optimisation of axon transport velocity (Perrot et al., 2008). Fast axonal transport (50–250 mm/d) is involved in the transport of organelles, including mitochondria, lysosomes and endosomes, in addition to channel proteins and neurotransmitters. Slow axonal transport (0.1–4 mm/d) is responsible for the transport of cytoskeletal and cytosolic proteins. Impairment of axonal transport is one of the key factors common to several neurodegenerative disorders (Chevalier-Larsen and Holzbaur, 2006; De Vos et al., 2008). NF-H is the dynamic and interchangeable component of the NF network, positioned primarily in the periphery of the core composed of NF-L subunits (Takeda et al., 1994). Phosphorylation of the NF-H carboxy-terminal tail domains regulates both axonal transport (Ackerley et al., 2003) and diameter or caliber (Perrot et al., 2008). As previously mentioned, Neurofilament-H is a substrate of JNK, which is negatively regulated by BDNF-induced expression of MKP-1 (Oliva et al., 2006).

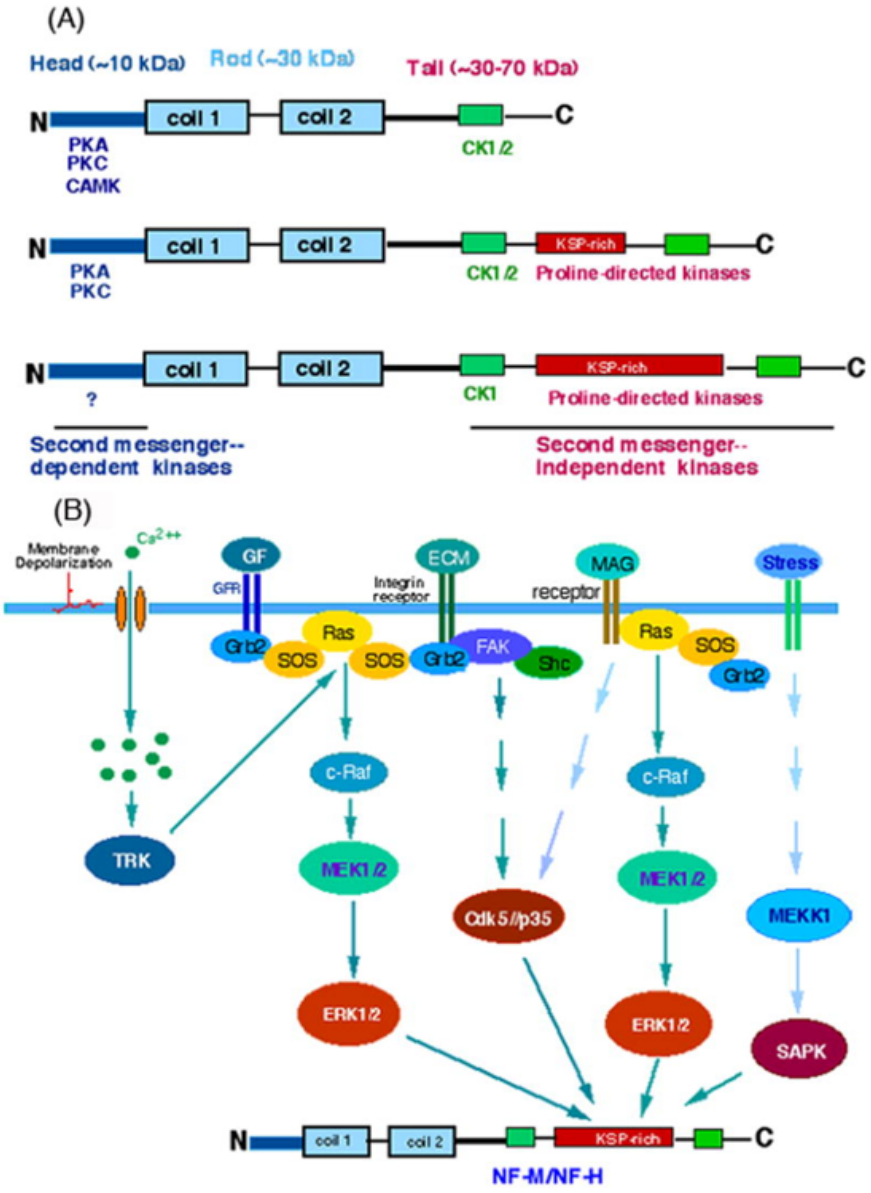


Figure 17.The role of phosphorylation on the structural dynamics of neurofilament proteins (*in* Sihag et al., 2007). (A) Schematic representation ofneurofilament (NF-L, NF-M and NF-H) protein phosphorylation, by different protein kinases, in the amino-terminal head and carboxyl-terminal tail domains. (B) Signaling transduction involved in the phosphorylation of 'KSP motifs' on the NF-M/NF-H proteins. Ca^{2+} influx, growth factors (GF), extracellular matrix (ECM), myelin-associated glycoprotein (MAG) and stress (osmotic, UV) were proposed to activate MAP kinases (ERK1/2, SAPK) or Cdk5, which are known to phosphorylate NF-M/NF-H *in vivo*.

Increasing NF-H phosphorylation changes its charge depended proton-binding capacity. Non-phosphorylated NF-H only binds two protons, whereas phosphorylated NF-H can bind up to 82 protons (Chang et al., 2009). NF-H phosphorylation causes lateral protrusion of the carboxyl-terminal tail regions from the filament backbone, forming 'sidearms'. The radial extension of sidearms out of the dense polymer structure results from charge repulsion and locally altered entropic and electrostatic interactions betweenneurofilaments, which controls the formation of a cytoskeletal lattice supporting the mature axon (Grant et al., 2000; Petzold, 2005; Kim et al., 2011). The abnormal accumulation of neurofilamentous inclusions, mainly composed by hyperphosphorylated NF-H, is a hallmark of numerous neurodegenerative disorders, including ALS, PD, Charcot-Marie-Tooth (CMT) disease, giant axonal neuropathy, progressive supranuclear palsy and spinal muscular atrophy (Ronald et al., 2009).

6. Mechanisms of synaptic plasticity induced by BDNF

BDNF plays an important role in several forms of synaptic plasticity (Vicario-abejon et al., 1998; Rutherford et al., 1998; Huang et al., 1999; Minichiello, 2009; Yoshii and Constantine-Patton, 2010). In the hippocampus, BDNF is involved in both learning (Linnarsson et al., 1997; Minichiello et al., 1999) and memory formation (Lee et al., 2004; Beckinschtein et al., 2008b), and is essential for a variety of adaptive neuronal responses dependent on short-term plasticity (Cirulli et al., 2004; Ipe et al., 2010).

Furthermore, the mature form of BDNF facilitates long-term potentiation (LTP) (Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996) and conversely attenuates long-term depression (LTD) (Akaneya et al. 1996; Huber et al. 1998; Kinoshita et al. 1999).

6.1- Hippocampal LTP

Hippocampal LTP is a long-lasting increase in synaptic efficacy between two neurons that are activated simultaneously (or in response to an adequate protocol of presynaptic stimulation), and akey molecular mechanisms involved in learning and memory. Hippocampal LTP occurs at different synapses, but has been studied to a larger extent at the synapses from CA3 afferents onto CA1 pyramidal neurons.

On the one hand, a weak, high frequency tetanus triggers an increase in synaptic efficacy lasting 1-2 hours, known as Early-Phase LTP (E-LTP) (Figure 18). This short-lasting form of LTP requires the activation of existing glutamate receptors in postsynaptic dendritic spines and their trafficking to synapses but not de novo protein synthesis (Malenka and Bear, 2004). The calcium influx through NMDA receptors causesa significant increase in intracellular calcium concentration and activation of CaMKII, as well as other protein kinases, increasing AMPA receptor phosphorylation and potentiation of ionic conductance (Malinow, 2003; Lynch, 2004; Tse, 2012). Threfore, E-LTPresults from this increased phosphorylation and membrane expression of AMPA receptors, in combination with metabolic changes resulting from the postsynaptic Ca^{2+} increases generated through NMDA receptors (Lauri et al., 2007).

On the other hand, repeated, strong, high frequency stimulations induce an increase in synaptic efficacy lasting over several hours or days (Abraham, 2003). L-LTP requires de novo protein synthesis and structural changes in synapses (Kandel, 2001; Yuste and Bonhoeffer, 2001; Bosch and Hayashi, 2012). L-LTP evokes cAMP-dependent PKA and ERK/MAPK activation, which lead to the downstream phosphorylation of

transcription factors, including CREB/CRE and Elk-1, increasing the expression of several genes, which are required for late-phase LTP (L-LTP) (Lu et al., 2008).

Decreased BDNF signaling in BDNF and TrkB knockout mice or upon application of endogenous BDNF scavangers, including TrkB-Fc (TrkB ligand binding sites fused to the Fc region of immunoglobulin G) and anti-BDNF antibodies (Yoshii and Constantine-Paton, 2007), attenuates hippocampal LTP (Korte et a., 1995; Patterson et al., 1996; Chen et al., 1999; Xu et al., 2000; Minichiello et al., 2002). Furthermore, exogenous BDNF application can rescue impaired LTP either through viral-mediated delivery (Korte et al., 1996) or upon direct application (Patterson et al., 1996; Pozzo-Miller et al., 1999).

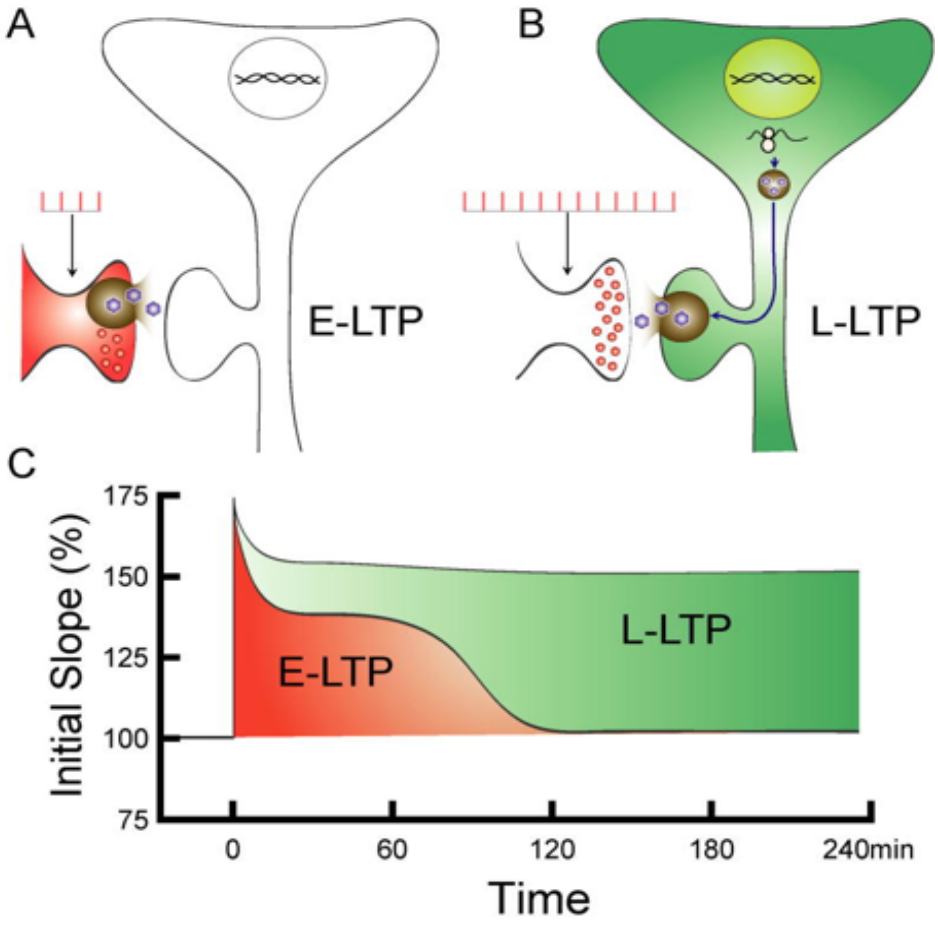


Figure 18. Early- and late-phase LTP (*in* Lu et al., 2008). E-LTP requires activity-dependent secretion of BDNF from presynaptic sites (red in A and C), while the long-term maintenance of L-LTP requires sustained supply of BDNF through activity-dependent transcription and translation in the postsynaptic neurons (green in B and C).

BDNF has a potentiating effect on E-LTP, as LTP is induced when BDNF is simultaneously applied with a synaptic stimulus, below the LTP threshold (Kovalchuk et al., 2002). BDNF enhances synaptic responses to high-frequency stimulation and synaptic vesicle docking at presynaptic active zones, thereby triggering its effect on E-LTP (Pozzo-Miller et al., 1999; Xu et al., 2000; Jovanovic et al., 2000). A brief application of BDNF also triggers postsynaptic calcium transients through calcium channels and NMDA receptors (Kovalchuk et al., 2002), which promote synaptic accumulation of PSD-95 (Yoshii and Constantine-Paton, 2007). PSD-95 scaffolds complexes of AMPA receptors with transmembrane AMPA receptor regulatory proteins (TARPs) at the synapse (Tomita et al., 2004), in a stoichiometry dependent on the neuronal population (Shi et al., 2009), thereby controling the trafficking and gating of AMPARs (Kato et al., 2010).

BDNF is also involved in L-LTP, as demonstrate by the effects of BDNF acute application, which induces synaptic potentiation in the hippocampal CA1 region (Kang et al., 1997) and sustained increases in field excitatory postsynaptic potentials (EPSP), lasting 2 to 10 hours, indicative of L-LTP (Messaoudi et al., 1998). Accordingly, in hippocampal slices, fast increases in BDNF enhanced basal synaptic transmission and slow, gradual increases induce LTP (Ji et al., 2010). Similarly, both endogenous BDNF scavengers TrkB-Fc and anti-BDNF antibodies block L-LTP (Kang et al., 1997), and BDNF perfusion in BDNF knockout mice rescues L-LTP (Korte et al., 1996; Pang et al., 2004).

6.2-Protein synthesis-dependent mechanisms of synaptic plasticity

The stimulation of transcription in postsynaptic neurons and local protein synthesis are both required for L-LTP, which is mimicked by BDNF application to hippocampal slices and in vivo (Soulé et al., 2006). The application of exogenous BDNF in the mature form is also sufficient to rescue L-LTP in the CA1 region of the hippocampus when protein synthesis is inhibited (Pang et al., 2004) suggesting that local translation of BDNF plays a key role in the late phase of LTP. BDNF induces the mTOR-dependent activation of local translation in neuronal dendrites (Takei et al., 2004), promoting the local expression of mainly synaptic proteins, including CaMKIIα, NMDA receptor subunits, Homer-2, and the PSD scaffolding protein (Schratt et al., 2004). The role of BDNF in synaptic potentiation is further supported by the results showing effects of the neurotrophin on protein synthesis in dendrites (Aakalu et al., 2001), as well as bythe synaptic potentiation observed upon injection of the neurotrophin into the rat dentate gyrus. Translation activation by BDNF is mediated by rapid phosphorylation of the eukaryotic initiation factor 4E (eIF4E) and elongation factor-2, and enhancement of eIF4E expression (Kanhema et al., 2006).

In synaptoneurosomes, a subcellular fraction containing the pre- and post-synaptic regions, BDNF selectively induces a transient phosphorylation of eIF4E and upregulates CaMKII (but not eEF2), in contrast with the BDNF-induced LTP, which induced a transient phosphorylation of eIF4E and eEF2, and enhanced expression of eIF4E protein in dentate gyrus homogenates. This suggests that BDNF-induced translation is initiated at synapses, whereas both initiation and elongation are regulated at non-synaptic sites (Kanhema et al., 2006; reviewed in Santos et al., 2010). BDNF further induces the translocation of the initiation factor eIF4E to mRNA granules, by an F-actin-dependent mechanism (Smart et al., 2003), and the activation of the dendritic translation machinery, which triggers the local synthesis of GluA1 (Schratt et al., 2004; Fortin et al., 2012), Arc/Arg3.1 (Yin et al., 2002; Takei et al., 2004) and CaMKII (Takei et al., 2004). Furthermore, BDNF activation of CAMKK, which controls mTOR activation, induces the synaptic delivery of GluA1-containing AMPA receptors, enhancing the synaptic strength, a BDNF effect mediated by TRPC5- and TRPC6-containing TRPC channels (Fortin et al., 2012). Conversely, Calpain-2knockdown by small interfering RNAin cultured cerebrocortical neurons fully suppresses the effect of BDNF on mTOR activation, precluding BDNF-induced local protein translation in dendrites (Briz et al., 2013).

In vivo studies have implicated different signaling pathways and mechanisms in BDNF-triggered translation required for the induction of L-LTP. In the dentate gyrus, BDNF-induced translation is apparently mediated by ERK signaling (Kanhema et al., 2006). However, the mTOR-PI3K-dependent pathway has also been shown to mediate the regulation of dendritic protein synthesis downstream BDNF activation of TrkB receptors (Schratt et al., 2004), including the synthesis of activity-regulated cytoskeleton-associated protein (Arc) and CaMKII, which is partly blocked by rapamycin in synaptoneurosomes (Takei et al., 2004). Upon BDNF-dependent upregulation of *Arc* mRNA, *Arc* transcripts are rapidly delivered to neuronal dendrites (Yin et al., 2002). Arc translation is then required for BDNF-induced LTP and its time-dependent consolidation (Soulé et al., 2005). Arc regulates actin polymerization and remodeling, thereby contributing for long-term LTP (Messaoudi et al., 2007). In addition, the PLCγ signaling pathway has been proposed to mediate the activity of TrkB receptors coupled to BDNF-induced synaptic potentiation because L-LTP is impaired in mice with a targeted mutation in the PLC-γ docking site, but not in the Shc site, on TrkB (Minichiello et al., 2002).

6.3-BDNF-induced synaptic tagging

The maintenance of L-LTP also requires activity-dependent gene transcription, which occurs in the nucleus, in addition to *de novo*protein synthesis or local protein translation. According to the synaptic "tagging" model, which accounts for the synapse-specificity of L-LTP, newly synthesized proteins are specifically targeted to, or "captured" by, tetanized synapses but not by nearby un-tetanized synapses (Frey and Morris, 1997; Redondo and Morris, 2011). In this mechanism, a strong tetanus induces the protein synthesis of "plasticity-related proteins" (PRPs) in the soma, which are then transported to dendrites. PRPs can only modify tetanized activated synapses, because only synaptic tags, previously created by tetanic stimulation, can capture PRPs (Martin and Kosic, 2002) but the nature and identity of PRPs and synaptic tags remain mostly unidentified (Reymann and Frey, 2007; Redondo and Morris, 2011).

BDNF is a potential PRP. Strong tetani enhance the expression of BDNF in the soma of CA1 pyramidal neurons (Castren et al., 1993; Dragunow et al., 1993; Patterson et al., 1992), likely through BDNF promoter III-dependent transcription (Lee et al., 2005). BDNF is among the only three genes upregulated by strong, L-LTP-inducing stimulation, statistically identified as L-LTP specific genes (Barco et al., 2005). Furthermore, BDNF perfusion can induce the transition from E-LTP to L-LTP when paired with a stimulus that would otherwise only elicit E-LTP, that is, a weak, tetanic stimulation that creates a synaptic tag but not PRP expression (Figurov et al., 1996; Kovalchuk et al., 2002) (Pang et al., 2004), which can be reversed by TrkB-IgG (Barco et al., 2005).

PKMζ has been shown to mediated the maintenance of BDNF-induced L-LTP in the absence of protein synthesis (Mei et al., 2011), specifically establishing synaptic tagging of long-term potentiation (Sajikumar and Korte, 2011). In addition, PKMζ regulatesthe synaptic localization of PSD-95 in combination with TrkB receptors (Yoshii et al., 2011), which likely represent synaptic tags corresponding to the role of BDNF as a PRP.

7. Glutamate excitotoxicity

The excitatory system in the CNS consists primarily of glutamatergic neurons, which play a key role in neurophysiological functions, including cognition and perception. Under normal conditions, glutamate is the main excitatory neurotransmitter, which is released from presynaptic terminals, activating postsynaptic ionotropic and metabotropic glutamate receptors (Figure 19). However, excessive activation of glutamate receptors results in neuronal dysfunction and death, in a process known as excitotoxicity (Dong et al., 2009).

7.1-Excitotoxicity as a unifying model of neurodegeneration

Excitotoxicity has been proposed as the primordial mechanism of neurodegeneration for several neuropathological conditions including Alzheimer´s disease (Miguel-Hidalgo et al., 2002; Hynd et al., 2004; Lesné et al., 2006; Yoshiyama et al., 2007; Zwilling et al., 2011; Camandola and Mattson, 2011), Parkinson's disease (Helton et al., 2008), Huntington's disease (McGeer and McGeer, 1976; Zeron et al., 2001; Stack et al., 2007; Zwilling et al., 2011), Amyotrophic Lateral Sclerosis (Lai et al., 2006; Kasai et al., 2011), Dementia with Lewy bodies (Kramer and Schulz-Schaeffer, 2007) and Prion disease (Mallucci et al., 2007). In addition, excitotoxicity has a pivotal role in acute and chronic mental disorders (Lipton and Rosenberg, 1994; Nonaka et al., 1998, Hashimoto et al., 2002; Blaylock and Strunecka, 2009), including cerebral ischemia (Hossmann, 1994; Siesjo et al., 1995; Gascón et al., 2008; Mustafa et al., 2010; Zhang et al., 2010), hypoxia (Schurr et al., 1995; Sun et al., 2010), traumatic brain (Brittain et al., 2011) and spinal cord (Ferguson et al., 2008) injuries, epileptic seizures (Ben-Ari, 1985; Baumeister et al., 1994; Vincent and Mulle, 2009), oxygen/glucose deprivation (Dennis et al., 2011) or neurodegeneration after stroke (Aarts et al., 2003; Sattler and Tymiansky, 2001; Zhou et al., 2010).

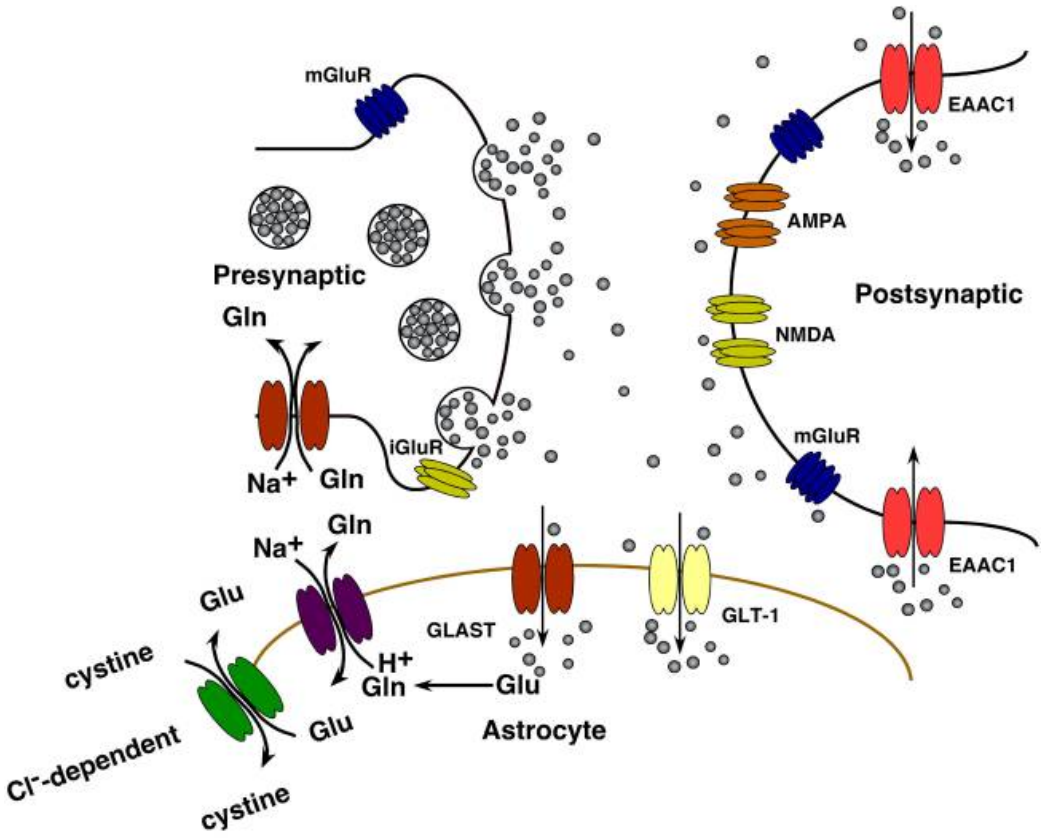


Figure 19. Schematic representation of an excitatory synapse (*in* Sheldon and Robinson, 2007). Glutamate released from pre-synaptic terminals activates ionotropic and metabotropic glutamate receptors. Na^+-dependent glutamate transporters mediate glutamate uptake. Glial cells express glutamine synthetase, enabling them to convert transported glutamate into glutamine, which can then be shuttled to neurons via glutamine transporters and converted to glutamate. System Xc− exchanges glutamate for cystine, providing cysteine as a precursor for glutathione synthesis.

During an excitotoxic event, neurons are exposed to toxic concentrations of excitatory neurotransmitters causing an excessive activation of calcium-permeable glutamate receptors (Noh et al., 2005) and the opening of gap junction hemichannels (Thompson et al., 2006), which account for an acute ionic disarray.

The collapse of transmembrane Na^+ and K^+ gradients not only induces Na^+-dependent glutamate transporterson neurons and astrocytes into ceassing glutamate uptake, but also reverses their glutamate transport, leading to glutamate efflux and accumulation in the extracellular space (Li et al., 2001; Romera et al., 2004; Grewer et al., 2008; Foran and Trotti, 2009; Brandon et al., 2011). The rise in intracellular Na^+ also accounts for the immediate neuronal swelling observed in hippocampal cultures, caused by passive Cl^- influx (Rothman, 1985; Beck et al., 2003), whereas calcium overload

triggers the activation of proteases, including calpains (Bano et al., 2005; Xu et al., 2009; Brustovetsky et al., 2010; Gomes et al., 2011) and caspases (Takano et al., 2005; Higuchi et al., 2005; Carlsson et al., 2011; Tornero et al., 2011). Alterations in the ubiquitin-proteasome system (UPS) were also shown to occur under excitotoxic conditions and in brain ischemia, whereby proteasome inhibition provides neuroprotection under the latter conditions (Phillips et al., 2000; Di Napoli and McLaughlin 2005) (section 7.4).

7.2-The role of calpains in glutamate excitotoxicity

Calpains are calcium-activated neutral proteases which target several proteins for degradation, in particular, spectrin (Vanderklish and Bahr, 2000) and actin microfilaments (Chung et al., 2005). The collapsed cytoskeleton proteins, together with vesicles and organelles, accumulate onto focal bead-like swellings or blebs at non-random sites (Bindokas and Miller, 1995), primarily at synaptic contacts enriched in mitochondria, on axons and dendrites, as a result of the impaired retrogade-anterograde transport and inhibition of the mitochondrial respiratory chain complex IV activity (Takeuchi et al., 2005). Although inhibiting mitochondrial ATP production does not affect vesicle motility during fast axonal transport, which is dependent on vesicular glycolysis for ATP production (Zala et al., 2013), mitochondrial transport and their membrane potential (ψ_m) have been correlated (Chang and Reynolds, 2006). The loss of ψ_m and drop in intracellular ATP levels (Abramov and Duchen 2008) following an excitotoxic insult might further aggravate the failure in the bi-directional transport mechanisms within the lysosome and ER compartments in dendrites (Greendwood et al., 2007). Alterations in mitochondrial transport may also affect the anterograde transport of neurofilaments in developing axons (Perrot and Julien, 2009), as observed in hippocampal neurons, which may account for the increased susceptibility of developing neurons to glutamate excitotoxicity (Johnson et al., 1999).

Calpain activation under excitotoxic conditions also contributes to neuronal deregulation due to the abnormal cleavage of several proteins. The plasma membrane Na^+/Ca^{2+} exchanger (NCX) is one of the calpain targets and the cleavage of the transporter impairs the extrusion of Ca^{2+} by the transporter contributing to a $[Ca^{2+}]_i$ overload (Bano et al., 2005). Several other critical proteins for neuronal function, including the NMDA receptor subunit GluN2B (Simpkins et al., 2003; Zhou and Baudry, 2006), the AMPA receptor subunit GluA1 (Glazner et al., 2000; Lu et al., 2002), and the mGluR1α metabotropic glutamate receptors (Xu et al., 2007) are also cleaved, as well as the vesicular GABA transporter (Gomes et al., 2011), to name a few.

7.3- Excitotoxicity-induced activation of caspases and apoptotic cell death

Downstream of the events involving mitochondrial electron transport defects (Luetjens et al., 2000), mitochondrial cytochrome c release is followed by an increase in caspase-9 and caspase-3 activation, and there is an increased production of superoxide, nitric oxide and other reactive oxygen species (ROS), leading up to apoptotic-like chromatin condensation and, finally, DNA fragmentation, in a panoply of neuronal cells subjected to glutamate excitotoxicty (Molz et al., 2008; Seo et al., 2009; Karmarkar et al., 2011). Other caspase-independent pathways of programmed cell death are also activated under excitotoxic conditions, including apoptosis triggered by Poly (ADP-ribose) polymerase-1 (PARP-1)-dependent translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus (Yu et al., 2002; Wang et al., 2004; Cheung et al., 2005), which requires Bax and calpains, but not caspases or cathepsins (Moubarak et al., 2007).

7.4- The Ubiquitin-proteasome system in excitotoxicity and ischemia

Recently, the ubiquitin proteasome system (UPS) has also been implicated in glutamate excitotoxicity, either through changes in UPS activity (Ge et al., 2007; Caldeira et al., 2013) or the proteasome-mediated cleavage of proteins, including the Bcl-2 interacting mediator of cell death (Bim) (Meller et al., 2006; Ordonez et al., 2010), Bcl-2 (Zhang et al., 2011) and DGKζ (Okada et al., 2012). Excitotoxic stimuli induce the nucleocytoplasmic translocation of the diacylglycerol kinase DGKζ followed by its degradation through the cytoplasmic UPS in hippocampal neurons. Although DGKζ-deficient neurons do not succumb directly to apoptosis, they are more vulnerable to excitotoxicity (Okada et al., 2012).

The role of the UPS in cell death caused by excitotoxicity and ischemia is quite complex because proteasome activity is downregulated in the ischemic brain (Asai et al., 2002; Ge et al., 2007; Suh et al., 2010) but both inhibition (Phillips et al., 2000; Zhang et al., 2001; Williams et al., 2003) and enhancement (Li et al., 2011; Hogins et al., 2011) of the proteasome activity was shown to be neuroprotective in ischemia. In addition, UPS inhibition attenuates the neuroprotective effect of both acute and delayed ischemic preconditioning (Rehni et al., 2011), while pharmacological preconditioning with adenosine induces the proteasomal degradation of Bim mediated by p42/44 MAPK (Ordonez et al., 2010). Presynaptic silencing, another neuroprotective strategy endogenously triggered by neurons during excitotoxic insults, also depends on proteasome function because an hypoxic insult increases the percentage of silent

glutamate synapses in a proteasome-dependent manner and proteasome inhibition prevents the hypoxia-induced silencing and exacerbates neuronal loss (Hogins et al., 2011).

In summary, generalized activation of different proteolytic mechanisms, downstream of NMDA receptors overactivation, sets off mitochondrial dysfunction, generation of ROS, cytoskeleton disarray and, ultimately, cell death under glutamate excitotoxicity. Concomitantly, protein synthesis arrest (reviewed in DeGracia and Hu, 2007) and endoplasmic reticulum stress (Sokka et al., 2007; Ruiz et al., 2009) are also found among the numerous consequences of delayed calcium deregulation as a result of excitotoxicity.

8. Neuroprotection mediated by BDNF

BDNF application rescues different neural tissues from excitotoxic, ischemic, traumatic and toxic injuries, in vitro and in vivo, upon acute or long-term neurotrophic treatment, both before and after the insult (Coughlan et al., 2009; Pereira et al., 2009; Murray and Holmes, 2011; Noble et al., 2011).

In vitro, BDNF protects cultured cerebrocortical (Hetman et al., 1999; Sun et al., 2008), cerebellar granule (Bazán-Peregrino et al., 2007; Wang et al., 2010) and hippocampal (Almeida et al., 2005; Johnson-Farley et al., 2007) neurons from apoptotic cell death through activation of the ERK and PI3K signaling pathways. BDNF also rescues cerebrocortical neurons from oxygen-glucose deprivation (Ferenz et al., 2012) and prevents N-methyl-D-aspartic acid (NMDA)-induced protein kinase C (PKC) inactivation, equally promoting maximal protection from cell death with BDNF pre-incubation, either continuously for 8 hours, or transiently between 8 and 4 hours, prior to NMDA excitotoxicity (Tremblay et al., 1999).

In vivo, BDNF promotes the survival of numerous neuronal populations, including embryonic primary sensory neurons (Barde et al., 1982; Lindsay et al., 1985; Davies et al., 1986), cholinergic neurons of the basal forebrain (Knusel et al., 1991), dopaminergic neurons of the substantia nigra (Hyman et al., 1991) and retinal ganglion cells (Johnson et al., 1986). Long-term (7-day) BDNF intraventricular infusion beginning 24 hours (Schabitz et al., 1997) or immediately before (Beck et al., 1994) the injury reduces infarct size and protects hippocampal CA1 neurons in a rat model of transient forebrain ischemia, respectively. Infusion (Yamashita et al., 1997) and vehicle-mediated intravenous (Wu and Pardridge, 1999; Schabitz et al., 2000) administration of BDNF shortly (up to 30 minutes) after medial cerebral artery occlusion (MCAO) produce similar neuroprotective results, mimicked even with delayed (up to 2 hours) BDNF intravenous application (Zhang and and Pardridge, 2001).

BDNF neuroprotection in vivo extends to other insults, namely hypoxic-ischemic (Han and Holtzman, 2000), traumatic and spinal cord (Oppenheim et al., 1992; Ikeda et al., 2002) injuries, in addition to kainate excitotoxicity (Gratacòs et al., 2001) and neonatal hypoxia (Galvin and Oorschot, 2003), and other further recent therapeutic strategies, including ex vivo gene therapy (Yasuhara et al., 2006; Shi et al., 2009; Takeshima et al., 2011). Adenoviral vector-mediated delivery of BDNF, expressed under the regulation of the hypoxia response element (HRE) from the vascular endothelial growth factor gene produces similar results, improving the recovery from brain injury in a mouse focal cerebral ischemia model (Shi et al., 2009).

Human neural stem cells (NSCs), genetically modified to overexpress BDNF, grafted into the cerebral cortex overlyingintracerebral hemorrhage (ICH) in mice induce behavioral improvement and produce a threefold increase in cell survival at 2 weeks and 8 weeks posttransplantation (Lee et al., 2010). Gain-of-function studies show that recombinant BDNF mimics the beneficial effects of NSC transplantation. Furthermore, loss-of-function studies show that depletion of NSC-derived BDNF fails to improve cognition or restore hippocampal synaptic density (Blurton-Jones et al., 2009)

Continuous intraventricular administration of BDNF also protects cerebrocortical neurons against apoptosis and reduces infarct size after 2-vein occlusion is rats (Takeshima et al., 2011). Moreover, an antisense BDNF oligonucleotide, which blocks the expression of *Bdnf* mRNA, precludes the beneficial effects of rehabilitation exercise on recovery of skilled reaching, upon infusion into the contralateral lateral ventricle for 28 days after ischemia in rats, with no effect on the reaching with the unaffected limb. This indicates that the treatment is specific to the relearning of motor skills after ischemia and shows a critical role for BDNF in rehabilitation-induced recovery after stroke (Ploughman et al., 2009). Furthermore, BDNF promotes regeneration of adult sensory neurons, retinal ganglion cells and basal forebrain cholinergic neurons following injury, in the adult brain (reviewed in Sohrabji and Lewis, 2006).

The use of BDNF in clinical applications is, however, limited by unfavorable pharmacokinetics, specifically its short plasma half-life (less than 1 minute in rats) and the low rate of transport across the blood-brain barrier (BBB) (Pardridge et al, 1994; Poduslo et al., 1996), poor intraparenchymal penetration (Morse et al., 1993) and adverse side-effects, mainly resulting from the low-affinity BDNF- $p75^{NTR}$ interaction, which can induce pain (Zhang et al., 2008), among other factors (Kingwell, 2010).

Recent advances through the development of alternative BDNF delivery methods, for example, pegylation (Pardridge et al., 1998), chimeric peptide approaches (Zhang and Pardridge, 2001), adeno-associated viral (AAV) vector-mediated gene delivery (Baumgartner and Shine, 1997; Martin et al., 2003; Kells et al., 2004; Shi et al., 2009), genetically engineered bone marrow mesenchymal stem cells (Kurozumi et al., 2004; Sasaki et al., 2009; Harper et al., 2009; Makar et al., 2009; Park et al., 2012) and the use of poly(ethylene vinyl acetate) (EVAc) polymers (Sirianni et al., 2010) as vehiclesforlong-term in vivo delivery of BDNF, in addition to partial (Schmid et al., 2012) or selective (Jang et al., 2010; Bai et al., 2010; Chen et al., 2011) TrkB agonists and BDNF peptidomimetics (O'Leary and Hughes, 2003; Fletcher et al., 2008; Massa et al., 2010), have enabled to overcome such drawbacks and successfully promote neurotrophic activities and neuroprotection aimed at designing new therapeutic strategies.

II. Aims

Excitotoxic injury is known to induce the proteolytic cleavage of numerous proteins in the soma and neurite compartments, which contributes to cell death. BDNF partially prevents excitotoxic cell death *in vitro* and *in vivo*as determined based on nuclear morphology analysis. However, it remains to be assessed whether BDNF also protects neurons from the loss of axons and dendrites, and synaptic activity, which would be required to maintain connectivity. In particular, the precise molecular mechanisms BDNF triggers following TrkB receptor activation and signal transduction towards inducing neuronal protection and/or recovery from a toxic glutamate insult, and its functional consequences, are still not fully understood.

Upon previous findings, we hypothesized that BDNF protects neurons against excitotoxicity either by stabilizing the existing morphology and physiology of neurons, or by promoting the restoration of normal morphology and synaptic function post-insult. In order to test this hypothesis, we aimed to examine the molecular and cellular mechanisms triggered by the neurotrophin, primarily in hippocampal neuronsin the early stages of development, but also in more mature neurons, with fully developed synapses. For this purpose, we aimed at using cell biology methods and pharmacological inhibitors of the key signalling effectors downstream TrkB receptor activation, in addition to functional assays to assess the synaptic activity of neurons before and after the toxic insult, with and without BDNF pre-incubation.

This study aimed at focusing on key protein markers of axons (Neurofilament H), dendrites (Microtubule-Associated Protein 2) and glutamatergic (Vesicular Glutamate Transporters 1 and 2) and GABAergic (Glutamate Decarboxylase 65 and 67) functions, among others, to further examine the spatiotemporal resolution of BDNF neuroprotective effects in cultured hippocampal neurons submitted to glutamate excitotoxicity. Furthermore, we hypothesized that three protease pathways were involved in excitotoxic mechanisms, calpains, caspases and the ubiquitin-proteasome system (UPS), and that BDNF could target one or more of these pathways in its mechanism of action in axons and dendrites.

Therefore, the aims of this studywere:

(i) To characterize *in vitro* and *in vivo* the effect of excitotoxicity on structural and functional key neuronal markers, including Glutamate Decarboxylase isoforms GAD65 and GAD67:

Several lines of evidence have suggested that multiple proteolytic systems would be involved in the cleavage of GAD isoforms under excytotoxic conditions, including calpain- and cathepsin-mediated proteolytic mechanisms (Wei et al, 2006; Sha et al., 2008; Monnerie & Le Roux, 2008). However, the ubiquitin-proteasome system had not been implicated in such mechanisms. In particular, the soluble isoform GAD67 is mainly found in the cytoplasm and, thus, may be a UPS target. Therefore, we aimed at assessing whether the UPS targets GAD isoforms under excitotoxic conditions and the inherent functional consequences for these proteins markers of GABAergic neurons.

(ii) To identify the mechanism of neurite protection by BDNF:

We aimed at analyzing the activation of calpain- and caspase-mediated proteolytic mechanisms, and the putative alterations in the UPS in cell lysates of cultured hippocampal neurons at different time points post-insult, with and without BDNF incubation, towards further characterizing the cellular damage induced by excitotoxic stimulation. Furthermore, we aimed at assessing the spatiotemporal regulation of BDNF-induced neuroprotective mechanisms using several key neurobiological markers, and the transient receptor potential channels TRPC3 and TRPC6, which are activated by calcium store depletion and required for BDNF-dependent survival of cerebellar granule neurons deprived of serum (Jia et al., 2007), towards further understanding the protective effect of BDNF in hippocampal neurons subjected to excitotoxic stimulation.

(iii) To elucidate the role of BDNF on the functional regulation of the vesicular glutamate transporters (VGLUT1 and VGLUT2) during the development and maturation of hippocampal synapses:

The control conditions of preliminary experiments assessing BDNF neuroprotection of VGLUT2 showed that incubation with the neurotrophin alone significantly upregulated the protein levels of the vesicular glutamate transporter, considerably above the control levels, without BDNF incubation, similarly to its effect on the protein markers of BDNF-induced neuroprotection, TRPC3 and TRPC6. Therefore, we aimed at examining whether BDNF could regulate the expression of VGLUTs, which would correlate its neuroprotective effect with a protein-synthesis dependent mechanism, concomitantly suggesting a likely link, or even overlap, between BDNF-induced mechanisms of synaptic plasticity, with VGLUTs as the molecular effectors of its mechanism of action.

Overall, these studies aimed at assessing whether BDNF is able to protect both structurally and functionally hippocampal neurons under excitotoxic conditions and elucidate some of the mechanisms involved in BDNF-induced neuroprotection.

Chapter 1

The role of the ubiquitin-proteasome system in excitotoxicity-induced cleavage of glutamic acid decarboxylase in cultured hippocampal neurons

Glutamic acid decarboxylase (GAD) is the key enzyme in the synthesis of γ-aminobutyric acid (GABA) and the functional protein marker of GABAergic neurons, which represent approximately 10% of total cells in cultured hippocampal neurons, but play a key role in regulating neuronal excitability, especially in hippocampal neurons. Consequently, any alterations in the activity of the enzyme will also have an impact on GABAergic synaptic transmission. GAD exists in two isoforms encoded by different genes, GAD65 and GAD67, with a molecular weight of 65 and 67 KDa, respectively. GAD65 represents 81% of total GAD in rat hippocampus and is found predominantly in association with synaptic vesicle membranes in nerve terminals, while GAD67 is evenly distributed throughout the cell, constitutively active, and accounts for the basal production of the cytosolic pool of GABA.

Several lines of evidences suggested that multiple proteolytic systems were involved in the cleavage of GAD under excitotoxic conditions, including calpain- and cathepsin-mediated proteolytic mechanisms, but the ubiquitin-proteasome system had not been previously implicated in such mechanisms. In particular, the soluble isoform GAD67 is present mainly in the cytoplasm and, thus, could constitute a target of the UPS. Therefore, we sought to assess whether the UPS would target the GAD isoforms under excitotoxic conditions and the inherent functional consequences.

In the present study, we aimed at examining the synthesis, cleavage and subcellular location of GAD65 and GAD67, and the role of calpain and the UPS in these modifications following excitotoxicity induced by exposure of hippocampal primary cultures to glutamate and subsequent analyses at different time periods. The results indicated that GAD65 and GAD67 are processed via UPS activity, albeit apparently without requiring ubiquitination for the process to occur, indicating that GAD molecules may be trapped by other ubiquitinated components that render the enzymes susceptible to UPS processing. In particular, this study enables to conclude that the UPSmost likely regulates GAD67 cleavage under excitotoxic conditions through modulation of an unknown GAD binding partner. In addition, GAD activity was measured in the cortex and cerebellum of adult rats after a 24 h post-mortem period to induce the cleavage of GAD. The cleavage of GADs diminishes the enzymatic activity and the characteristic punctate distribution of GAD65 along neurites in cultured hippocampal neurons was also affected under excitotoxic conditions, thereby indicating that the GABAergic function is significantly affected under these conditions.

OPEN ACCESS Freely available online

PLoS one

Role of the Proteasome in Excitotoxicity-Induced Cleavage of Glutamic Acid Decarboxylase in Cultured Hippocampal Neurons

Márcio S. Baptista[1☯], Carlos V. Melo[1*☯¤a], Mário Armelão[1], Dennis Herrmann[1], Diogo O. Pimentel[1], Graciano Leal[1], Margarida V. Caldeira[1], Ben A. Bahr[2], Mário Bengtson[3¤b], Ramiro D. Almeida[1], Carlos B. Duarte[1]

1 Center for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Coimbra, Portugal, **2** Biotechnology Research and Training Center, University of North Carolina, Pembroke, North Carolina, United States of America, **3** Department of Cancer and Cell Biology, Genomics Institute of the Novartis Research Foundation (GNF), San Diego, California, United States of America

Abstract

Glutamic acid decarboxylase is responsible for synthesizing GABA, the major inhibitory neurotransmitter, and exists in two isoforms—GAD65 and GAD67. The enzyme is cleaved under excitotoxic conditions, but the mechanisms involved and the functional consequences are not fully elucidated. We found that excitotoxic stimulation of cultured hippocampal neurons with glutamate leads to a time-dependent cleavage of GAD65 and GAD67 in the N-terminal region of the proteins, and decrease the corresponding mRNAs. The cleavage of GAD67 was sensitive to the proteasome inhibitors MG132, YU102 and lactacystin, and was also abrogated by the E1 ubiquitin ligase inhibitor UBEI-41. In contrast, MG132 and UBEI-41 were the only inhibitors tested that showed an effect on GAD65 cleavage. Excitotoxic stimulation with glutamate also increased the amount of GAD captured in experiments where ubiquitinated proteins and their binding partners were isolated. However, no evidences were found for direct GADs ubiquitination in cultured hippocampal neurons, and recombinant GAD65 was not cleaved by purified 20S or 26S proteasome preparations. Since calpains, a group of calcium activated proteases, play a key role in GAD65/67 cleavage under excitotoxic conditions the results suggest that GADs are cleaved after ubiquitination and degradation of an unknown binding partner by the proteasome. The characteristic punctate distribution of GAD65 along neurites of differentiated cultured hippocampal neurons was significantly reduced after excitotoxic injury, and the total GAD activity measured in extracts from the cerebellum or cerebral cortex at 24h postmortem (when there is a partial cleavage of GADs) was also decreased. The results show a role of the UPS in the cleavage of GAD65/67 and point out the deregulation of GADs under excitotoxic conditions, which is likely to affect GABAergic neurotransmission. This is the first time that the UPS has been implicated in the events triggered during excitotoxicity and the first molecular target of the UPS affected in this cell death process.

Citation: Baptista MS, Melo CV, Armelão M, Herrmann D, Pimentel DO, et al. (2010) Role of the Proteasome in Excitotoxicity-Induced Cleavage of Glutamic Acid Decarboxylase in Cultured Hippocampal Neurons. PLoS ONE 5(4): e10139. doi:10.1371/journal.pone.0010139

Editor: Rafael Linden, Universidade Federal do Rio de Janeiro (UFRJ), Brazil

Received October 10, 2009; **Accepted** March 16, 2010; **Published** April 12, 2010

Funding: This work was supported by Fundação para a Ciência e a Tecnologia (FCT) and FEDER (Fundo Europeu de Desenvolvimento Regional) (PTDC/SAU-FCF/72283/2006; to CBD) and by the Calouste Gulbenkian Foundation (Proc. 71949; to CVM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: cvmelo@stanford.edu

☯ These authors contributed equally to this work.

¤a Current address: Nancy Priztker Laboratory, Department of Psychiatry and Behavioral Sciences, Stanford University, Palo Alto, California, United States of America

¤b Current address: Department of Cell Biology, The Scripps Research Institute, La Jolla, California, United States of America

Introduction

In traumatic brain injury, epilepsy, and following episodes of hypoxia-ischemia the excessive release of glutamate and the consequent overactivation of glutamate receptors leads to cell death by excitotoxicity [1–4]. Brain ischemia also has a strong impact in GABAergic neurotransmission. The Ca^{2+}-dependent exocytotic release of GABA appears to account for the initial phase of neurotransmitter release at the onset of ischemia, while the reversal of the plasma membrane transporters is responsible for much of the subsequent efflux [5,6]. However, the decrease in surface expression of post-synaptic $GABA_A$ receptors, in part due to their internalization, decreases GABAergic synaptic transmission [7]. Following transient focal ischemia there is also a decrease in the expression of the vesicular GABA transporter, which may have a delayed impact on the exocytotic release of the neurotransmitter [8]. The plasma membrane GABA transporter GAT1 is a calpain substrate [9], and calpain activation in the postischemic brain [10] may contribute to the deregulation of the transporter.

Glutamic acid decarboxylase (GAD) is the key enzyme in the synthesis of γ-aminobutyric acid (GABA) [11] and any alterations in the activity of the enzyme will also have an impact on the GABAergic synaptic transmission. GAD exists in two isoforms encoded by different genes, GAD65 and GAD67, with a

molecular weight of 65 and 67 KDa, respectively [12]. GAD65 represents 81% of total GAD in rat hippocampus [13], and is found predominantly in association with synaptic vesicle membranes in nerve terminals [14–16]. This GAD isoform synthesizes mainly the vesicular pool of GABA [17,18], and is responsible for the fine tuning of inhibitory transmission [19]. In contrast, GAD67 is evenly distributed throughout the cell [20], being constitutively active and accounting for the basal production of the cytosolic pool of GABA [21]. Both isoforms of GAD are cleaved in cerebrocortical neurons subjected to excitotoxic conditions by a mechanism that is sensitive to inhibitors of calpain [22–25], a non-lysosomal, calcium-activated protease that has been implicated in excitotoxic neuronal damage [26], and recombinant GAD65 and 67 are cleaved in vitro by calpain [22,23]. Cathepsin inhibitors also inhibited the cleavage of GAD65 and 67 in cerebrocortical neurons exposed to a toxic concentration of glutamate, and recombinant GAD was cleaved by cathepsin L in an in vitro assay [24]. These evidences suggest that multiple proteolytic systems are involved in the cleavage of GAD under excitotoxic conditions.

The ubiquitin-proteasome system (UPS) is the major extralysosomal system for protein degradation in the cells [27,28]. Proteins targeted to be degraded by this system are first conjugated by polyubiquitin chains and then degraded by the proteasomes. The role of the UPS in cell death in the ischemic brain is rather complex since the activity of the proteasome is downregulated in the ischemic brain [29,30] but inhibition of the proteasome was found to be neuroprotective in focal brain ischemia [31–33]. Furthermore, the effect of proteasome deregulation on the turnover of specific proteins in the ischemic brain remains to be investigated. Hence, in the present study we investigated the putative role of the UPS in GAD cleavage under excitotoxic conditions. In particular the soluble isoform GAD67 is present mainly in the cytoplasm [34] and, therefore, may constitute a target of the UPS. We found that excitotoxic stimulation of hippocampal neurons with glutamate downregulates GADs, both at mRNA and protein levels. Our results indicate that the UPS does regulate GAD67 cleavage under excitotoxic conditions, possibly through modulation of an unknown GAD binding partner. Cleavage of GADs diminished the activity of the enzyme and the characteristic punctate distribution of GAD65 along neurites was also affected under excitotoxic conditions.

Materials and Methods

Hippocampal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, for 15min at 37°C; GIBCO-Invitrogen, Paisley, UK) and deoxyribonuclease I (5.36 mg/ml), in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO-Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO-Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO-Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6 well plates (870,000 cells/well) coated with poly-D-lysine (0.1 mg/mL), or on poly-D-lysine coated glass coverslips, at a density of 150,000 cells/well (12 well plates). The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37°C, for 7 or 10 days. Excitotoxic stimulation was performed with 125 μM glutamate in supplemented Neurobasal medium, for 20 min at 37°C, in a humidified incubator. After stimulation with glutamate the cells were further incubated with the original culture medium for the indicated periods of time. When appropriate, 50 μM UBEI-41 (ubiquitin-activating enzyme inhibitor; Biogenova Corp., Maryland, USA), 1 μM MG132 (Calbiochem, Darmstadt, Germany), 10 μM Lactacystin (Sigma, MO, USA) or 10 μM YU102 (Biomol, Exeter, UK) were added to the incubation medium 30 min (or 1 h for UBEI-41) before stimulation.

Animals used in the preparation of cell cultures and in the GAD activity experiments (see below) were handled according to National and Institutional guidelines. Experiments conducted at the Center for Neuroscience and Cell Biology were performed according to the European Union Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. These experiments did not require approval by an Institutional Animal Care and Use Committee (IACUC). The work performed at GNF adhered to the Animal Behavior Society Guidelines for the Use of Animals in Research, and was approved by the Institutional Animal Care IACUC.

Preparation of extracts

Hippocampal neurons (DIV7) were washed twice with ice-cold PBS and once more with PBS buffer supplemented with 1 mM DTT and a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma-Aldrich Química, Sintra, Portugal). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with the cocktail of protease inhibitors. After centrifugation at 16,100 g for 10 min, protein in the supernatants was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL), and the samples were denaturated with 2x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue), at 95°C for 5 min.

Total RNA isolation

Total RNA was extracted from 7 DIV cultured hippocampal neurons using TRIzol® Reagent (Invitrogen), following the manufacturer's specifications. The content of 2 wells from a 6 well plate, with 870,000 cells/well (DIV7), was collected for each experimental condition. After the addition of chloroform and phase separation, the RNA was precipitated by the addition of isopropanol. The precipitated RNA was washed once with 75% ethanol, centrifuged, air-dried and resuspended in 60 μl of RNase-free water (GIBCO-Invitrogen). The whole procedure was performed at 4°C.

RNA Quality and RNA Concentration

RNA quality and integrity was assessed using the Experion automated gel-electrophoresis system (Bio-Rad, Amadora, Portugal), as previously described [35]. A virtual gel was created for each sample, allowing the detection of degradation of the reference markers, RNA 18S and 28S. Samples showing RNA degradation or contamination by DNA were discarded. RNA concentration was determined using both the fluorescent dye RiboGreen (Invitrogen-Molecular Probes, Leiden, The Netherlands) and NanoDrop 1000 (Thermo Scientific). The samples were aliquoted and stored at -80°C to further use.

Reverse Transcription reaction

For first strand cDNA synthesis 1000 ng of total RNA was mixed with Random Hexamer Primer $p(dN)_6$ followed by 10 min

denaturation at 65°C to ensure loss of secondary structures that may interfere with the annealing step. The samples were chilled on ice, and the template-primer mix was then supplemented with Reaction Buffer (50 mM Tris/HCl, 30 mM KCl, 8 mM $MgCl_2$, pH 8.5), Protector RNase Inhibitor (20U), dNTPs (1 mM each) and finally AMV Reverse Transcriptase (10U; Roche, Carnaxide, Portugal), in a 20 µl final volume. The reaction was performed at 25°C for 10 min, followed by 30 min at 55°C, for primer annealing to the template and cDNA synthesis, respectively. The Reverse Transcriptase was then denatured during 5 min at 85°C, and the samples were then cooled to 4°C for 5 min, and finally stored at −80°C until further use.

Primer Design

Primers for real-time PCR were designed using the "Beacon Designer 7" software (Premier Biosoft International, CA, USA), and the following considerations were taken: (1) GC content about 50%; (2) annealing temperature (T_a) between 55±5°C; (3) secondary structures and primer-dimers were avoided; (4) Primer length between 18–24 bp; (5) Final product length between 100–200 bp. The primers used for amplification of GAD65 and GAD67 were, respectively, NM 012563 (accession number to mRNA sequence) – 5′GCT CAT TGC CCG CTA TAA G3′ and 5′ATC ACG CTG TCT GTT CCG3′; NM 017007 – 5′ACA CTT GAA CAG TAG AGA C3′ and 5′GCA GGT TGG TAG TAT TAG G3′. The primers used for the amplification of endogenous controls GAPDH and Tubulin alpha 1a were, respectively, NM 017008 –5′AAC CTG CCA AGT ATG ATG3′ and 5′ GGA GTT GCT GTT GAA GTC3′ ; NM 022298 –5′CAT CCT CAC CAC CCA CAC3′ and 5′GGA AGC AGT GAT GGA AGA C3′. Following the first experiment all sets of primers were tested for their specificity in an agarose gel that allows determination of the product size and possible non-specific products.

Real-Time PCR

For gene expression analysis 2 µl of 1:100 diluted cDNA was added to 10 µl 2x SYBR Green Master Mix (Bio-Rad) and the final concentration of each primer was 250 M in 20 µl total volume. The thermocycling reaction was initiated with activation of the Taq DNA Polymerase by heating at 95°C during 30 s, followed by 45 cycles of a 10 s denaturation step at 95°C, a 30 s annealing step, and a 30 s elongation step at 72°C. The fluorescence was measured after the extension step, using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction the melting step was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 s, up to 95°C, with continuous measurement of fluorescence, allowing detection of possible non-specific products. The assay included a non-template control and a standard curve (in 10-fold steps) of cDNA for assessing the efficiency of each set of primers. All reactions were run in duplicate to reduce confounding variance [36].

Real Time PCR Data Processing

The threshold cycle (C_t) represents the detectable fluorescence signal above background resulting from the accumulation of amplified product, and is a proportional measure of the starting target sequence concentration. C_t was measured in the exponential phase and, therefore, was not affected by possible limiting components in the reaction. For every run performed C_t was set at the same fluorescence value. Data analysis was performed using the GenEx (MultiD Analyses, Sweden) software for Real-Time PCR expression profiling, and the results were normalized with a set of two internal control genes. Statistical analysis was performed using the Student's *t* test.

Immunoblotting

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels (or 7.5% gels when spectrin products were detected), transferred to polyvinylidene (PVDF) membranes (Millipore Corp., Billerica, MA), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4°C), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20000 dilution; 1 h at room temperature) or exposed directly to ECL in the ubiquitin-conjugates detection which films were scanned and the optical densities of the bands were measured with appropriate software. Alkaline phosphatase activity was visualized by ECF on the Storm 860 Gel and Blot Imaging System (GE Healthcare, Buckinghamshire, UK). The following primary antibodies were used: anti-GAD65/67 (1:5000, Sigma), anti-GAD67 (1:250; BD Biosciences, Erembodegem, Belgium), antibody against calpain-mediated fragment of spectrin/fodrin nSBDP $_N$SBDPs [1:300 [37,38]] and anti-β-Actin (1:5000, Sigma).

Recombinant GAD65 cleavage assay

0.75 µg of recombinant GAD65 (Diamyd Diagnostics, Stockholm, Sweden) were incubated with 1.5 µg of 20S or 26S proteasome (Biomol) at 37°C for 2 h, in a total volume of 20 µl of buffer (30 mM TrisHCl pH 7.6, 100 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM ATP, 1 mM DTT, 5% (v/v) glycerol), with or without 10 µM MG132. A pre-incubation of 5 min with the proteasome inhibitor was performed. Reactions were stopped by addition of 20 µl of 2x concentrated denaturating buffer (same for immunoblot), resolved by 12% PAGE and probed with a GAD65 antibody by Western blot.

Purified proteasome activity

To test for the activity of the purified proteasome activity, the 20S and 26S proteasome preparations were incubated in the presence of the chymotrypsin-like fluorogenic peptide suc-LLVY-MCA (Peptide Institute, Inc., Osaka, Japan). The proteasome preparations were incubated with the substrate (50 µM) in the presence or in the absence of the proteasome inhibitor MG132 (10 µM), in a medium containing 1 mM EDTA, 10 mM tris-HCl (pH 7.5), 20% glycerol, 4 mM DTT, 2 mM ATP (100 µl final volume). Substrate degradation by the proteasome was monitored every 5 min during 1 h at 37°C in a fluorescence-luminescence detector (Synergy^{TM} HT Multi-Mode Microplate Reader, BioTek, Winooski, VT), set to 380 and 460 nm, excitatory and emission wavelengths, respectively.

Immunoprecipitation Assay

Immunoprecipitation of ubiquitin-conjugated proteins was performed using the Ubiqapture – Q Kit (Biomol, Exeter, UK), as described by the manufacturer. A total of 50 µg lysates from cultured hippocampal neurons were used per assay. Samples were added to the tubes containing 80 µl UbiQapture-Q matrix and incubated overnight at 4°C in an horizontal rotor mixer. The matrix was then carefully washed and the ubiquitin-protein conjugates were eluted by addition of 160 µl of PBS and 50 µl of 5X concentrated denaturating buffer (same for immunoblot). Samples were quenched by incubation during 15 min at 4°C on an horizontal rotor and then denaturated by heating during at 95°C for 10 min. The eluted fraction was clarified from the matrix pellet by centrifugation at 16,100 g during 10 min. Western blot analysis was performed as previously described using an anti-

GAD65/67 antibody and the ubiquitin-conjugate antibody supplied by the kit, applying equal sample volumes (approximately 60 µl).

Measurement of GAD activity

Wistar adult rats were decapitated and each head was covered and kept at room temperature (approximately 21°C) for 24 h. Brains were then dissected and placed on an ice-cold plate for dissection of the cerebellum and cerebral cortex. Samples were then resuspended in 50 mM TrisHCl and 0.02% Triton X-100, sonicated with a probe sonicator in 5 pulses of 5 seconds, and centrifuged at 16,100 g for 10 min. The supernatants were diluted (1:30–1:100) and the protein content was measured using the BCA method. Activity of glutamate decarboxylase (GAD) was measured by the [^{14}C]CO2 trapping method, using L-[1-^{14}C]-glutamic acid (60 mCi/mmol, GE Healthcare, Buckinghamshire, UK) as a substrate [39]. Enzyme activity was expressed as nmol of product/h/mg of protein. Reactions contained 40 µg of extract protein and 0.5 M KH_2PO_4, 5 mM ethylenediamineteraacetic acid (EDTA), 1 mM 2-aminoethyliso-thiouronium bromide (AET), 10 mM glutamate, 1 mM pyridoxal phosphate and L-[1-^{14}C]-glutamic acid in a total volume of 100 µl. Samples were incubated for 1 h at 37°C in test tubes containing #32 glass fiber filters (Schleider and Schuell, Keene, NH, USA) coated with 0.5 M Solvable (Packard Instruments, CT, USA). Each filter was suspended at the top of the tube, just underneath a rubber stopper, which sealed the tube. The reaction was stopped by the injection of 15% trichloroacetic acid through the stopper. The tubes were incubated at room temperature for another 120 min to ensure complete release and absorption of [^{14}C]CO_2 into the filter paper. The filter papers were then removed from the tubes and placed in scintillation vials for measurement of the [^{14}C]CO_2 product in a Packard 2000 spectrometer provided with dpm correction. The scintillation cocktail used contained 5.84 g 2,5-diphenyloxazole (PPO) and 133.6 mg of 1,4-Bis(5-phenyl-2-oxazolyl)benzene (POPOP), 800 ml toluene and 200 ml of Triton X-100. Sample extracts were also analysed by Western Blot using the anti-GAD65/67 antibody.

Immunocytochemistry

For immunocytochemistry, cultured hippocampal neurons were grown on poly-D-lysine coated glass coverslips, at a density of 45×10^3 cells/cm^2, and were then fixed in PBS supplemented with 4% paraformaldehyde/4% sucrose, for 30 min at 4°C. After fixation the cells were permeabilized with 0.25% Triton X-100 in PBS, for 5 min at room temperature, washed three times in PBS, and then blocked with 20% normal goat serum, for 1 h at room temperature, and stained against VGLUT1 (1:1000; Synaptic Systems) + VGLUT2 (1:500; Synaptic Systems) or GABA (1:2000; SIGMA) overnight at 4°C. Next, the cells were washed six times and incubated for 1 h at room temperature with the secondary antibody (Alexa Fluor® 488 goat anti-rabbit, 1:500 to 1:1000; Barcelona, Spain). The cells were washed three times, mounted on glass slides with the Dako mounting medium and viewed on an Axiovert 200 fluorescence microscope coupled to an Axiocam HRm digital camera (Zeiss) (Figure 1).

Immunocytochemistry experiments for localization of GAD65 were performed using hippocampal neurons maintained in culture for 10 days. The cells were fixed with 4% formaldehyde, 4% sucrose in PBS for 12 min at room temperature and were subsequently permeabilized with 0.25% Triton X-100 in PBS for 3 min, washed 3 times in PBS and incubated in blocking solution (2% bovine serum albumin, 2% glycine, and 0.2% gelatin in 50 mM NH_4Cl) for 1 h at room temperature. Afterwards, the neurons were incubated for 1 h with a mouse monoclonal antibody anti-$GABA_A$Receptor β2/3 (Upstate Biotechnology) at a dilution of 1:200, and a rabbit polyclonal antibody anti-GAD65 (SIGMA) at a dilution of 1:1000, in blocking solution. Following the incubation with the primary antibodies, the cells were rinsed 4 times in PBS during 15 min periods, incubated for 1 h at room temperature with secondary antibodies Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Invitrogen), at a dilution of 1:500 in blocking solution, rinsed 3 times in PBS followed by a final rinse in deionized water, dried, and mounted in Vectashield mounting solution (Vector Laboratories, Inc.). The neurons were imaged at the Garner Laboratory (Stanford University) with a Zeiss Axovert 200M microscope using a 63x objective. The image emission was directed through a CSU10 spinning disk confocal unit (Yokogawa) and collected by a 512B-CCD camera (Roper Scientific). Image acquisition and analysis was conducted with Metamorph software. For each field of view, stacks of 2 images with a Z step size of 0.2 mm were collected and a Metamorph 3D reconstruction tool was used to create a projection image. For each condition, 15 images were collected from two different cover slips.

For quantitative assessment of $GABA_A$Receptor β2/3 and GAD65 protein co-localization, the 3D reconstruction stacked images were submitted to threshold using the MetaMorph Inclusive Threshold application, in order to include only the puncta labeled by the GAD65 antibody. The total number of GAD65 puncta was determined using Integrated Morphometry Analysis of the image and selecting the Area parameter setup for measurement. After proceeding to perform an identical 3D reconstruction and threshold of the $GABA_A$Receptor β2/3 corresponding image, the two reconstructed images were overlayed and a color threshold was set. Finally, the overlay image was used to quantify the number of puncta positive for both $GABA_A$Receptor β2/3 and GAD65, using the Integrated Morphometry Analysis tool. This procedure was repeated for each field of view, and the ratio of $GABA_A$Receptor β2/3 subunits and GAD65 positive puncta per total number to GAD65 puncta was determined in percentage. The average number of 15 images, per condition, was calculated for 3 independent experiments. Likewise, the number of GAD65 puncta per unit length of axon was determined by selecting the option Trace Region on MetaMorph to delineate segments of axons with at least 100 µm, and measuring the number of GAD65 puncta on the image previously submitted to a threshold, using Integrated Morphometric Analysis. The length of each axonal segment was determined selecting the Multi-line tool and uniting consecutive puncta along the delineated neurite. This procedure was repeated in each field of view, for 10–15 images per condition, in each of the 3 independent neuronal preparations.

Determination of the viability of GABAergic neurons with Hoescht 33342

Determination of cell viability was performed by fluorescence microscopy, using the indicator Hoechst 33342 as previously described [40]. The cells were stimulated with 125 µM glutamate for 20 min, in Neurobasal medium supplemented with the GABA transporter inhibitor SKF89976 (10 µM). After the excitotoxic insult hippocampal neurons were further incubated in culture conditioned medium supplemented with 10 µM SKF89976 for 12 h. Incubation of the cells with SKF89976 during stimulation with glutamate and after the excitotoxic insult prevents the depletion of GABA through reversal of the plasma membrane transporter [41]. GABAergic neurons were stained using an anti-GABA polyclonal antibody (see above), and the nuclear morphol-

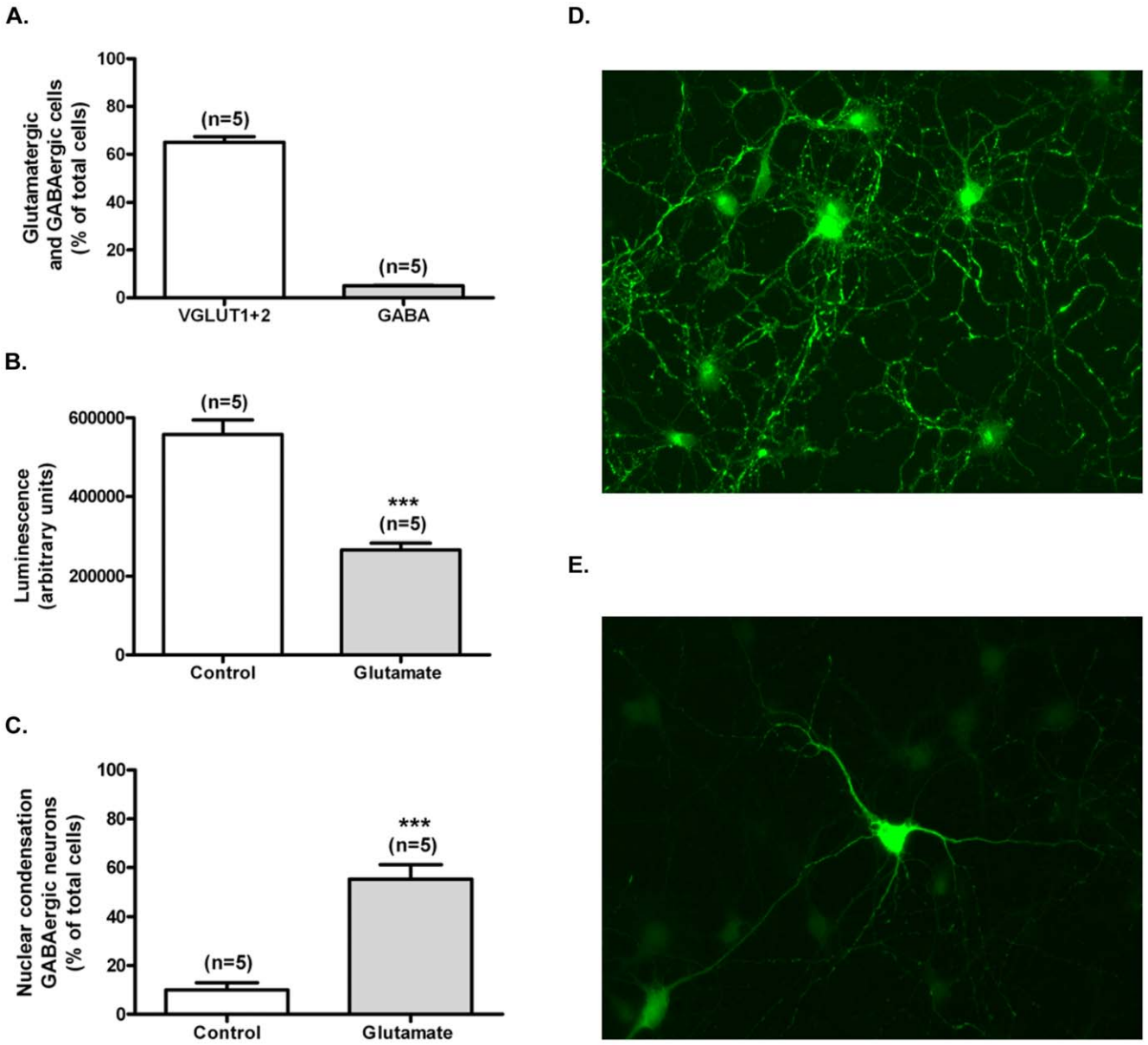


Figure 1. Glutamate excitotoxicity decreases viability of cultured hippocampal GABAergic neurons. GABAergic and glutamatergic neurons in the cultures (DIV7) were identified by immunocytochemistry, using antibodies against GABA (A, E) and VGLUT1+2 (A, D). The total number of cells present in the analysed fields was calculated based on the number of nuclei, stained with the fluorescent dye Hoechst 33342. Data are presented as mean±SEM of 5 independent preparations (A). Excitotoxic stimulation of hippocampal neurons was performed by incubation with 125 μM glutamate, for 20 min, in fresh Neurobasal medium containing B27 supplement, and the cells were further incubated in the original medium for 14 h. Cell death was assessed with the recombinant Luciferase chemoluminescence assay with CellTiterGlo (B), or by fluorescence microscopy using the fluorescence dye Hoechst 33342 (C). In the latter condition GABAergic cells were identified by immunocytochemistry, using an antibody against GABA. Data are presented as mean±SEM of 5 independent experiments. Statistical analysis was performed using Student's *t*-test. ***$p<0.001$.

doi:10.1371/journal.pone.0010139.g001

ogy was assessed through staining with Hoechst 33342. Analysis of the nuclear morphology was limited to GABAergic neurons, stained with the anti-GABA antibody.

Measurement of metabolic activity with CellTiter-Glow

Rat hippocampal neurons cultured in 384 micro-titter plates, coated with poly-D-lysine, at a density of 91.6×10^3 cells/cm^2 were incubated with 125 iM glutamate in supplemented Neurobasal medium, for 20 min at 37°C. After stimulation with glutamate, the cells were further incubated with the original culture medium, for 14 h at 37°C. The viability of the cells was then measured by analysing the levels of ATP as an indicator of cellular metabolic activity, using CellTitter-Glo (Promega) according to manufacturer's instructions. Briefly, in each well, 50 il of PBS/CellTitter-Glo (1:1) were dispensed with a Multidrop 384 stacker (Titertek), after removal of the growth media, at room temperature. The plate was then placed on an orbital shaker for 2 minutes, at maximum speed and further incubated for 10 minutes at room temperature, without shaking. Luciferase luminescence was measured immediately afterwards, using an Acquest plate reader (Molecular Devices).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA analysis of variance followed by the Bonferroni test, or using the Student's *t* test, as indicated in the figure captions.

Results

Excitotoxic damage of cultured GABAergic hippocampal neurons

Dissociated cultures of hippocampal neurons contain glutamatergic and GABAergic neurons, expressing the vesicular glutamate transporter 1 (VGLUT1) and glutamic acid decarboxylase, respectively [42]. Immunocytochemistry experiments using antibodies against VGLUT1+VGLUT2 (Fig. 1D) and against GABA (Fig. 1E) showed that 65% of the cells present in hippocampal cultures are glutamatergic and 5% are GABAergic, respectively (Fig. 1A).

Excitotoxic stimulation of cultured hippocampal neurons with 125 μM glutamate for 20 min reduced cell viability as determined using the CellTiter-Glo Luciferase chemiluminescence assay, a method based on the quantification of the ATP present in the cells. Luciferase activity was reduced to 48% of the control 14 h after the toxic insult (Fig. 1B), in agreement with the results obtained in experiments where cell survival was determined using fluorescence microscopy with the indicator Hoechst 33342 [40]. Under these conditions damaged hippocampal neurons display an apoptotic-like morphology. Since GABAergic neurons represent a minor fraction of the cells present in the cultures, we have specifically assessed the effects of excitotoxic stimulation with glutamate on the neuronal population displaying GABA immunoreactivity. In these experiments the cells were stimulated with glutamate in the presence of the GABA transporter inhibitor SKF89976 in order to prevent the release of the neurotransmitter through reversal of the plasma membrane transporter [41]. The number of GABAergic cells displaying apoptotic-like morphology 12 h after glutamate stimulation was about 54% (Fig. 1C).

Excitotoxicity-induced cleavage of GAD and down-regulation of gene expression

To test the effect of glutamate stimulation on glutamic acid decarboxylase, a marker of GABAergic neurons, GAD protein levels were evaluated after the excitotoxic insult, using an antibody that recognizes both forms of the enzyme in a common C-terminal region (Fig. 2C). Under control conditions the antibody allows identifying the two GAD isoforms, with 67 kDa and 65 kDa. Glutamate stimulation induced a time-dependent decrease in the abundance of both isoforms, and this effect was correlated with the upregulation of a truncated form with an apparent molecular mass of 55–58 kDa (Fig. 2A). The truncated form still bound the C-terminus directed antibody, but no smaller immunoreactive forms of GAD were detected in the blots (not shown). These results indicate that glutamate-induced cleavage of the two GAD isoforms occurs at the N-terminal region and gives rise to truncated forms with similar apparent molecular weights.

In order to further characterize the cleavage of GAD under excitotoxic conditions, we tested a GAD67 specific antibody that binds its N-terminus (amino acids 17–130). The immunoreactivity pattern in extracts prepared from cells incubated for 14 h after the toxic insult with glutamate was similar to that obtained using the antibody directed against the C-terminal region of GAD (Fig. 2B). This indicates that GAD67 is cleaved before amino acid 130.

Besides its effect in inducing the cleavage of GAD, excitotoxic stimulation with glutamate may also have delayed effects on GAD by acting at the transcription level. This was tested by Real-Time PCR, in cells subjected to excitotoxic stimulation with glutamate for 20 min and further incubated in culture medium for 4 h. Under these conditions there was a 58% and 71% downregulation of GAD65 and GAD67 mRNA, respectively, relative to unstimulated cells (Fig. 3).

Proteasome inhibitors protect GAD65/67 from cleavage under glutamate-induced excitotoxicity in hippocampal neurons

Multiple proteolytic systems have been shown to participate in the cleavage of GAD under excitotoxic conditions, including calpains and cathepsins [22–25]. Despite the key role of the ubiquitin-proteasome system (UPS) in protein degradation in the CNS, no studies have addressed its role in the down-regulation of full-length GAD isoforms under excitotoxic conditions. To test for the effect of inhibiting different proteolytic activities of the proteasome, we used the chymotrypsin-like activity directed inhibitor MG132 and the post-glutamyl peptide hydrolyzing-activity (PGPH) directed inhibitor YU102. We also tested the effect of lactacystin which shows a slight preference for the trypsin-like and caspase-like activities [43]. MG132 is a synthetic peptide aldehyde that binds reversibly to the 20S proteasome active site forming a covalent hemiacetal adduct [44,45]. The effect of proteasome inhibitors was tested 5 h after the toxic insult with glutamate since long incubation periods with these compounds causes neuronal cell death [46,47]. MG132 abrogated glutamate-induced cleavage on both isoforms of GAD, as determined 5 h after the toxic insult (Fig. 4A). Lactacystin is a *Streptomyces lactacystinaeus* metabolite that targets the 20S proteasome by an irreversible modification of the amino terminal threonine of β-subunits, while YU102 is a α′, β′-epoxyketine, the only peptidyl-glutamylpeptidehydrolyzing (PGPH)-specific peptide used in this study [45,48]. Both YU102 and lactacystin inhibited glutamate-evoked GAD65 cleavage, but were without effect on GAD67 (Fig. 4A).

To further characterize the role of the UPS in the glutamate-evoked cleavage of GAD we tested the effect of the ubiquitin-activating enzyme (E1) inhibitor, UBEI-41 [49]. It has been assumed that only a single activating enzyme for ubiquitin exists, which operates at the initial step of the ubiquitin-proteasome pathway. Therefore, if the UPS plays a role in the cleavage of GAD, inhibitors of E1 should abrogate the excitoxicity-induced cleavage of the GAD. Accordingly, inhibition of the ubiquitin-activating (E1) enzyme prevented the effect of excitotoxic stimulation on the cleavage of GAD67 and GAD65 (Fig. 4B).

In previous studies the cleavage of GAD under excitotoxic conditions was found to be abrogated by calpain inhibitors [22–25]. Furthermore, recombinant GAD65 and 67 are cleaved in vitro by calpain [22,23]. Accordingly, incubation of hippocampal neurons with ALLN, a chemical inhibitor that targets preferentially calpains when used at lower concentrations, prevented the glutamate-induced cleavage of GAD65/67 (Fig. 4B). Therefore, we determined whether inhibition of calpains could account for the effect of the UPS inhibitors on glutamate-induced cleavage of GADs. Activation of calpain was measured by western blot, using an antibody that binds specifically to the product resulting from the cleavage of spectrin by calpains (SBDPs) [37,38,50]. Glutamate stimulation increased the formation of N-terminal SBDPs ($_{N}$SBDPs), and this effect was only slightly inhibited by MG132 (16.6%; $p<0.05$) and by UBEI-41 (28.3%; $p>0.05$) (Fig. 4C). These results indicate that calpain inhibition is not likely to account for the effects of MG132 and UBEI-41 on glutamate-evoked cleavage of GAD.

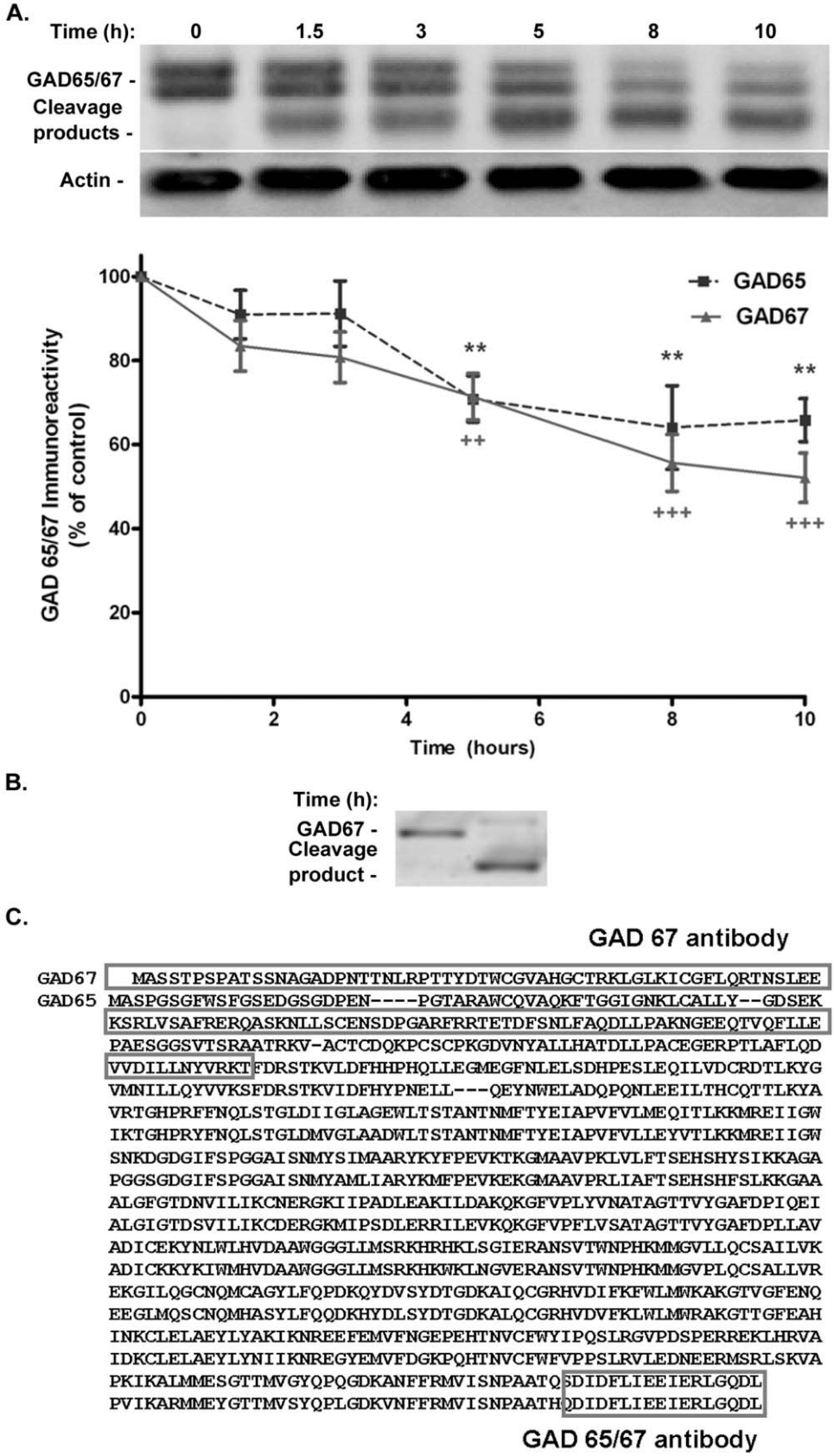


Figure 2. Glutamate excitotoxicity induces a time-dependent decrease in GAD65 and GAD67 protein levels in cultured hippocampal neurons. Neurons were stimulated with 125 μM glutamate, for 20 min, and further incubated in culture conditioned medium for the indicated period of time. Full length GAD 65/67 protein levels were determined by Western Blot with an antibody that recognizes both isoforms.

Control protein levels of GAD65/67 were set to 100%. Actin was used as loading control (A). Panel A shows a representative experiment and mean±SEM of 9 independent experiments. The cleavage of GAD67 was also analysed with an antibody directed against amino acids 70–130 of this isoform (B). In this case the results obtained under control conditions were compared with the immunoreactivity in extracts prepared 14 h after the toxic insult. The amino acid sequence of GAD65 (lower sequence) and 67 (top sequence) are aligned in panel C, which also show the binding sites for the antibodies used in this study. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's multiple comparison test. $^{**}p<0.01$; $^{***}p<0.001$.
doi:10.1371/journal.pone.0010139.g002

GAD65/67 interact with ubiquitinated proteins in primary hippocampal cultures

The results shown above suggest that the proteasome plays a direct role in GAD67 cleavage under excitotoxic conditions. In particular, the inhibition of GAD67 cleavage by the E1 inhibitor (Fig. 4C) suggests that the enzyme is ubiquitinated before cleavage by the proteasome. To test for the possible ubiquitination of GADs we used the UbiQapture^{TM}-Q Kit which allows isolating both mono- and poly-ubiquitinylated proteins (and their binding partners), independent of lysine residue chain linkage. Ubiquitinated proteins (and their binding partners) were isolated from extracts of hippocampal neurons stimulated or not with glutamate (with or without MG132), and the results were analysed by Western Blot with an anti-GAD65/67 antibody. GAD65/67 was immunoprecipated in similar amounts in all experimental conditions tested, but stimulation with glutamate in the presence or in the absence of MG132 increased the capture of GAD (Fig. 5A, top panel). However, in all experimental conditions the mobility of the immunoprecipitated GAD65/67 was the same as the mobility of the protein present in extracts directly loaded on the gel, suggesting that there is no change in GAD ubiquitination following glutamate stimulation. Taken together these results suggest that GAD67 interacts with another protein(s) that is ubiquitinated, and capture of this protein by an anti-ubiquitin antibody allows co-purification of the enzyme. The increased co-immunopurification of GAD67 in extracts from cells stimulated with glutamate may suggest that the excitotoxic insult increases the ubiquitination of the GAD67 interacting protein(s).

Since the 20S proteasome is able to cleave substrates without ubiquitination [51,52], an in vitro system was used to determine whether this could account for the observed inhibitory effect of MG132 on the excitotoxicity-induced cleavage of GAD65. Recombinant GAD65 was incubated with 20S and 26S proteasomes using the protocol previously described [51], which allowed characterizing the ubiquitin- and ATP-independent cleavage of YB-1 (a DNA/RNA-binding nucleocytoplasmic shuttling protein) by the 20S proteasome in vitro. No cleavage of recombinant GAD65 was observed following incubation with the 20S or 26S proteasome (Fig. 5B), suggesting that this GAD isoform does not undergo a ubiquitin-independent proteasomal cleavage, as described for YB-1. Control experiments using fluorogenic substrates showed that the 20S and 26S proteasome preparations were active (Fig. 5C), further suggesting that the proteasome does not act directly on GAD65.

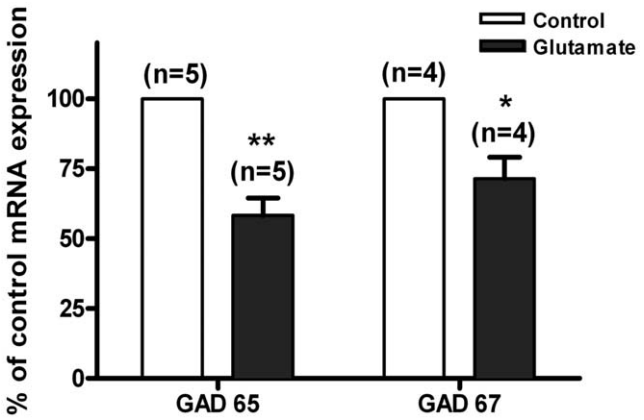


Figure 3. Glutamate excitotoxicity decreases GAD65/67 mRNA. Gene expression was analysed in cultured hippocampal neurons (7 DIV) exposed or not to 125 μM Glutamate, for 20 min, and then returned to the original culture medium for 4 h. For the reverse transcription reaction 1 μg of total RNA was used. The results were normalized with two internal control genes, GAPDH and Tubulin. Data are presented as mean±SEM of four to five independent transcription reactions, performed in independent preparations. Statistical analysis was performed using Student's *t*-test. $^{*}p<0.05$; $^{**}p<0.01$.
doi:10.1371/journal.pone.0010139.g003

GADs cleavage is correlated with decreased enzyme activity and changes the subcellular distribution

Since GADs play a key role in the synthesis of GABA from glutamate, we investigated how the cleavage of the enzyme affects its activity. The assay of GAD activity using the $[^{14}C]CO_2$ trapping method requires the use significant amounts of protein that cannot be obtained using hippocampal cultures. Therefore, the effect of GAD cleavage on the activity of the enzyme was investigated using brain tissue from decapitated rats. Previous studies have shown that under these conditions GAD is cleaved with a pattern similar to that observed under excitotoxic conditions, particularly in the cerebellum and in the cerebral cortex [53]. The post-mortem cleavage of GAD65 and GAD67 in these brain regions was confirmed in the present study (Fig. 6B), and 24 h after death there was a decrease in the total full length GAD protein levels both in the cerebellum and in the cerebral cortex (Fig. 6A). At this time point the activity of GAD was decreased to 68.8% in the cerebral cortex and to 33.1% in the cerebellum, while the total amount of full-length protein was reduced to 73% and 58%, respectively. The total GAD protein levels (full-length + cleaved protein) at 24 h post-mortem was not significantly different from the amount of protein detected under control conditions (see representative western blot in the top panel of Fig. 6B).

GAD65 is anchored to synaptic vesicles through its N-terminus [54,55]. Since glutamate stimulation cleaves GAD near the N-terminal region, we hypothesized that the cleavage of the enzyme could affect its sub-cellular localization. Under control conditions GAD65 displays a partially punctate distribution along neurites (Fig.7A, arrowheads), but this pattern is altered 4 h after excitotoxic stimulation with glutamate. Under the latter conditions some neurites show a more homogeneous distribution of GAD65, diffuse along the neuronal processes (Fig. 7A, B), and the number of GAD65 puncta is significantly reduced in comparison to the control conditions (Fig. 7C). Colocalization of GAD65 with the β2/3 $GABA_A$ receptor subunits was also significantly decreased (Fig. 7C), showing a loss of synaptic distribution of GAD65 under excitotoxic conditions.

Discussion

Previous studies have shown the cleavage of the glutamic acid decarboxylase isoforms GAD65 and GAD67 under excitotoxic

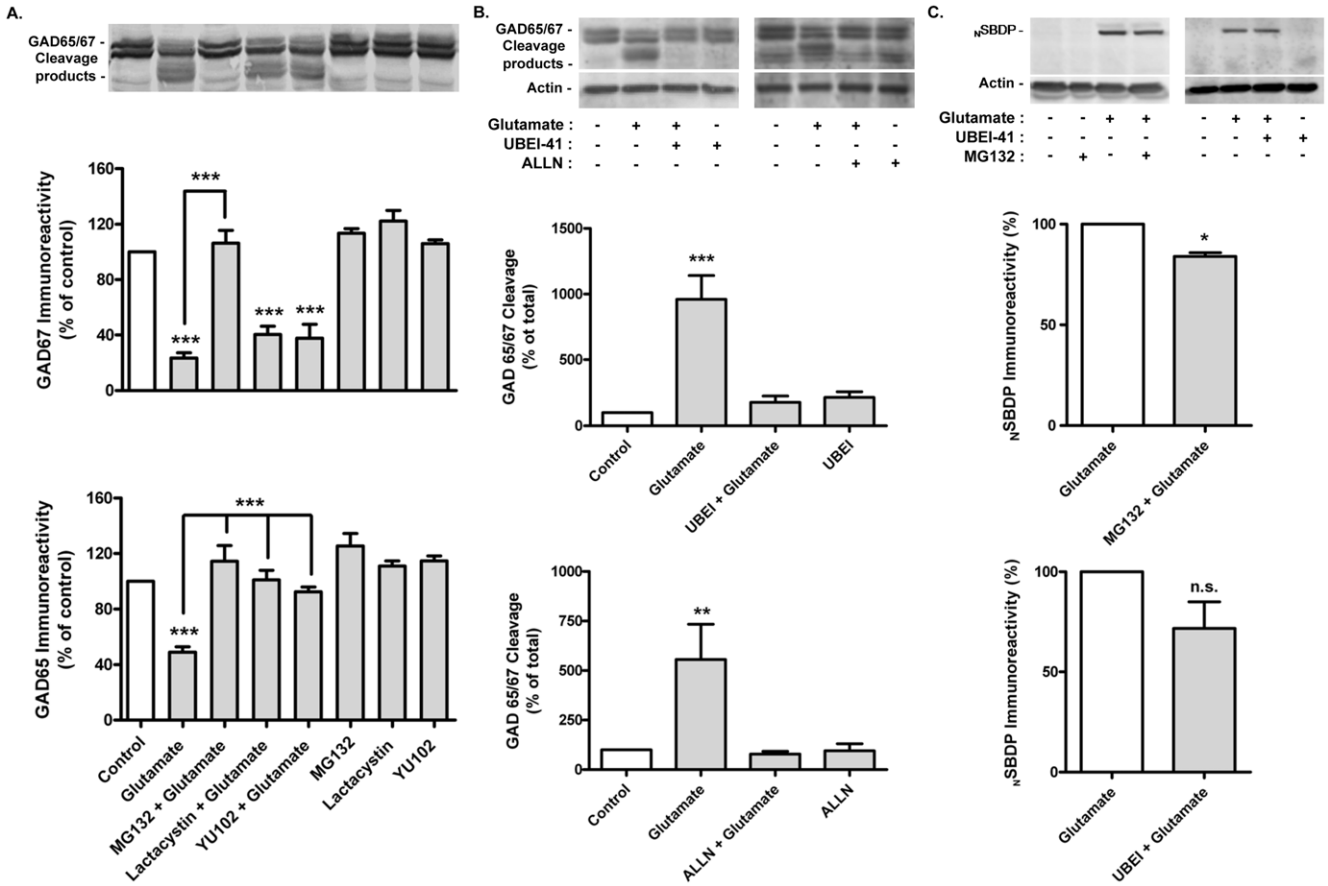


Figure 4. Proteasome and ubiquitin-activating enzyme (E1) inhibitors prevent glutamate–induced GAD65/67 cleavage. Cultured hippocampal neurons were pre-incubated or not with 50 μM of UBEI-41 (E1 inhibitor), for 1h, or with the proteasome inhibitors MG132 (1 μM), lactacystin (10 μM) or YU102 (10 μM), for 30 min, before excitotoxic stimulation with glutamate (125 μM), for 20 min. The cells were further incubated in culture conditioned medium (with or without chemical inhibitors) for 5h, and the GAD65/67 immunoreactivity was assessed by western blot (A and B). Incubation with the calpain inhibitor ALLN (10 μM) was performed under the same conditions. The average results in (A) represent the changes in GAD65 or GAD67 immunoreactivity. In panel (B) GAD cleavage was calculated as a percentage of the total enzyme content (GAD65/67). When calpain activity was evaluated through formation of N-terminal spectrin breakdown products ($_N$SBDPs) the cells were incubated for 30 min after the toxic insult (C). The effect of MG132 and UBEI-41 on calpain activation is expressed as a percentage of the activity measured in the absence of the protease inhibitors. Results are means±SEM of 3–4 different experiments, performed in independent preparations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test (A and B) or the Student's *t* test (C). ***$p<0.001$.
doi:10.1371/journal.pone.0010139.g004

conditions [23,25], and pointed out a key role for calpains in this process [22–24]. In this work we show that the activity of E1 ubiquitin ligase and the proteasome are required for glutamate-evoked cleavage of GAD67, although no clear evidences were obtained showing a direct ubiquitination of the enzyme. Furthermore, cleavage of GAD65/67 was found to decrease enzyme activity and changed the characteristic punctate distribution of GAD65 along neurites. Both effects are likely to downregulate the activity of GABA as a neurotransmitter under excitotoxic conditions.

Glutamate-induced cleavage of GAD protein levels and downregulation of mRNA

Excitotoxic stimulation of cultured hippocampal neurons induced the cleavage of GAD65 and 67 by a mechanism sensitive to the calpain inhibitor ALLN, similarly to what was observed in neuronal cultures prepared from the whole brain or from the cerebral cortex [23,24]. The full-length proteins were cleaved into a truncated form with approximately 55–58 kDa, which was detected by an antibody directed against the N-terminal of GAD65 and GAD67. Since no immunoreactive bands with low apparent molecular weight were identified, the results indicate that both GAD isoforms are cleaved in a sequence close to the N-terminal region of the proteins. Accordingly, an antibody directed against amino acids 17–130 of GAD67 also detected the cleavage product of the enzyme, showing that the cleavage site is located before amino acid 130. The sequence after amino acid 100 in GAD67 shows high homology with GAD65 (Fig. 2C), and this explains the similarity in the apparent molecular weight of the cleavage products of GAD65 and GAD67. Much of the available evidences suggest that the N-terminal segment of GAD is exposed and flexible [56], and this may make this region available for cleavage by proteases. In vitro studies showed that recombinant human GAD67 lacking the first 70 or the first 90 amino acids is not cleaved by calpain, in contrast with the full length protein [22], suggesting that under excitotoxic conditions GAD67 may be cleaved between amino acids 90 and 130. If this is the case, the dimerization of GADs required for their activity is likely not affected by enzyme cleavage since dimer formation occurs through interaction of C-terminal portions of GAD molecules [56]. GAD is a pyridoxal 5′-phosphate (PLP)-dependent enzyme, but the co-enzyme binding site is not contained within the N-terminal regions [57]. Therefore,

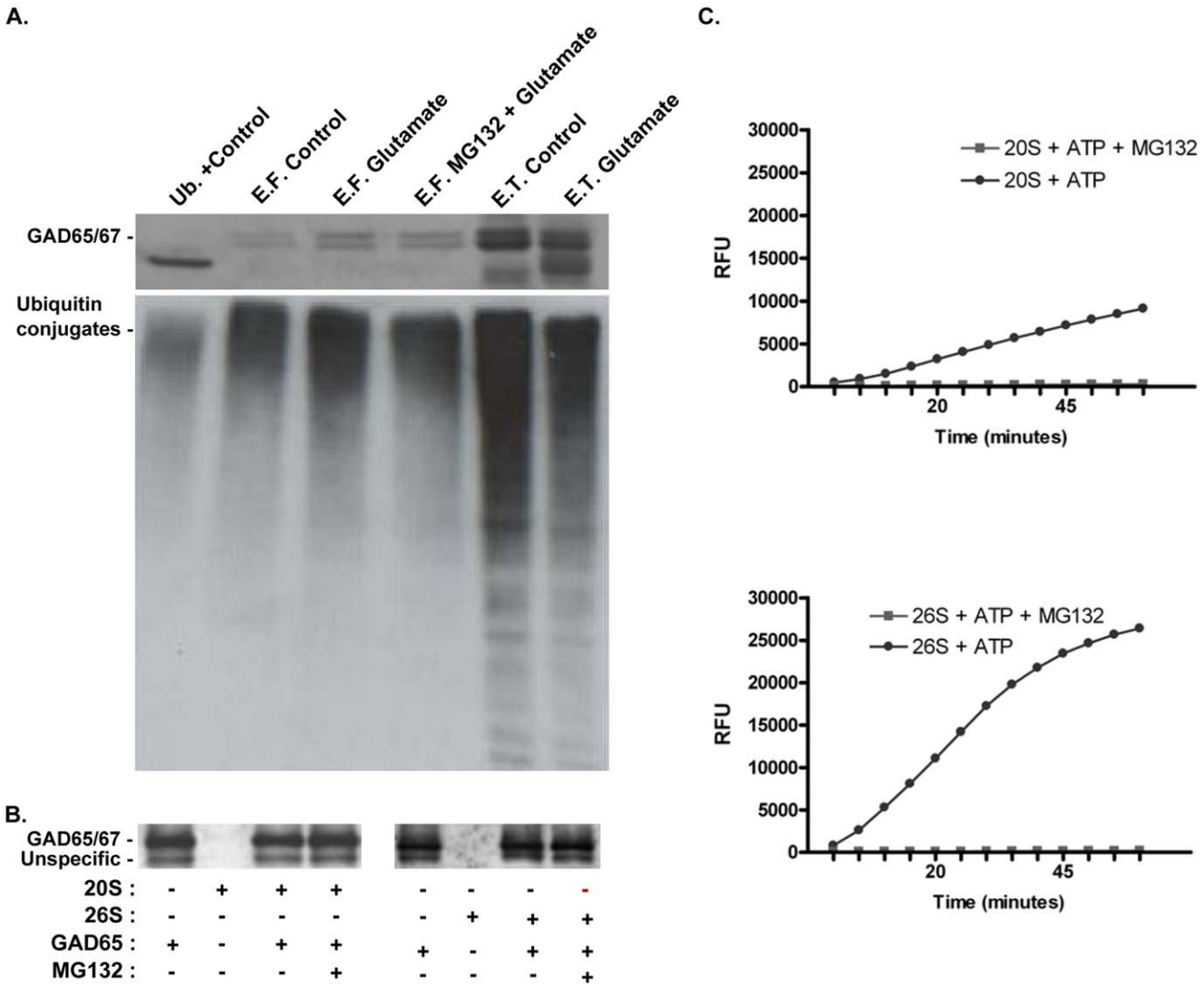


Figure 5. GAD 65/67 are captured with an anti-ubiquitin antibody in hippocampal cultures. (A) Cultured hippocampal neurons were stimulated or not with 125 μM glutamate for 20min, in the presence or in the absence of 10 μM MG132, and the cells were further incubated with culture conditioned medium for 4h before preparation of the extracts. In the top panel, mono- and poly-ubiquitinylated proteins were isolated using the UbiQapture^{TM}-Q Kit, and the eluted fraction [46] was subjected to western blot, using an antibody against GAD65/67. GAD65/67 total immunoreactivity in the extracts prepared from control cells and from hippocampal neurons stimulated with glutamate is shown on the right (E.T.). The left lane was loaded with a control provided in the kit, consisting in ubiquitinated protein lysate. The same membranes were probed for mono- and poly-ubiquitin using an antibody included in the kit (A, middle). (B) Human recombinant GAD65 (0.75 μg) were incubated with 20/26S proteasomes (1.5 μg) for 2h at 37°C with or without MG132. The extracts were then probed with GAD65/67 antibody. The activity of the 20S/26S proteasomes used in the experiments was confirmed using the fluorogenic substrate suc-LLVY-MCA. The increase in fluorescence resulting from the cleavage of the substrate was measured in relative fluorescence units (RFU) (C). The results of the capture of ubiquitinated proteins and the assay of the recombinant GAD65 cleavage are representative of two and three independent experiments, respectively.
doi:10.1371/journal.pone.0010139.g005

changes in PLP binding are not likely to account for the changes in GAD activity following enzyme cleavage.

In addition to the cleavage of GAD we also observed a decrease in the mRNA levels for both isoforms of the enzyme in hippocampal neurons subjected to an excitotoxic insult with glutamate (Fig. 3). This is likely to limit the de novo synthesis of GAD, which could otherwise compensate for the observed dowregulation of the full-length protein. It remains to be determined whether the observed decrease in the mRNA for GAD is due to a reduction in transcription activity and/or to an active degradation of the existing transcripts. The rapid down-regulation of GAD mRNA following excitotoxic stimulation of cultured hippocampal neurons contrasts with the delayed effects of ischemic injury on GAD67 mRNA [58]; unilateral ischemic lesions of the frontoparietal cortex in adult rats increased GAD67 mRNA levels in the striatum, lasting up to 3 months after surgery. Inhibition of NMDA receptors also downregulated GAD mRNA in various brain regions, starting at day 2 after treatment [59], suggesting that glutamate receptors are directly coupled to the activation of GAD67 expression.

UPS system activity is essential for excitotoxicity-induced GAD cleavage

Previous studies have shown that calpain inhibitors fully block [23] [or inhibit to a great extent [24]] the glutamate evoked cleavage of GAD65/67 (see also Figure 4B), and in vitro experiments showed that calpains cleave recombinant GAD67 [22]. Taken together these

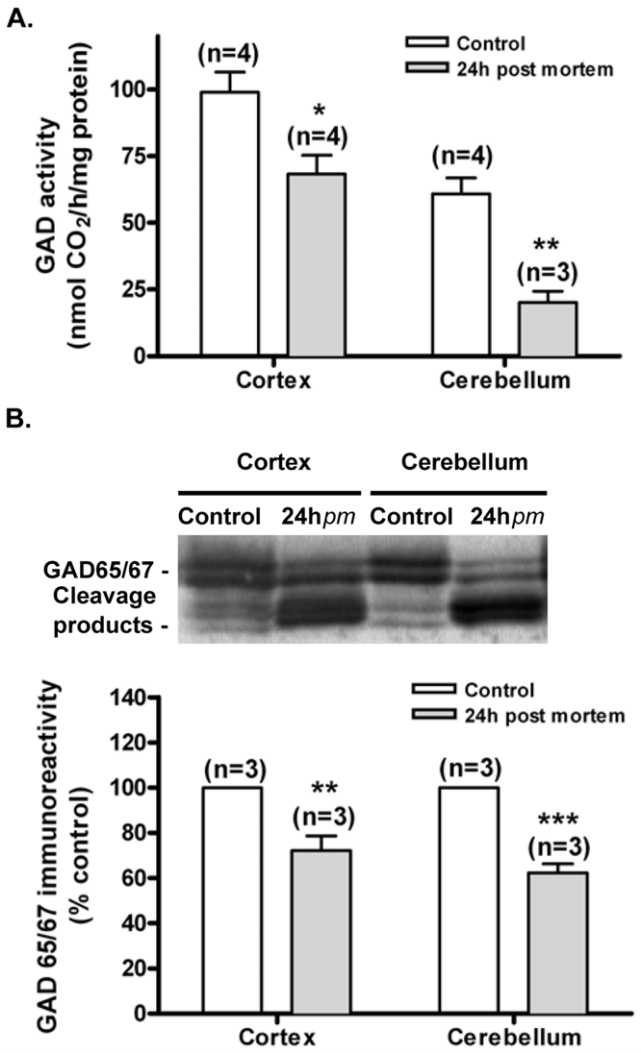


Figure 6. Excitotoxicity-induced decrease in GAD65/67 activity. Heads of adult Wistar rats were decapitated and processed immediately or kept for 24 h at room temperature. The extracts were used for both GAD activity measurements and Western Blot analysis. GAD activity was determined using a trapping technique for radiolabelled [^{14}C]CO_2 brought by GAD65/67 activity, and was expressed as nmol CO_2/hr/mg of protein (A). Full-length GAD65/67 protein levels from the same extracts were determined by Western Blot using an anti-GAD65/67 antibody, and control protein levels of GAD65/67 were set to 100% (B). Data are presented as mean±SEM of 3 to 4 independent experiments. Statistical analysis was performed using Student's *t*-test. *$p<0.05$; **$p<0.01$: ***$p<0.001$.
doi:10.1371/journal.pone.0010139.g006

evidences strongly suggest that calpains play a key role (if not exclusive) in GAD cleavage under excitotoxic conditions. Surprisingly, we observed that inhibition of the proteasome with MG132, lactacystin or YU102 fully abrogated the cleavage of GAD67 in hippocampal neurons subjected to excitotoxic conditions, and MG132 had the same effect on the cleavage of GAD65. Furthermore, inhibition of the ubiquitin-activating enzyme (E1) with UBEI-41 also prevented the cleavage of GAD67 and GAD65. The effects of MG132 and UBEI-41 cannot be attributed to inhibition of calpains since the inhibitors had a small (MG132) or no effect (UBEI-41) on the glutamate-evoked calpain activation, as determined by measuring the formation of spectrin breakdown products (Fig. 4C). Since the molecular weight of the GAD truncated forms observed in the present studies is similar to that observed in previous studies where calpains were shown to participate in the cleavage of the enzyme, it is likely that the UPS and calpains act in a co-ordinated manner to cleave GADs. If this is the case, the UPS is likely to act upstream of calpains since no evidences were found for a direct effect of proteasome in GAD cleavage.

Interaction between calpains and the UPS is also physiologically relevant in other scenarios. Sequential activity of calpains and the UPS has been proposed to contribute to the degradation of myofibrils; calpains release myofibrils form the contractile apparatus, which allows initiating ubiquitination and degradation by the proteasome [60,61]. Also, degradation of IκB through phosphorylation-dependent ubiquitination or following cleavage by calpains is thought to release NFκB, and the transcription factor migrates to the nucleus where it binds DNA [62–64]. There are reports suggesting that proteasome inhibition could be neuroprotective after stroke [31,65], namely through stabilization of IκB and thereby preventing NF-κB activation. Inhibition of calpains can also provide functional neuroprotection in various animal models of cerebral ischemia [66].

The role of ubiquitination in GADs cleavage

The glutamate-evoked cleavage of GAD65/67 was sensitive to proteasome inhibition, but incubation of GAD65 with the 20S proteasome did not give rise to the cleavage product of the enzyme. Although some proteins are cleaved by the 20S proteasome without ubiquitination [51,52], this is not the case of GAD65.

Inhibition of the E1 ubiquitinating enzyme also abrogated the cleavage of GAD65/67 under excitotoxic conditions, indicating that protein ubiquitination plays a key role in the process. Separation of mono and poly-ubiquitinated proteins with the UbiQapture^{TM}-Q Kit allowed recovering GAD65 and GAD67, but although there was an increase in the amount of GAD isolated with the kit under excitotoxic conditions the apparent molecular weight of the proteins isolated was similar to that observed in whole cell extracts. This strongly suggests that GAD is not ubiquitinated, but instead interacts with a protein which state of ubiquitination is increased following excitotoxic stimulation with glutamate. This may be related with an increase in ubiquitin mRNA transcripts, as observed following ischemia [67,68], with an impairment of the proteasome activity [29,30,69], and/or to a signalling cascade induced by excitotoxicity that may ultimately leads to the ubiquitination of the GAD binding partner. Excitotoxicity also regulates the NF-κB transcription factor after cerebral ischemia through ubiquitination and degradation of its binding partner IκB [70]. Similarly, the cleavage of GAD may follow the increase in ubiquitination and degradation of a binding partner, which may allow cleavage of the enzyme by calpains. This hypothesis explains the effect of both E1 inhibition and proteasome inhibition on GAD cleavage, and the results showing no apparent ubiquitination of the enzyme (present work), and the role of calpains [24]. The GAD binding partner that may be involved in the regulation of the protein under excitotoxic conditions remains to be identified.

Interestingly, the proteasome inhibitors showed differential effects on GAD65 and GAD67 cleavage induced by excitotoxic stimulation with glutamate. MG132 abrogated the cleavage of both GAD isoforms, in contrast with YU102 and lactacystin which were only effective against the cleavage of GAD67. The difference in the effects of the inhibitors tested may be due to their specificities: MG132 and YU102 act preferentially on the cheymotrypsin-like and caspase-like activities of the proteasome, respectively, whereas lactacystin targets preferentially the trypsin-like and caspase-like activities [43,48]. Assuming that under excitotoxic conditions the proteasome targets a GAD binding partner before cleavage of the

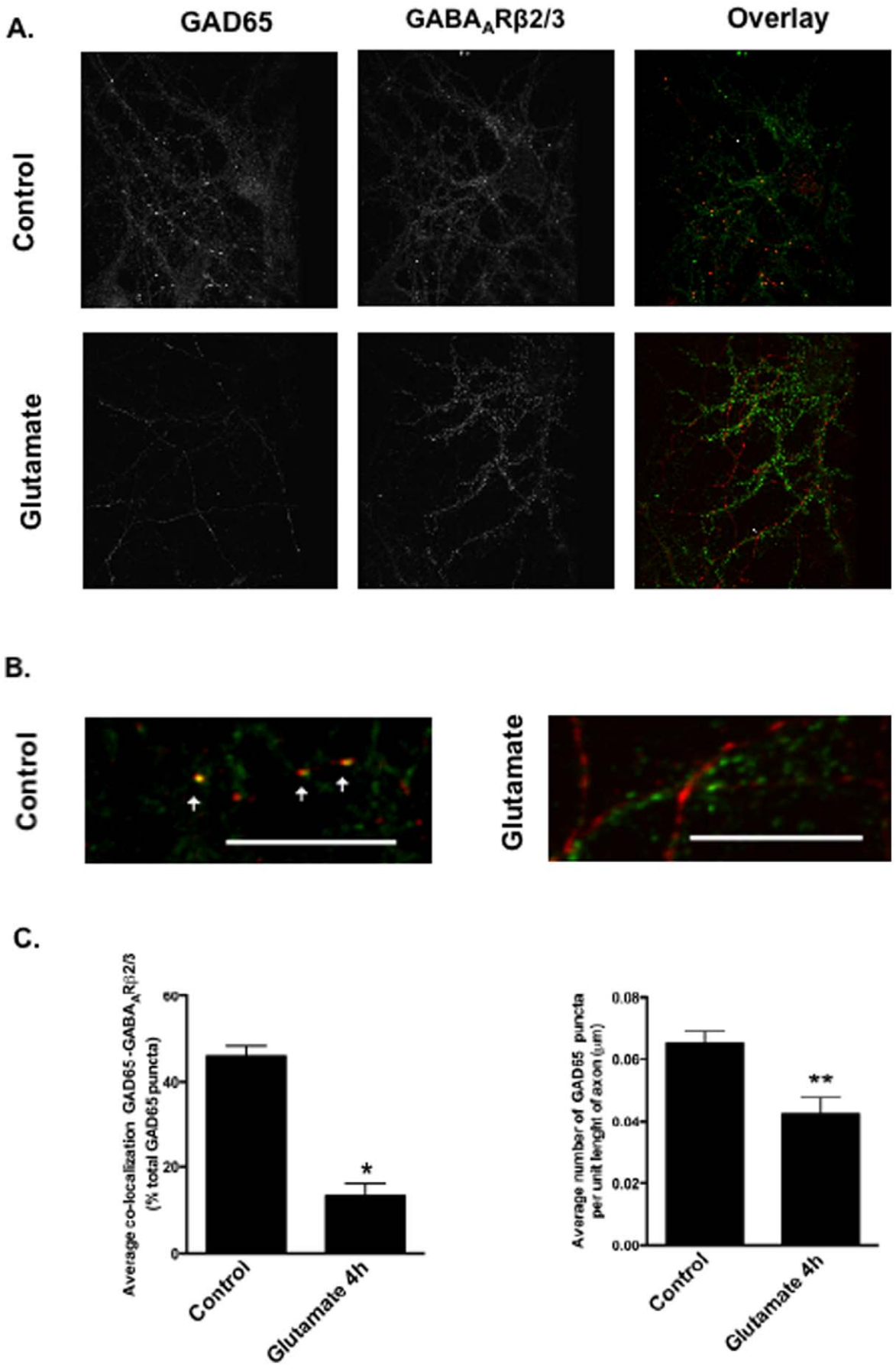

A.
GAD65
GABA$_A$Rβ2/3
Overlay
Control
Glutamate
B.
Control
Glutamate
C.
Average co-localization GAD65-GABA$_A$Rβ2/3 (% total GAD65 puncta)
60
40
20
0
*
Control
Glutamate 4h
Average number of GAD65 puncta per unit lenght of axon (µm)
0.08
0.06
0.04
0.02
0.00
**
Control
Glutamate 4h

Figure 7. Glutamate changes the subcellular distribution of GAD65 along neurites. 10 DIV hippocampal neurons were incubated with or without glutamate (125 μM) for 20 min, and then returned to the original culture medium for 4 h.(A) Cells were fixed, permeabilized and probed with specific antibodies to GAD65 and $GABA_A$ receptor subunits β2/3. (B) Arrows indicate GAD65 clustering (red), a pattern that is changed in glutamate treated cells. Images are representative of three different experiments performed in independent preparations. Images in (B) show colocalization of the immunoreactivity for GAD65 (red) and $GABA_A$ receptor subunits β2/3 (green) under control conditions, and the redistribution of GAD65 in the axons of hippocampal neurons subjected to excitotoxic conditions. The scale bar corresponds to 10 μm. (C) Quantification of the images for the colocalization of GAD65 and $GABA_A$ subunits β2/3, expressed as percentage of total GAD65 puncta (left), and the average number of GAD65 puncta per axon unit length (right). Data are presented as mean±SEM of 3 independent experiments performed in different preparations. Statistical analysis was performed using Student's *t*-test. *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pone.0010139.g007

enzymes by calpains, these results suggest that the proteasome substrates bound to each of the GAD isoforms are distinct.

Alterations of GADs activity and localization under excitotoxic conditions

The decrease in GADs activity observed in *post mortem* cerebral cortices to 74% was correlated with a decrease to 73% of the full-length protein found in the extracts analysed by Western Blot; in the cerebellum the GAD activity decrease to 48% in *post mortem* extracts relative to control, and a down-regulation of the full-length protein to 58% was observed. This decrease in enzyme activity following N-terminal cleavage is in agreement with previous results obtained using a similar experimental paradigm [53] and with the effect of calpain cleavage at the N-terminal region on the activity of recombinant GAD67 [22]. In contrast, truncation of the N-terminal region of recombinant GAD65 increased enzyme activity [71] and trypsin cleavage of recombinant GAD65 and GAD67 at their N-terminal region was shown to increase enzyme activity [56]. The difference between the effects observed in brain extracts and in *in vitro* experiments may be due to interaction of GAD with regulatory proteins (see below), which are absent when recombinant proteins are used. Post-translational modifications of GAD, such as phosphorylation, may also contribute to the differences in the effect of N-terminal cleavage on the activity of the enzyme measured in brain extracts or using recombinant protein [72]. A decrease in enzyme activity in neurons subjected to excitotoxic conditions may activate compensatory mechanisms in surviving neurons since the expression of GAD is regulated by the abundance of GABA by a mechanism independent of the activation of GABA receptors [73,74]. However, within the time frame analysed after the excitotoxic insult we found no evidences for an upregulation of GAD protein levels from de novo protein synthesis (Fig. 2).

In this work we also found that excitotoxic stimulation of cultured hippocampal neurons changes the subcellular distribution of GAD65, with a loss of protein clustering along neurites. This is in agreement with the results showing a role for palmitoylation of Cys30 and Cys45 in GAD65 in the post-Golgi trafficking of the protein to presynaptic clusters [75,76]. The N-terminal truncation under excitotoxic conditions is likely to separate this targeting sequence from the catalytic domain of GAD65, dissociating the enzyme from synaptic vesicles as suggested in the results of the immunocytochemistry experiments shown in Fig. 7. Furthermore, the decrease in colocalization of GAD65 and the β2/3 $GABA_A$ receptor subunits suggests that the cleaved protein becomes more diffuse, moving away from the synapse.

It was proposed that association of GAD with membranes and the anchoring of the enzyme to synaptic vesicles occur first through formation of a complex with the heat shock protein 70 family member HSC70 (heat shock cognate 70), followed by interaction with cysteine string protein (CSP), an integral protein of the synaptic vesicle [54]. Cleavage or degradation of the GAD anchoring proteins may release the enzymes anchored to synaptic vesicles and may contribute to change the subcellular distribution of the enzyme under excytotoxic conditions. If the N-terminal region of GAD65 plays a role in the interaction with the anchoring proteins, the cleavage of the enzyme under excitotoxic conditions may also explain the observed changes in immunoreactivity after the toxic insult with glutamate. The interaction of GAD with HSC70 and synaptic vesicles also promotes the activity of the enzyme [54]. The release of GAD65 from synaptic vesicles that may occur under excitotoxic conditions would explain, at least in part, the decrease in enzyme activity observed in cerebellar and cerebrocortical extracts containing cleaved GAD.

The anchoring of GAD65 to synaptic vesicles through interaction with the vesicular GABA transporter may allow coupling the synthesis of GABA to the packaging of the neurotransmitter into the vesicles [17]. The cleavage of the N-terminal region of GAD65 and the consequent dissociation of the enzyme from synaptic vesicles and from the synapse may decrease the accumulation of GABA in the vesicles and, therefore, may deregulate GABAergic synapses. This is particularly relevant considering that GAD65 is the isoform responsible for the synaptically released GABA [77].

In conclusion, we showed that excitotoxic conditions lead to the cleavage of GAD65/67 in cultured hippocampal neurons in a UPS-dependent manner. GAD cleavage decreased enzyme activity and changed the subcellular distribution of the 65KDa isoform, which should decrease GABA production and may affect the accumulation of the neurotransmitter in synaptic vesicles.

Acknowledgments

We thank Dr. K.M. Frick (Yale University, New Haven, CT) for the help provided in setting up the assay for measuring GAD activity and Elisabete Carvalho for assistance in the preparation of cell cultures. The help provided by Cláudio A. Joazeiro (Genomics Institute of the Novartis Research Foundation) with experimental design and the technical assistance of Angela Luzader with the neuronal cultures at the Aninal vivarium at GNF is also greatly appreciated. The mousse monoclonal antibody anti-$GABA_A$Receptor β2/3 was a generous gift from Ami Citri (R. Malenka Lab). We would also like to acknowledge Christoph Mass (C. Garner Lab) for the help with the MetaMorph analysis and Dr. Craig C. Garner for allowing us to perform some of the imaging experiments in his laboratory.

Author Contributions

Conceived and designed the experiments: MSB CVM MB RDA CBD. Performed the experiments: MSB CVM MA DH DOP GL MVC RDA CBD. Analyzed the data: MSB CVM MB CBD. Contributed reagents/materials/analysis tools: BAB. Wrote the paper: MSB CVM CBD.

References

1. Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330: 613–622.
2. Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system, Neuron 1: 623–634.

3. Gardoni F, Di Luca M (2006) New targets for pharmacological intervention in the glutamatergic synapse. Eur J Pharmacol 545 (2006): 2–10.
4. Lewen A, Li GL, Olsson Y, Hillered L (1996) Changes in microtubule-associated protein 2 and amyloid precursor protein immunoreactivity following traumatic brain injury in rat: influence of MK-801 treatment. Brain Res 719: 161–171.
5. Carvalho AP, Ferreira IL, Carvalho AL, Duarte CB (1995) Glutamate receptor modulation of [^{3}H]GABA release and intracellular calcium in chick retina cells. Ann N Y Acad Sci 757: 439–456.
6. Saransaari P, Oja SS (2008) GABA release under normal and ischemic conditions. Neurochem Res 33: 962–969.
7. Schwartz-Bloom RD, Sah R (2001) gamma-Aminobutyric acid$_A$ neurotransmission and cerebral ischemia. J Neurochem 77: 353–371.
8. Vemuganti R (2005) Decreased expression of vesicular GABA transporter, but not vesicular glutamate, acetylcholine and monoamine transporters in rat brain following focal ischemia. Neurochem Int 47: 136–142.
9. Baliova M, Knab A, Franekova V, Jursky F (2009) Modification of the cytosolic regions of GABA transporter GAT1 by calpain. Neurochem Int 55: 288–294.
10. Bevers MB, Neumar RW (2008) Mechanistic role of calpains in postischemic neurodegeneration. J Cereb Blood Flow Metab 28: 655–673.
11. Martin DL, Rimvall K (1993) Regulation of gamma-aminobutyric acid synthesis in the brain, J Neurochem 60: 395–407.
12. Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ (1991) Two genes encode distinct glutamate decarboxylases. Neuron 7: 91–100.
13. Sheikh SN, Martin SB, Martin DL (1999) Regional distribution and relative amounts of glutamate decarboxylase isoforms in rat and mouse brain. Neurochem Int 35: 73–80.
14. Hsu CC, Thomas C, Chen W, Davis KM, Foos T, et al. (1999) Role of synaptic vesicle proton gradient and protein phosphorylation on ATP-mediated activation of membrane-associated brain glutamate decarboxylase. J Biol Chem 274: 24366–24371.
15. Nathan B, Floor E, Kuo CY, Wu JY (1995) Synaptic vesicle-associated glutamate decarboxylase: identification and relationship to insulin-dependent diabetes mellitus. J Neurosci Res 40: 134–137.
16. Reetz A, Solimena M, Matteoli M, Folli F, Takei K, et al. (1991) GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. EMBO J 10: 1275–1284.
17. Jin H, Wu H, Osterhaus G, Wei J, Davis K, et al. (2003) Demonstration of functional coupling between gamma -aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles, Proc Natl Acad Sci U S A 100: 4293–4298.
18. Wu H, Jin Y, Buddhala C, Osterhaus G, Cohen E, et al. (2007) Role of glutamate decarboxylase (GAD) isoform, GAD65, in GABA synthesis and transport into synaptic vesicles-Evidence from GAD65-knockout mice studies. Brain Res 1154: 80–83.
19. Asada H, Kawamura Y, Maruyama K, Kume H, Ding R, et al. (1996) Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. Biochem Biophys Res Commun 229: 891–895.
20. Kaufman DL, Houser CR, Tobin AJ (1991) Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem 56: 720–723.
21. Lernmark A (1996) Glutamic acid decarboxylase–gene to antigen to disease. J Intern Med 240: 259–277.
22. Sha D, Jin Y, Wu H, Wei J, Lin CH, et al. (2008) Role of mu-calpain in proteolytic cleavage of brain L-glutamic acid decarboxylase. Brain Res 1207: 9–18.
23. Wei J, Lin CH, Wu H, Jin Y, Lee YH, et al. (2006) Activity-dependent cleavage of brain glutamic acid decarboxylase 65 by calpain. J Neurochem 98: 1688–1695.
24. Monnerie H, Le Roux PD (2008) Glutamate alteration of glutamic acid decarboxylase (GAD) in GABAergic neurons: the role of cysteine proteases. Exp Neurol 213: 145–153.
25. Monnerie H, Le Roux PD (2007) Reduced dendrite growth and altered glutamic acid decarboxylase (GAD) 65- and 67-kDa isoform protein expression from mouse cortical GABAergic neurons following excitotoxic injury in vitro. Exp Neurol 205: 367–382.
26. Vanderklish PW, Bahr BA (2000) The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 81: 323–339.
27. Herrmann J, Lerman LO, Lerman A (2007) Ubiquitin and ubiquitin-like proteins in protein regulation. Circ Res 100: 1276–1291.
28. Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67: 425–479.
29. Ge P, Luo Y, Liu CL, Hu B (2007) Protein aggregation and proteasome dysfunction after brain ischemia. Stroke 38: 3230–3236.
30. Asai A, Tanahashi N, Qiu JH, Saito N, Chi S, et al. (2002) Selective proteasomal dysfunction in the hippocampal CA1 region after transient forebrain ischemia. J Cereb Blood Flow Metab 22: 705–710.
31. Phillips JB, Williams AJ, Adams J, Elliott PJ, Tortella FC (2000) Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia. Stroke 31: 1686–1693.
32. Williams AJ, Hale SL, Moffett JR, Dave JR, Elliott PJ, et al. (2003) Delayed treatment with MLN519 reduces infarction and associated neurologic deficit caused by focal ischemic brain injury in rats via antiinflammatory mechanisms involving nuclear factor-kappaB activation, gliosis, and leukocyte infiltration. J Cereb Blood Flow Metab 23: 75–87.
33. Zhang L, Zhang ZG, Zhang RL, Lu M, Adams J, et al. (2001) Postischemic (6-Hour) treatment with recombinant human tissue plasminogen activator and proteasome inhibitor PS-519 reduces infarction in a rat model of embolic focal cerebral ischemia. Stroke 32: 2926–2931.
34. Solimena M, Dirkx Jr. R, Radzynski M, Mundigl O, De Camilli P (1994) A signal located within amino acids 1-27 of GAD65 is required for its targeting to the Golgi complex region. J Cell Biol 126: 331–341.
35. Santos AR, Duarte CB (2008) Validation of internal control genes for expression studies: effects of the neurotrophin BDNF on hippocampal neurons. J Neurosci Res 86: 3684–3692.
36. Kubista K, Sindelka R, Tichopad A, Bergkvist A, Lindh D, et al. (2007) The prime technique: real-time PCR data analysis, G.I.T. Laboratory Journal 9: 2.
37. Bahr BA (2000) Integrin-type signaling has a distinct influence on NMDA-induced cytoskeletal disassembly. J Neurosci Res 59: 827–832.
38. Munirathinam S, Rogers G, Bahr BA (2002) Positive modulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. Toxicol Appl Pharmacol 185: 111–118.
39. Frick KM, Burlingame LA, Delaney SS, Berger-Sweeney J (2002) Sex differences in neurochemical markers that correlate with behavior in aging mice. Neurobiol Aging 23: 145–158.
40. Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, et al. (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 12: 1329–1343.
41. Duarte CB, Ferreira IL, Santos PF, Oliveira CR, Carvalho AP (1993) Glutamate increases the $[Ca^{2+}]_i$ but stimulates Ca^{2+}-independent release of [^{3}H]GABA in cultured chick retina cells. Brain Res 611: 130–138.
42. Swanwick CC, Harrison MB, Kapur J (2004) Synaptic and extrasynaptic localization of brain-derived neurotrophic factor and the tyrosine kinase B receptor in cultured hippocampal neurons. J Comp Neurol 478: 405–417.
43. Kisselev AF, Goldberg AL (2001) Proteasome inhibitors: from research tools to drug candidates. Chem Biol 8: 739–758.
44. Rock KL, Gramm C, Rothstein L, Clark K, Stein R, et al. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78: 761–771.
45. Myung J, Kim KB, Crews CM (2001) The ubiquitin-proteasome pathway and proteasome inhibitors. Med Res Rev 21: 245–273.
46. Rideout HJ, Stefanis L (2002) Proteasomal inhibition-induced inclusion formation and death in cortical neurons require transcription and ubiquitination. Mol Cell Neurosci 21: 223–238.
47. Butts BD, Hudson HR, Linseman DA, Le SS, Ryan KR, et al. (2005) Proteasome inhibition elicits a biphasic effect on neuronal apoptosis via differential regulation of pro-survival and pro-apoptotic transcription factors. Mol Cell Neurosci 30: 279–289.
48. Myung J, Kim KB, Lindsten K, Dantuma NP, Crews CM (2001) Lack of proteasome active site allostery as revealed by subunit-specific inhibitors. Mol Cell 7: 411–420.
49. Yang Y, Kitagaki J, Dai RM, Tsai YC, Lorick KL, et al. (2007) Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. Cancer Res 67: 9472–9481.
50. Caba E, Brown QB, Kawasaki B, Bahr BA (2002) Peptidyl alpha-keto amide inhibitor of calpain blocks excitotoxic damage without affecting signal transduction events. J Neurosci Res 67: 787–794.
51. Sorokin AV, Selyutina AA, Skabkin MA, Guryanov SG, Nazimov IV, et al. (2005) Proteasome-mediated cleavage of the Y-box-binding protein 1 is linked to DNA-damage stress response. EMBO J 24: 3602–3612.
52. Baugh JM, Pilipenko EV (2004) 20S proteasome differentially alters translation of different mRNAs via the cleavage of eIF4F and eIF3. Mol Cell 16: 575–586.
53. Martin SB, Waniewski RA, Battaglioli G, Martin DL (2003) Post-mortem degradation of brain glutamate decarboxylase, Neurochem Int 42: 549–554.
54. Hsu CC, Davis KM, Jin H, Foos T, Floor E, et al. (2000) Association of L-glutamic acid decarboxylase to the 70-kDa heat shock protein as a potential anchoring mechanism to synaptic vesicles, J Biol Chem 275: 20822–20828.
55. Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR (1994) Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms, J Neurosci 14: 1834–1855.
56. Battaglioli G, Liu H, Hauer CR, Martin DL (2005) Glutamate decarboxylase: loss of N-terminal segment does not affect homodimerization and determination of the oxidation state of cysteine residues, Neurochem Res 30, 989–1001.
57. Fenalti G, Law RH, Buckle AM, Langendorf C, Tuck K, et al. (2007) GABA production by glutamic acid decarboxylase is regulated by a dynamic catalytic loop. Nat Struct Mol Biol 14: 280–286.
58. Salin P, Chesselet MF (1993) Expression of GAD (M_r 67,000) and its messenger RNA in basal ganglia and cerebral cortex after ischemic cortical lesions in rats. Exp Neurol 119: 291–301.
59. Qin ZH, Zhang SP, Weiss B (1994) Dopaminergic and glutamatergic blocking drugs differentially regulate glutamic acid decarboxylase mRNA in mouse brain. Brain Res Mol Brain Res 21: 293–302.
60. Jackman RW, Kandarian SC (2004) The molecular basis of skeletal muscle atrophy. Am J Physiol Cell Physiol 287: C834–843.

61. Bartoli M, Richard I (2005) Calpains in muscle wasting. Int J Biochem Cell Biol 37: 2115–2133.
62. Alkalay I, Yaron A, Hatzubai A, Orian A, Ciechanover A, et al. (1995) Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 92: 10599–10603.
63. Scherer DC, Brockman JA, Chen Z, Maniatis T, Ballard DW (1995) Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proc Natl Acad Sci U S A 92: 11259–11263.
64. Chen F, Lu Y, Kuhn DC, Maki M, Shi X, et al. (1997) Calpain contributes to silica-induced I kappa B-alpha degradation and nuclear factor-kappa B activation. Arch Biochem Biophys 342: 383–388.
65. Di Napoli M, McLaughlin B (2005) The ubiquitin-proteasome system as a drug target in cerebrovascular disease: therapeutic potential of proteasome inhibitors. Curr Opin Investig Drugs 6: 686–699.
66. Ray SK (2006) Currently evaluated calpain and caspase inhibitors for neuroprotection in experimental brain ischemia. Curr Med Chem 13: 3425–3440.
67. Noga M, Hayashi T (1996) Ubiquitin gene expression following transient forebrain ischemia. Brain Res Mol Brain Res 36: 261–267.
68. Vannucci SJ, Mummery R, Hawkes RB, Rider CC, Beesley PW (1998) Hypoxia-ischemia induces a rapid elevation of ubiquitin conjugate levels and ubiquitin immunoreactivity in the immature rat brain. J Cereb Blood Flow Metab 18: 376–385.
69. Liu CL, Martone ME, Hu BR (2004) Protein ubiquitination in postsynaptic densities after transient cerebral ischemia. J Cereb Blood Flow Metab 24: 1219–1225.
70. Shah IM, Lees KR, Pien CP, Elliott PJ (2002) Early clinical experience with the novel proteasome inhibitor PS-519. Br J Clin Pharmacol 54: 269–276.
71. Wei J, Jin Y, Wu H, Sha D, Wu JY (2003) Identification and functional analysis of truncated human glutamic acid decarboxylase 65. J Biomed Sci 10: 617–624.
72. Wei J, Wu JY (2008) Post-translational regulation of L-glutamic acid decarboxylase in the brain. Neurochem Res 33: 1459–1465.
73. de Almeida OM, Gardino PF, Loureiro dos Santos NE, Yamasaki EN, de Mello MC, et al. (2002) Opposite roles of GABA and excitatory amino acids on the control of GAD expression in cultured retina cells, Brain Res 925: 89–99.
74. de Mello FG, Hokoc JN, Ventura AL, Gardino PF (1991) Glutamic acid decarboxylase of embryonic avian retina cells in culture: regulation by gamma-aminobutyric acid (GABA). Cell Mol Neurobiol 11: 485–496.
75. Kanaani J, el-HusseiniAel-D, Aguilera-Moreno A, Diacovo JM, Bredt DS, et al. (2002) A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65. J Cell Biol 158: 1229–1238.
76. Kanaani J, Patterson G, Schaufele F, Lippincott-Schwartz J, Baekkeskov S (2008) A palmitoylation cycle dynamically regulates partitioning of the GABA-synthesizing enzyme GAD65 between ER-Golgi and post-Golgi membranes, J Cell Sci 121: 437–449.
77. Soghomonian JJ, Martin DL (1998) Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci 19: 500–505.

Chapter 2

Spatiotemporal Resolution of BDNF neuroprotection in cultured hippocampal neurons

This study applied a pharmacological approach to examine the role of different protease systems in the degeneration of soma and neurites, and neuroprotection by BDNF, towards further characterizing the cellular damage induced by excitotoxic stimulation with glutamate. Therefore, we analyzed the activation of calpain and caspase-3, and putative alterations in the ubiquitin-proteasome system (UPS), in cell lysates of cultured hippocampal neurons, at different time points after the insult. Calpain activation was the fastest, peaking at 30 min after the excitotoxic insult, gradually decreasing at subsequent time points, whereas caspase activation only reached maximal values at the latest time-points tested. Maximal accumulation of ubiquitin-conjugated proteins was found at intermediate time points, suggesting that the deregulation of the UPS is preceded by calpain activation and decreases upon caspase activation. Furthermore, the spatiotemporal regulation of the neuroprotective mechanisms induced by BDNF was assessed using several key neurobiological markers was assessed, after a toxic glutamate insult, in a time-dependent manner. The neurobiological markers chosen included MAP-2, Neurofilament H, and the vesicular glutamate transporters 1 and 2, enabling the spatial segregation of dendrites and axons, and the assessment of synaptic markers of glutamatergic neurons, which represent approximately 80% of the total neurons in hippocampal cultures. There was a time-dependent decrease in the protein levels and immunoreactivity of the tested markers, prevented upon proteasome and calpain inhibition with specific chemical inhibitors. However, pre-incubation with the pan-caspase inhibitor z-VAD-FMK showed no significant effect in reducing axon and dendritic damage in neurons exposed to glutamate excitotoxicity, unlike its neuroprotective effect by precluding chromatin condensating. Together, these results indicate that distinct degenerative mechanisms are activated in different subcellular locations of the neuron, when comparing soma and neurites.

However, BDNF significantly protected both axons and dendrites from the excitotoxic injury, further suggesting its ability to abrogate different neurodegenerative mechanism. Accordingly, BDNF attenuated calpain activation while simultaneously protecting the vesicular glutamate transporters and glutamatergic function assessed with a glutamate FRET nanosensor, which measured the activity-dependent glutamate exocytotic release. PI3-K and PLCγ chemical inhibitors significantly blocked BDNF protective action, suggesting an activity-dependent mechanism of neuroprotection. The results indicate that neuronal repair after a degenerative insult may start at the synaptic level and BDNF activates different signalling mechanisms to induce neuroprotection in different subcellular compartments. The results also indicate that BDNF may selectively protect glutamatergic neurons, as BDNF failed to protect the protein levels of GAD65 and GAD67, although futher functional studies will be required to confirm this hypothesis.

Neuroscience 237 (2013) 66–86

SPATIOTEMPORAL RESOLUTION OF BDNF NEUROPROTECTION AGAINST GLUTAMATE EXCITOTOXICITY IN CULTURED HIPPOCAMPAL NEURONS

C. V. MELO,[a] S. OKUMOTO,[b†] J. R. GOMES,[a] M. S. BAPTISTA,[a] B. A. BAHR,[c] W. B. FROMMER[b] AND C. B. DUARTE[a,d*]

[a] *CNC – Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal*

[b] *Department of Plant Biology, Carnegie Institution of Washington, 260 Panama Street, Stanford, CA 94305, USA*

[c] *Biotechnology Research and Training Center, University of North Carolina-Pembroke, Pembroke, NC 28372, USA*

[d] *Department of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal*

Abstract—Brain-derived neurotrophic factor (BDNF) protects hippocampal neurons from glutamate excitotoxicity as determined by analysis of chromatin condensation, through activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3-K) signaling pathways. However, it is still unknown whether BDNF also prevents the degeneration of axons and dendrites, and the functional demise of synapses, which would be required to preserve neuronal activity. Herein, we have studied the time-dependent changes in several neurobiological markers, and the regulation of proteolytic mechanisms in cultured rat hippocampal neurons, through quantitative western blot and immunocytochemistry. Calpain activation peaked immediately after the neurodegenerative input, followed by a transient increase in ubiquitin-conjugated proteins and increased abundance of cleaved-caspase-3. Proteasome and calpain inhibition did not reproduce the protective effect of BDNF and caspase inhibition in preventing chromatin condensation. However, proteasome and calpain inhibition did protect the neuronal markers for dendrites (MAP-2), axons (Neurofilament-H) and the vesicular glutamate transporters (VGLUT1-2), whereas caspase inhibition was unable to mimic the protective effect of BDNF on neurites and synaptic markers. BDNF partially prevented the downregulation of synaptic activity measured by the KCl-evoked glutamate release using a Förster (Fluorescence) resonance energy transfer (FRET) glutamate nanosensor. These results translate a time-dependent activation of proteases and spatial segregation of these mechanisms, where calpain activation is followed by proteasome deregulation, from neuronal processes to the soma, and finally by caspase activation in the cell body. Moreover, PI3-K and PLCγ small molecule inhibitors significantly blocked the protective action of BDNF, suggesting an activity-dependent mechanism of neuroprotection. Ultimately, we hypothesize that neuronal repair after a degenerative insult is initiated at the synaptic level.

Key words: brain-derived neurotrophic factor (BDNF), excitotoxicity, dendrites, axons, synapses, neuroprotection.

INTRODUCTION

Excitotoxicity has been proposed as the primordial mechanism of neurodegeneration in chronic neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and others. Moreover, excitotoxicity has a pivotal role in neuronal death induced by acute CNS, insults such as ischemia and traumatic brain injury, and chronic mental disorders (Salinska et al., 2005). Therefore, the clinical and biomedical relevance of excitotoxicty, as well as the need for a deeper understanding of its cellular mechanisms, in order to develop new therapeutic approaches, are unquestionable.

During excitotoxicity, neurons are exposed to toxic concentrations of excitatory neurotransmitters causing an excessive activation of calcium-permeable glutamate receptors (Choi, 1988) and the opening of gap junction hemichannels (Thompson et al., 2006), which account for an acute ionic disarray. Activation of the Na–K–Cl cotransporter isoform 1 (NKCC1) is also thought to play a role in the disruption of ion homeostasis in cerebral ischemia (Beck et al., 2003). The resulting intracellular elevation of Na^+ and Cl^- is responsible for the immediate neuronal swelling, whereas calcium overload leads to protease up-regulation, including activation of calpains (e.g., Bano et al., 2005; Xu et al., 2009; Gomes et al., 2011) and caspases (e.g., Higuchi et al., 2005; Carlsson et al., 2011). Alterations in the ubiquitin–

*Correspondence to: C. B. Duarte, Center for Neuroscience and Cell Biology, University of Coimbra, Largo Marquês de Pombal, 3004-517 Coimbra, Portugal. Tel: +351-304-502-910; fax: +351-239-822-776. E-mail address: cbduarte@ci.uc.pt (C. B. Duarte).

† New address: Department of Plant Pathology, Physiology and Weed Science, 549 Latham Hall, Virginia Tech, Blacksburg, VA 24061, USA.

Abbreviations: BDNF, brain-derived neurotrophic factor; DTT, Dithiothreitol; EGTA, ethylene glycol tetraacetic acid; ERK, extracellular signal-regulated kinase; FRET, Förster (Fluorescence) resonance energy transfer; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAPK, mitogen activated protein kinase; NF-H, neurofilament-H; NMDA, *N*-methyl-D-aspartate; PI3-K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase C-γ; SBDPs, spectrin breakdown products; SEM, standard error of the mean; TBOA, DL-threo-β-benzyloxyaspartic acid; TRPCs, transient receptor potential channels; UPS, ubiquitin-proteasome system; VGLUT1-2, vesicular glutamate transporters.

0306-4522/12 $36.00 © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuroscience.2013.01.054

proteasome system (UPS) were also shown to occur under excitotoxic conditions and in brain ischemia, whereby proteasome inhibition provides neuroprotection under the latter conditions (Di Napoli and McLaughlin, 2005; Caldeira et al., 2013).

Calpains are calcium-activated neutral proteases which target several proteins for degradation, in particular, spectrin (Vanderklish and Bahr, 2000) and actin microfilaments (Chung et al., 2005). The collapsed cytoskeleton proteins, together with vesicles and organelles, accumulate onto focal bead-like swellings or blebs at non-random sites (Bindokas and Miller, 1995), primarily at synaptic contacts enriched in mitochondria, on axons and dendrites, as a result of the impaired retrograde–anterograde transport and inhibition of the mitochondrial respiratory chain complex IV activity (Takeuchi et al., 2005). Downstream of these events involving mitochondrial electron transport defects (Luetjens et al., 2000), mitochondrial cytochrome c release is followed by an increase in caspase-9 and caspase-3 activation, and there is an increased production of superoxide, nitric oxide and other reactive oxygen species (ROS), leading up to apoptotic-like chromatin condensation and, finally, DNA fragmentation, in a panoply of neuronal cells subjected to glutamate excitotoxicty (Dawson and Dawson, 2004). Calpain activation under excitotoxic conditions also contributes to neuronal deregulation due to the abnormal cleavage of several proteins. The plasma membrane Na^+/Ca^{2+} exchanger (NCX) is one of the calpain targets and the cleavage of the transporter impairs the extrusion of Ca^{2+} by the transporter contributing to a $[Ca^{2+}]_i$ overload (Bano et al., 2005). Several other critical proteins for neuronal function, including the *N*-methyl-D-aspartate (NMDA) receptor subunit GluN2B (Simpkins et al., 2003; Zhou and Baudry, 2006), the AMPA receptor subunit GluA1 (Glazner et al., 2000; Lu et al., 2002), and the mGluR1α metabotropic glutamate receptors (Xu et al., 2007) are also cleaved, as well as the vesicular GABA transporter (Gomes et al., 2011), to name a few. Recently, the UPS has also been implicated in glutamate excitotoxicity, through changes in its activity (Ge et al., 2007; Caldeira et al., 2013) or cleavage of proteins by the proteasome such as glutamic acid decarboxylase 65 and 67 isoforms (Baptista et al., 2010).

Brain-derived neurotrophic factor (BDNF) signaling has been studied as a potential therapeutic approach for its TrkB-mediated activation of phosphatidylinositol 3-kinase (PI3-K), Ras/mitogen-activated protein kinase (MAPK) and phospholipase C intracellular signaling pathways, given the widespread cellular implications of glutamate excitotoxicity, as well as the lack of efficacy and side effects of glutamate receptor antagonists in clinical trials aiming to preclude the toxic effects of excessive ionotropic receptor activation (Sheldon and Robinson, 2007). BDNF has been shown to protect hippocampal neurons from glutamate excitotoxicity *in vitro* and *in vivo*, preventing the condensation of chromatin (Almeida et al., 2005; Baptista et al., 2010; Gomes et al., 2012). This protective effect can be mimicked by z-VAD-FMK, a general caspase inhibitor. However, it is not known whether the neurotrophin also prevents the degeneration of axons and dendrites, which would be required to preserve neuronal function. In fact, a single toxic stimulus can activate different degenerative programs in the cell body and in neurites (Berliocchi et al., 2005), being calpains more relevant for the loss of neuronal processes (Higuchi et al., 2005). This clearly indicates that the neuroprotective mechanisms in the cell body may not overlap with the ones observed in axons and dendrites. In this work we used key protein markers of the glutamatergic (VGLUT1 and VGLUT2) and GABAergic (GAD65/67) function, axons (NF-H) and dendrites (MAP-2) in order to further examine the spatiotemporal resolution of BDNF neuroprotective effects in cultured hippocampal neurons subjected to glutamate excitotoxicity.

EXPERIMENTAL METHODS

Hippocampal cultures

Experiments were performed according to the European Union Directive 86/609/EEC for the protection of animals used for experimental and other scientific purposes. Dams were sacrificed by cervical dislocation. Embryos were then surgically removed and sacrificed by decapitation. Animals used in the preparation of cell cultures at the Garner Laboratory, Stanford University, for the glutamate release experiments with the FRET glutamate sensor, were handled according to National and Institutional guidelines.

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18–E19 Wistar rat embryos, after treatment with trypsin (0.06%, for 15 min at 37 °C; GIBCO-Invitrogen) and deoxyribonuclease I (5.36 mg/ml), in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4{\cdot}2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO-Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO-Invitrogen) supplemented with B27 serum-free supplement containing antioxidants (1:50 dilution; GIBCO-Invitrogen #17504044), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6-well plates (870,000 cells/well) coated with poly-D-lysine (0.1 mg/mL), or on poly-D-lysine coated glass coverslips, at a density of 150,000 cells/well (12-well plates). The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37 °C, for 7 or 14 days. Excitotoxic stimulation was performed with 125 μM glutamate in supplemented Neurobasal medium, for 20 min at 37 °C, in a humidified incubator. After stimulation with glutamate the cells were further incubated with the original culture medium for the indicated periods of time. When appropriate, 100 ng/ml BDNF was added to the incubation medium 24 h before stimulation. Fifty micromolar PD150606 (calpain chemical inhibitor; SIGMA) and 50 μM z-VAD-FMK (pan-caspase inhibitor; SIGMA) were added 2 h before the excitotoxic insult whereas 1 μM Lactacystin (proteasome inhibitor; Sigma) was added to the incubation medium 30 min before stimulation.

Preparation of extracts

Hippocampal neurons (DIV7) were washed twice with ice-cold PBS and once more with PBS buffer supplemented with 1 mM Dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF); CLAP: 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml

pepstatin; Sigma–Aldrich Química). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris–HCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with the cocktail of protease inhibitors. After centrifugation at 16,100*g* for 10 min, protein in the supernatants was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific), and the samples were denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue), at 95 °C for 5 min.

Immunoblotting

Protein samples were separated by SDS–PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis), in 12% polyacrylamide gels (or 7.5% gels when spectrin cleavage products were detected), transferred to polyvinylidene (PVDF) membranes (Millipore Corp.), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4 °C), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20000 dilution; 1 h at room temperature) or exposed directly to ECL in the detection of ubiquitin-conjugates. The films were scanned and the optical densities of the bands were measured with appropriate software. Alkaline phosphatase activity was visualized by ECF on the Storm 860 Gel and Blot Imaging System (GE Healthcare). The following primary antibodies were used: anti-VGLUT1 and anti-VLGUT2 (1:1000, Synaptic Systems); anti-neurofilament-H (NF-H) and anti-MAP-2 (1:1000, SIGMA); antibody against calpain-mediated fragment of spectrin/fodrin SBDPs [1:300, (Bahr, 2000; Munirathinam et al., 2002)]; anti-β-Tubulin I (1:10000, Sigma); HRP-conjugated FK2 antibody (1:1000, BIOMOL); anti-cleaved-caspase-3 antibody (1:1000, Cell Signalling); anti-transient receptor potential channels (TRPC) 3 and 6 antibodies (dilution 1:1000, SIGMA).

Immunocytochemistry

For immunocytochemistry, cultured hippocampal neurons were grown on poly-D-lysine coated glass coverslips, at a density of 45×10^3 cells/cm^2, and were then fixed in PBS supplemented with 4% paraformaldehyde/4% sucrose, for 30 min at 4 °C. After fixation the cells were permeabilized with 0.25% Triton X-100 in PBS, for 5 min at room temperature, washed three times in PBS, and then blocked with 20% normal goat serum, for 1 h at room temperature. Incubation with primary antibodies was performed overnight at 4 °C. Next, the cells were washed six times and incubated for 1 h at room temperature with the appropriate secondary antibodies (Alexa Fluor® 488/594 goat anti-rabbit, 1:500 to 1:1000 and/or Texas Red® goat anti-mouse, 1:150; Invitrogen), and finally with Hoechst 33342 (0.5 μg/ml; Sigma) for 10 min at room temperature. The cells were washed three times, mounted on glass slides with the Dako mounting medium and viewed on an Axiovert 200 fluorescence microscope coupled to an Axiocam HRm digital camera (Zeiss).

Image processing and analysis

16-bit images, recorded with an Axiovert 200 fluorescence microscope coupled to an Axiocam HRm digital camera (Zeiss), were converted into 8-bit images before the respective analysis protocol. The camera controller settings (gain, offset and sensitivity) were not altered during image recording from each field of view, and the signal intensity range was optimized in the preparations with hippocampal neurons exposed to excitotoxic stimulation with glutamate. The mean dendritic and axonal segments per neuron, in each condition tested, were assessed using the Scholl analysis plugin on Image J. The mean VGLUT1- and VGLUT2-positive puncta per neuron were assessed using the cell counter and analyze particles plugins on Image J. Briefly, each image was analyzed according to the following sequence of steps: background subtraction, threshold setting, quantification of the total number of neurons on the field of view (cell counter plugin), and quantification of the total number of puncta above the threshold setting (analyze particles plugin). Mean puncta per neuron corresponds to the total number of puncta divided by the total number of cells, averaged from 3 to 5 different fields of view, from four independent experiments.

Determination of the viability of cultured hippocampal neurons with Hoescht 33342

Determination of cell viability was performed by fluorescence microscopy, using the indicator Hoechst 33342 as previously described (Almeida et al., 2005). The cells were stimulated with 125 μM glutamate for 20 min, in Neurobasal medium. After the excitotoxic insult hippocampal neurons were further incubated in conditioned culture medium for 14 h. The nuclear morphology was assessed through staining with Hoechst 33342.

Quantification of activity-dependent exocytotic glutamate release using a glutamate FRET sensor

BL21 (DE3-gold) cells expressing pRSET-FLIPE600n were cultured in LB at room temperature for 2 days (Okumoto et al., 2005). Cells were harvested by centrifugation, re-suspended in extraction buffer (50 mM Sodium Phosphate, 300 mM NaCl, pH7.2), and disrupted by ultrasonication. The FRET glutamate sensor was purified by Talon His-affinity chromatography (Clontech). Binding to the resin was performed in batch at 4 °C, washed in a column with extraction buffer, and then eluted with extraction buffer containing 150 mM imidazole. FRET glutamate sensor was dialysed against Tyrode's buffer prior to use. Emission spectra and ligand titration curves were obtained by using a spectrofluorometer (excitation 433/12 nm; emission 485/12 and 528/12 nm).

Quantification of the exocytotic glutamate release in cultured hippocampal neurons was performed in cultured hippocampal neurons plated in 12-well dishes, at DIV7. Cells were initially washed with Tyrode's solution at 37 °C and depolarized for 60 s with high-K^+ Tyrode's solution (90 mM KCl and 31.5 mM NaCl) supplemented with 10 μM TBOA (DL-threo-β-benzyloxyaspartic acid, Tocris), a competitive non-transportable blocker of excitatory amino acid transporters. The depolarizing solution was replaced immediately after with normal Tyrode's solution containing the titered glutamate FRET sensor and 10 μM TBOA. Quantification of the exocytotic glutamate release was performed by a fluorescence plate reader at an excitation wavelength of 433/12 nm and emission wavelengths of 485/12 and 528/12 nm. The ratio between the fluorescence measured at the two wavelengths was calculated and the results were expressed as a percentage of the control glutamate release, measured in cells not subjected to excitotoxic stimulation.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's test, or Bonferroni test, at a significance level $\alpha = 0.01$ (99% confidence intervals), as indicated in the figure captions.

RESULTS

Excitotoxic damage of cultured hippocampal neurons is mediated by different proteolytic mechanisms

Cultures of rat hippocampal neurons have been widely used as an *in vitro* model of glutamate excitotoxicity. Excitotoxic stimulation with 125 μM glutamate for 20 min, followed by replacement with culture conditioned medium for different periods of time (Fig. 1A), has been previously shown to reduce significant cell viability as assessed by fluorescence microscopy analysis of chromatin condensation using the indicator Hoechst 33342 (Almeida et al., 2005). Under these conditions, about 50% of the hippocampal neurons display an apoptotic-like morphology, as determined 7–14 h after the insult, and neuronal death was blocked by the pan-caspase inhibitor z-VAD-FMK (Fig. 1B), in agreement with previous reports (e.g., Almeida et al., 2005; Ray et al., 2006). A significant neuroprotection was also observed in neurons pre-incubated with BDNF for 24 h before the excitotoxic insult, and in these experiments the neurotrophin was present during the period of the insult and after glutamate stimulation. In contrast, neither the proteasome inhibitor lactacystin (Fig. 1C) nor the calpain inhibitor PD 150606 (Fig. 1D) affected glutamate-induced neuronal death. Therefore, the protective effect of the pan-caspase inhibitor z-VAD-FMK cannot be attributed to calpain inhibition (Wolf et al., 1999).

In order to further characterize the cellular damage induced by excitotoxic stimulation with glutamate, we analyzed the activation of calpain and caspase-3, and the alterations in the ubiquitin–proteasome system (UPS), in cell lysates of cultured hippocampal neurons, at different time points after the insult. Calpain activation was measured by western blot, using an antibody that binds specifically to the product resulting from the cleavage of spectrin by calpains (SBDPs) (Bahr, 2000; Munirathinam et al., 2002). The results show that calpain activation was the fastest, peaking at 30 min after the excitotoxic insult (Fig. 2B), gradually decreasing at subsequent time points. Conversely, caspase-3 activation, assessed by immunoreactivity of the cleaved protease (Fig. 2C), was statistically significant only 5 h after glutamate stimulation, reaching a maximum 14 h after the insult, approximately between 2.5- and 3-fold the % of control. Interestingly, the maximum of FK2 immunoreactivity, a measure of ubiquitin-conjugated proteins, was observed at intermediate time points of 5–8 h after the insult (Fig. 2D). This time course for accumulation of ubiquitinated proteins is in agreement with the reported time-dependent decrease of the proteasome activity in hippocampal neurons subjected to excitotoxic stimulation, as determined with fluorogenic substrates

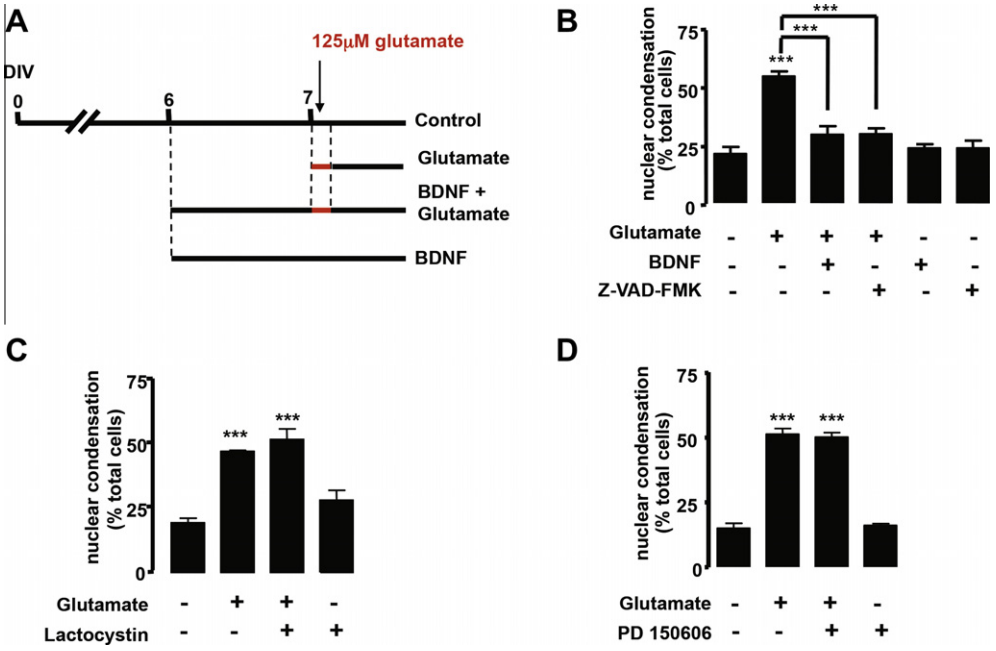


Fig. 1. BDNF protects cultured hippocampal neurons from chromatin condensation induced by glutamate excitotoxicity. Cultured hippocampal neurons were pre-incubated or not with BDNF (100 ng/ml), during 24 h (A), the pan-caspase inhibitor z-VAD-FMK (50 μM), for 2 h (B), the specific proteasome inhibitor Lactacystin (1 μM), 30 min (C), or the calpain inhibitor PD 150606, 50 μM, 2 h (D), before stimulation, or not, with 125 μM glutamate, for 20 min, in fresh Neurobasal medium containing B27 supplement. After excitotoxic stimulation, the cells were returned to the original medium, and further incubated for 14 h (B, D) or 5 h (C), according to the schematic representation. Therefore, the protease inhibitors were also present in the culture media after excitotoxic stimulation (A). A shorter post-incubation with lactacystin was used given the toxicity of the proteasome inhibitor. The total number of cells present in the analyzed fields was calculated based on the number of nuclei stained with the fluorescent dye Hoechst 33342 (B–D). Cell death was assessed 14 h after the insult by fluorescence microscopy using the fluorescence dye Hoechst 33342. Data are presented as mean ± standard error of the mean (SEM) of four independent preparations. Statistical analysis was performed using Bonferroni's Multiple Comparison Test. $^{***}p < 0.001$.

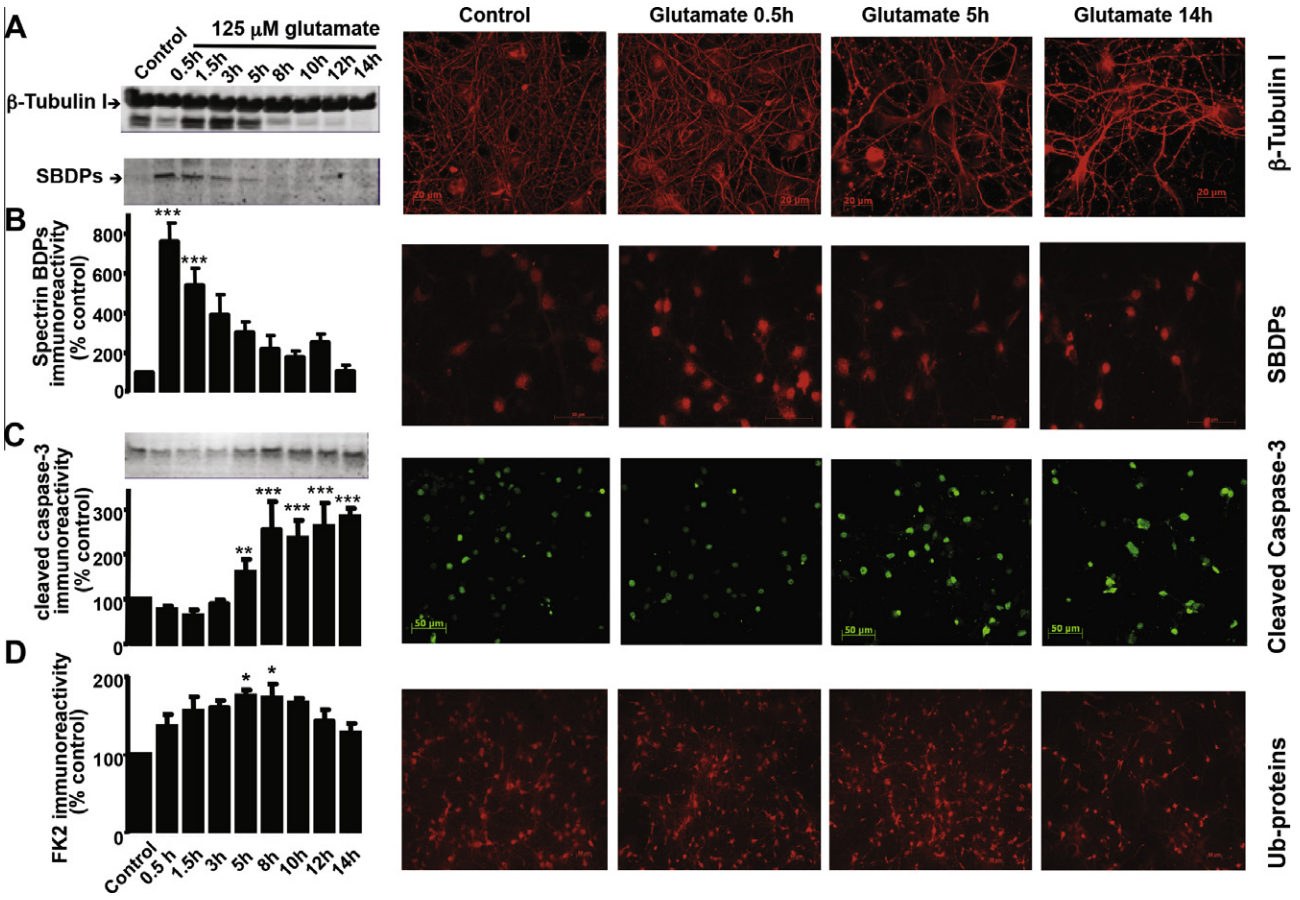


Fig. 2. Glutamate-induced neurodegeneration is linked to a differential regulation of proteolytic mechanisms: Calpain, caspase-3 and ubiquitin–proteasome system. Cultured hippocampal neurons were subjected to a toxic insult of 125 μM glutamate (in Neurobasal medium), for 20 min, and then lysed (western blot experiments) or fixed (immunocytochemistry experiments) after different incubation periods. The extracts were analyzed by immunoblotting with specific antibodies for (A) β-Tubulin I, (B) calpain-mediated formation of spectrin breakdown products (SBDPs), (C) Cleaved caspase-3 or (D) FK2 (monoclonal antibody that recognizes ubiquitin-conjugated proteins). The protein localization was visualized with (B) rabbit Alexa 594-, (C) rabbit Alexa 488- and (B, D) mouse Alexa 594-conjugated secondary antibodies, using a Zeiss Axiovert fluorescence microscope (B) 63×; (A, C) 40×; (D) 20× objectives. Data are presented as mean ± SEM of four independent experiments (B–D). Statistical analysis was performed using the Dunnett's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(Caldeira et al., 2013). The early formation of SBDPs following glutamate stimulation, before caspase-3 activation and downregulation of the proteasome activity, confirms that analysis of spectrin cleavage is an appropriate strategy to investigate the differential activation of proteases in hippocampal neurons subjected to excitotoxic injury. Taken together, these results suggest that the deregulation of the UPS is preceded by calpain activation and decreases at later time points when caspase activation peaks.

Considering the inability of specific calpain and proteasome inhibitors to preclude glutamate-evoked chromatin condensation (Fig. 1C, D), an event occurring in the soma, as opposed to the effect of the caspase inhibitor Z-VAD-FMK (Fig. 1B), and the different periods of activation of the three proteolytic mechanisms (Fig. 2B–D), we hypothesized that the activation of these proteases could occur at different sub-cellular locations in cultured neurons under glutamate excitotoxicity. This was confirmed by visualization of the proteins using fluorescence microscopy in cells fixed at different time points after the insult. Cleaved (active) caspase-3 was only visualized in the cell body, with increasing immunoreactivity from 30 min to 5 h, and an additional increase at 14 h after the insult. These results do not allow ruling out an activation of caspase-3 in neurites, but clearly show a differential effect in the soma and neurite compartments. The increase in SBDPs immunoreactivity and expression of ubiquitin-conjugated proteins in neurites and, to a lesser extent, in the soma, was noted at earlier time points, suggesting a spatiotemporal differentiation of these proteolytic mechanisms. We used β-Tubulin I as protein loading control for immunoblotting, since it is cleaved to a low extent under glutamate excitotoxic conditions (Fig. 2A), but simultaneously allows the visualization of neuritic disarray as a result of the toxic glutamate insult by immunocytochemistry.

Glutamate excitotoxicity downregulates the protein levels of dendritic (MAP-2), axonal (NF-H), and synaptic markers (VGLUT1–2)

The results of the previous section, suggesting temporal and spatial differences in proteolytic activation following excitotoxic stimulation, prompted us to examine the effects of glutamate excitotoxicity on protein levels and

subcellular location of specific neuronal markers for synapses (VGLUT1 and VGLUT2) (Fremeau et al., 2004) and neurites (MAP2, a dendritic marker [Dotti et al., 1988], and NF-H, an axonal marker [Mori et al., 2012]), and whether or not pre-incubation with BDNF would protect these marker proteins in cultured hippocampal neurons.

Cell extracts from cultured hippocampal neurons subjected to glutamate excitotoxicity and analyzed by immunoblotting with specific antibodies for MAP-2 and NF-H (Fig. 3A), as well as VGLUT1 and VGLUT2 (Fig. 3B), showed a time-dependent decrease in abundance of the protein markers. In all cases, a rapid decrease was observed, concomitant with fast activation of calpains (Fig. 2B), with their respective immunoreactivity reaching values close to (for NF-H and VGLUT2) or even below (for MAP-2 and VLGUT1) 50% of the control value (i.e., before the toxic insult) only 5 h after excitotoxic stimulation with glutamate. The fast decrease in the levels of these neuritic and synaptic protein markers clearly precedes caspase activation (Fig. 2C).

Immunocytochemistry experiments were performed to characterize the alterations in neurite length induced by excitotoxic stimulation with glutamate, based on NF-H and MAP2 immunoreactivity, and the effect of inhibitors of the proteasome (lactacystin), calpains (PD150606) and caspases (z-VAD-FMK). The number of VGLUT1 and VGLUT2 puncta were also determined under the same experimental conditions, at 5 h and 14 h after the excitotoxic insult. There was a time-dependent decrease in the length of neurites labeled with the anti-MAP2 or anti-NF-H antibodies (Figs. 3C, D and 4A–C), and a reduction in the number of VGLUT1 and VGLUT2 puncta was also observed (Figs. 3E, F and 4A, D, E). Inhibition of the proteasome and calpain by pre-incubation with lactacystin or PD150606, respectively, significantly reduced the loss of immunofluorescence of all markers tested, when compared with the control (Fig. 4). On the other hand, pre-incubation with the pan-caspase inhibitor z-VAD-FMK showed no significant effect in reducing axon and dendritic damage in neurons exposed to glutamate excitotoxicity.

Taken together these results indicate a temporal and spatial segregation of the proteolytic effects, wherein calpain activation and proteasome deregulation are found in neurites and, to a lesser extent, in the soma, at earlier time periods following the toxic insult, whereas caspase activation is found in the cell body at later stages of the neurodegenerative process triggered by glutamate excitotoxicity.

BDNF protects axonal and dendritic markers from glutamate-induced excitotoxicity

We aimed at determining whether pre-incubation with BDNF prevents or significantly decreases the glutamate-evoked loss of synaptic and neuritic markers, given the results indicating spatiotemporal differences in the activation of the various proteolytic systems in neurites and in the soma. Hippocampal neurons pre-incubated with BDNF before excitotoxic stimulation with glutamate showed significantly higher levels of the dendritic (MAP-2) and axonal (NF-H) markers, when compared with cells subjected to the toxic insult in the absence of BDNF, at 5 h (for MAP-2) or 14 h (for NF-H) after the insult (Fig. 5D, E), as determined by western blot. In contrast, incubation of hippocampal neurons with the neurotrophin for 5 or 14 h under control conditions did not affect MAP-2 and NF-H protein levels.

We visualized populations of fixed hippocampal neurons, cultured under the same conditions and labeled by immunocytochemistry with specific antibodies for the neuritic markers, using low-magnification fluorescence microscopy (as shown in Fig. 4), to further understand the protective effect of BDNF under excitotoxic conditions. We found that primary dendrites and axons are still abundant in neurons pre-incubated with BDNF, 14 h after the insult, in contrast with neurons that were not pre-incubated with BDNF, which displayed severe loss of neurites (Fig. 5A), at both timepoints. In comparison with the control condition (cells not subjected to glutamate excitotoxicity), the conditions in which BDNF preceded the glutamate insult clearly show similar number of primary neurites, although with a decreased intensity of the signal, when visualized with the same imaging parameters.

We sought to determine whether pre-incubation with BDNF modulates the glutamate-evoked activation of proteases, given the spatiotemporal differentiation between calpains and caspases (Fig. 2), and the predominantly protective effect of calpain inhibition over the conservation of neurites (Fig. 4 as opposed to Fig. 1). In order to answer this question, we analyzed the protein levels of SBDPs (Fig. 5F) under the experimental conditions used to analyze alterations in MAP-2 and NF-H protein levels. We found that pre-incubation with BDNF does reduce the formation of SBDPs as determined 30 min (not shown) or 5 h after excitotoxic stimulation (Fig. 5F), which is relevant considering that calpains are the first of all proteases analyzed to reach the peak activation following the toxic glutamate insult. Under control conditions, in the absence of glutamate, no SBDPs immunoreactivity was detected. In control experiments, we tested the effect of Z-VAD-FMK on glutamate-evoked accumulation of SBDPs, 5 and 14 h after the toxic insult. Surprisingly, the pan-caspase inhibitor increased the formation of spectrin breakdown products, both at 5 and 14 h after the toxic insult (Fig. 5G), possibly due to inhibition of the enzymes responsible for further degradation of calpain-generated spectrin breakdown products.

Overall, this indicates that intracellular protective mechanisms triggered by exogenous application of BDNF may, directly or indirectly, interfere with calpain activation, decreasing the extent to which axons and dendrites are lost after the toxic insult, since pre-incubation with BDNF reduces glutamate-evoked calpain activation.

BDNF selectively preserves glutamatergic markers after an excitotoxic injury

The abundance of VGLUT1 and VGLUT2, key markers of excitatory synaptic activity, which showed a time-

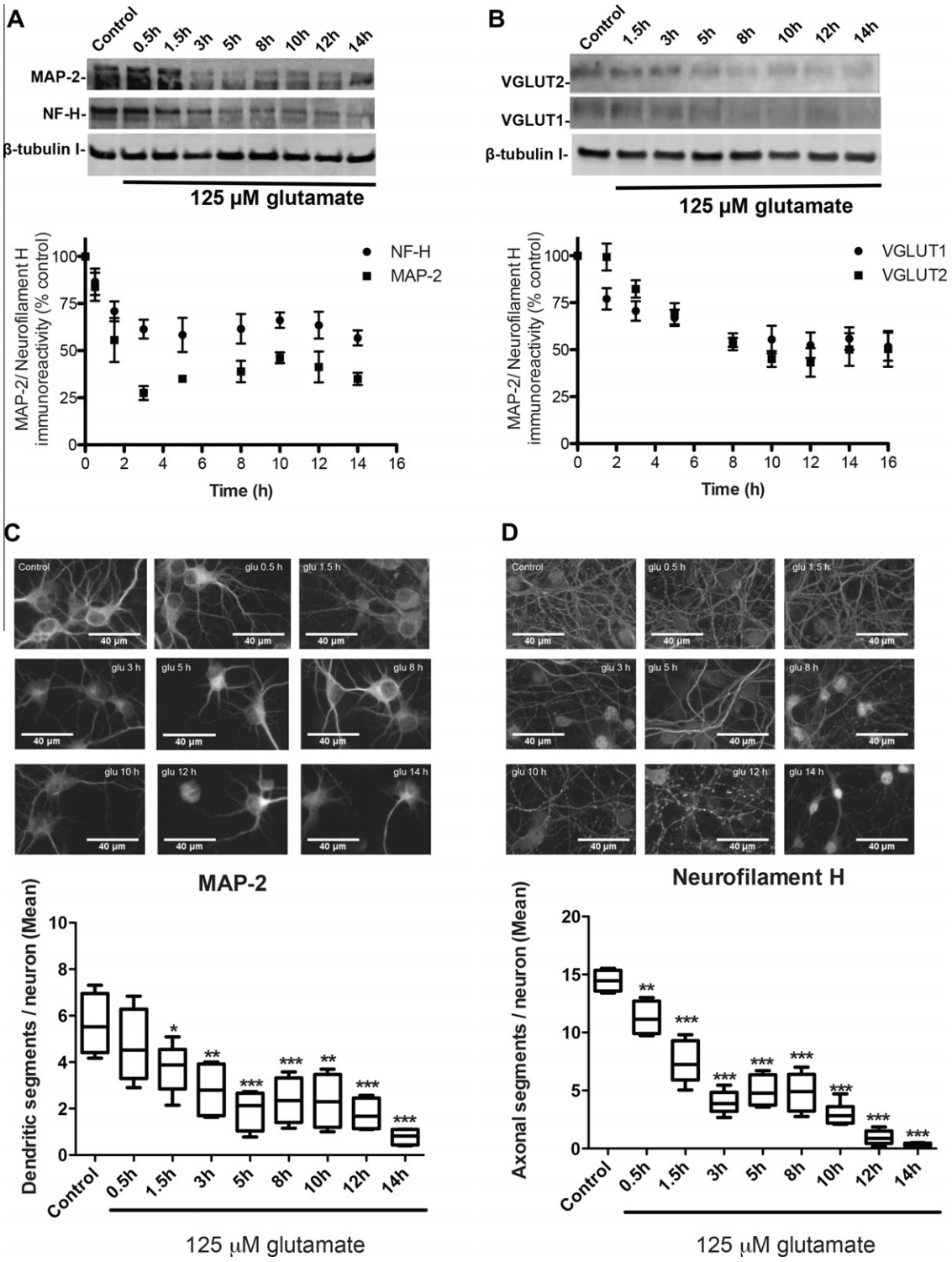


Fig. 3. Glutamate excitotoxicity induces a time-dependent decrease in the protein levels of the neuronal markers for dendrites (MAP-2), axons (Neurofilament H), and synaptic markers (VGLUT1-2). (A, B) Cultured hippocampal neurons were challenged with 125 μM glutamate, for 20 min, and then lysed after different incubation periods. The extracts were analyzed by immunoblotting with specific antibodies for (A) MAP-2 and Neurofilament-H, (B) VGLUT1 and VGLUT2. The upper panels show the results of representative experiments, and the lower panels show the quantification of the immunoreactivity. Data are presented as mean ± SEM of at least four independent experiments. (C–F) Hippocampal neurons were challenged with 125 μM glutamate, for 20 min, and then fixed in PBS supplemented with 4% paraformaldehyde/4% sucrose, for 30 min at 4 °C, after different incubation periods. Following permeabilization, cells were incubated overnight at 4 °C with anti-MAP-2 (C), anti-NF-H (D), anti-VGLUT1 (E) or anti-VGLUT2 (F) antibodies. Assessment of the mean dendritic (C) and axonal (D) segments per neuron was performed using the Sholl analysis plugin on Image J. Mean VGLUT1 (E) and VGLUT2 (F) puncta per neuron were assessed using the cell counter and analyze particles plugins on Image J (C–F). Data are presented as mean ± SEM of four different experiments, performed in independent preparations (B–D). Statistical analysis was performed using the Dunnett's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

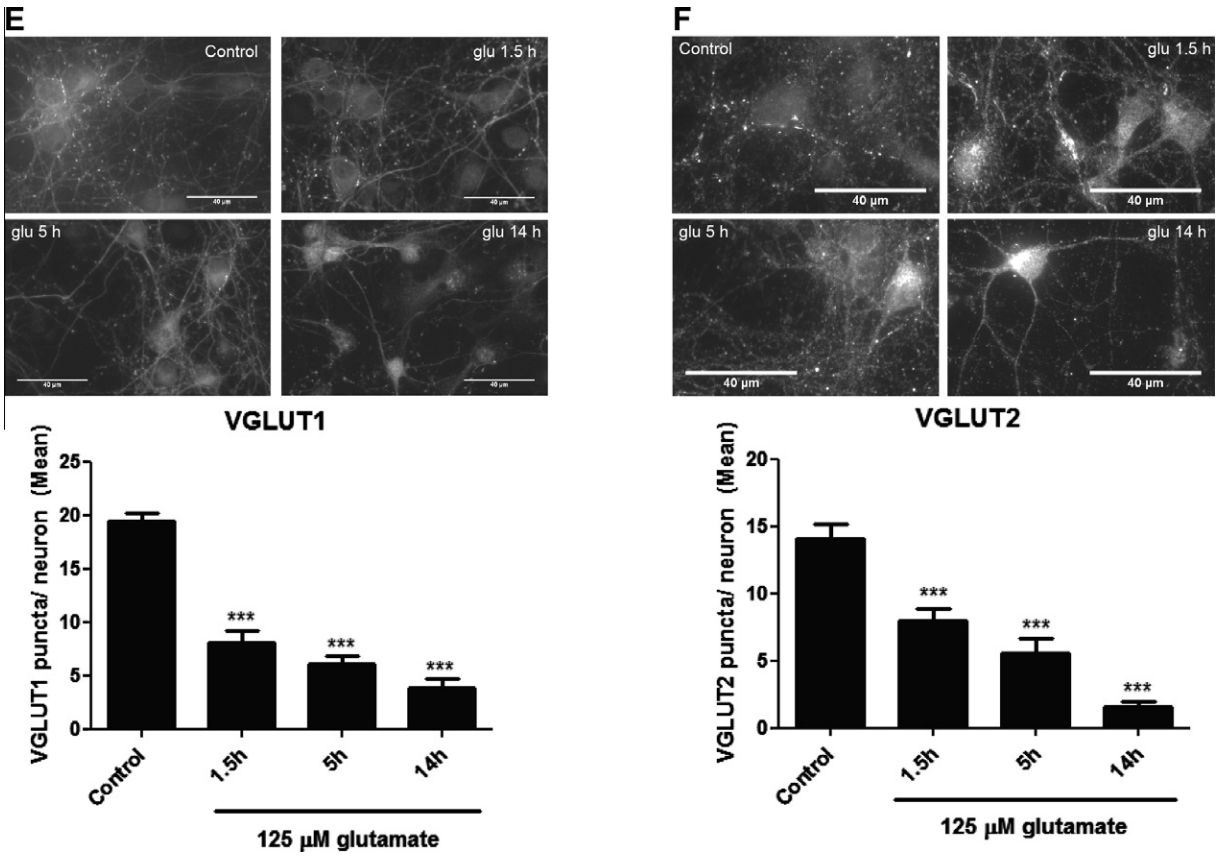


Fig. 3. (*continued*)

dependent decrease in their protein levels after a toxic glutamate insult (Fig. 3B), was also significantly affected in hippocampal neurons pre-incubated with BDNF before excitotoxic stimulation. Under the latter conditions, glutamate stimulation was less effective in down-regulating VGLUT proteins levels, as determined 14 h after the insult (Fig. 6D, E, respectively). Interestingly, incubation of hippocampal neurons with BDNF under control conditions, for 5 or 14 h, upregulated VGLUT2 proteins levels (Fig. 6E).

Immunocytochemistry experiments showed a loss of axonal labeling (Fig. 6A) and VGLUT1/2 puncta (Fig. 6B, C) in cultured hippocampal neurons 14 h after excitotoxic stimulation with glutamate, when compared to the respective controls. This loss in VGLUT labeling was not as significant in neurons pre-incubated with BDNF. Conversely, pre-incubation with BDNF did not prevent the decrease in protein levels of GABAergic markers GAD65 and GAD67 as a consequence of the glutamate toxic insult (authors' unpublished observations), suggesting that this neurotrophin has a differential protective effect on glutamatergic and GABAergic neurons, selectively preserving the protein levels VGLUT1 and VGLUT2 markers, albeit only partially.

BDNF protects survival (TRPC3 and TRPC6) markers from glutamate excitotoxicity

To further understand the protective effect of BDNF in hippocampal neurons subjected to excitotoxic stimulation, we analyzed the protein levels of TRPC3 and TRPC6, which are activated by calcium store depletion and required for BDNF-dependent survival of cerebellar granule neurons deprived of serum (Jia et al., 2007). Similarly to the markers described above, we observed a time-dependent decrease in the protein levels of the TRPCs under glutamate excitotoxicity (Fig. 7A, B) albeit with different kinetics. Whereas TRPC3 showed a gradual reduction of its protein levels, stabilizing after 12–14 h following the toxic insult, well below 40% of the control levels (Fig. 7A), TRPC6 appears to show a two-step reduction of its protein levels, as determined by immunoblotting (Fig. 7B), with an initial decrease of the protein levels between 3 and 5 h after the toxic insult, followed by a slight, transient recovery between 8 and 10 h and a subsequent secondary decrease to approximately 60% of the control protein levels at 12–14 h after the insult. This difference is suggestive of alternative regulatory mechanisms of the TRPC protein levels under glutamate excitotoxicity. However, in both cases, we found a protective effect of BDNF on the protein levels of TRPC3 (Fig. 7D) and TRPC6 (Fig. 7E), 5 h and 14 h after the insult, when compared with the same time points without pre-incubation with BDNF. This effect was particularly relevant at 5 h after glutamate stimulation, when TRPC protein levels were about 50% or 80% of the control when the cells were incubated in the absence or in the presence of BDNF, respectively. This effect of BDNF correlates with a transient increase in TRPC6 protein

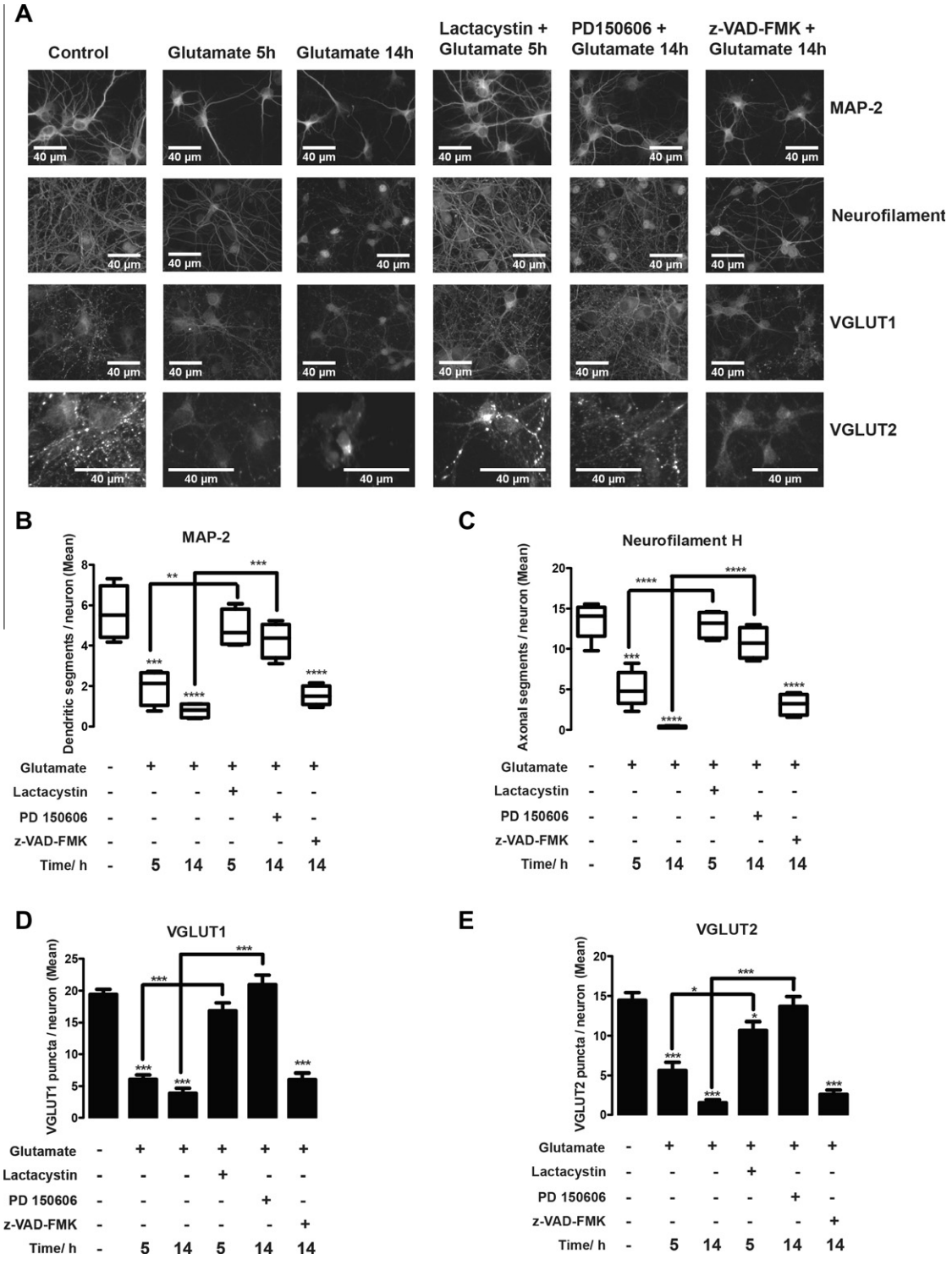


Fig. 4. Neuronal markers for dendrites (MAP-2) and axons (NF-H) are downregulated under excitotoxic conditions by proteasome- and Calpain-dependent mechanisms. (A–E) Cultured hippocampal neurons were challenged at DIV7 with 125 μM glutamate, for 20 min, after pre-incubation or not with general caspase inhibitor z-VAD-FMK (50 μM), for 2 h, specific proteasome inhibitor Lactacystin (1 μM), 30 min, or calpain inhibitor PD150606 (50 μM), during 2 h, and then incubated with the conditioned Neurobasal medium. Cells were fixed 5 or 14 h after the toxic insult and labeled with specific antibodies raised against MAP-2 (A, B) and Neurofilament H (A, C), VGLUT1 (A, D) and VGLUT2 (A, E). The cellular distribution of proteins was visualized with rabbit Alexa 488 (VGLUT1 and VGLUT2) or mouse Alexa 594-conjugated secondary antibody (MAP-2 and Neurofilament H) using a Zeiss Axiovert fluorescence microscope (40× objective) coupled to a digital camera. The images are representative of four independent experiments, performed in distinct preparations. Mean dendritic (B) and axonal (C) segments per neuron were quantified using the Sholl analysis plugin on Image J. Mean VGLUT1 (D) and VGLUT2 (E) puncta per neuron were assessed using the cell counter and analyze particles plugins on Image J. Data are presented as mean ± SEM of four independent experiments (B–E). Statistical analysis was performed using the Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

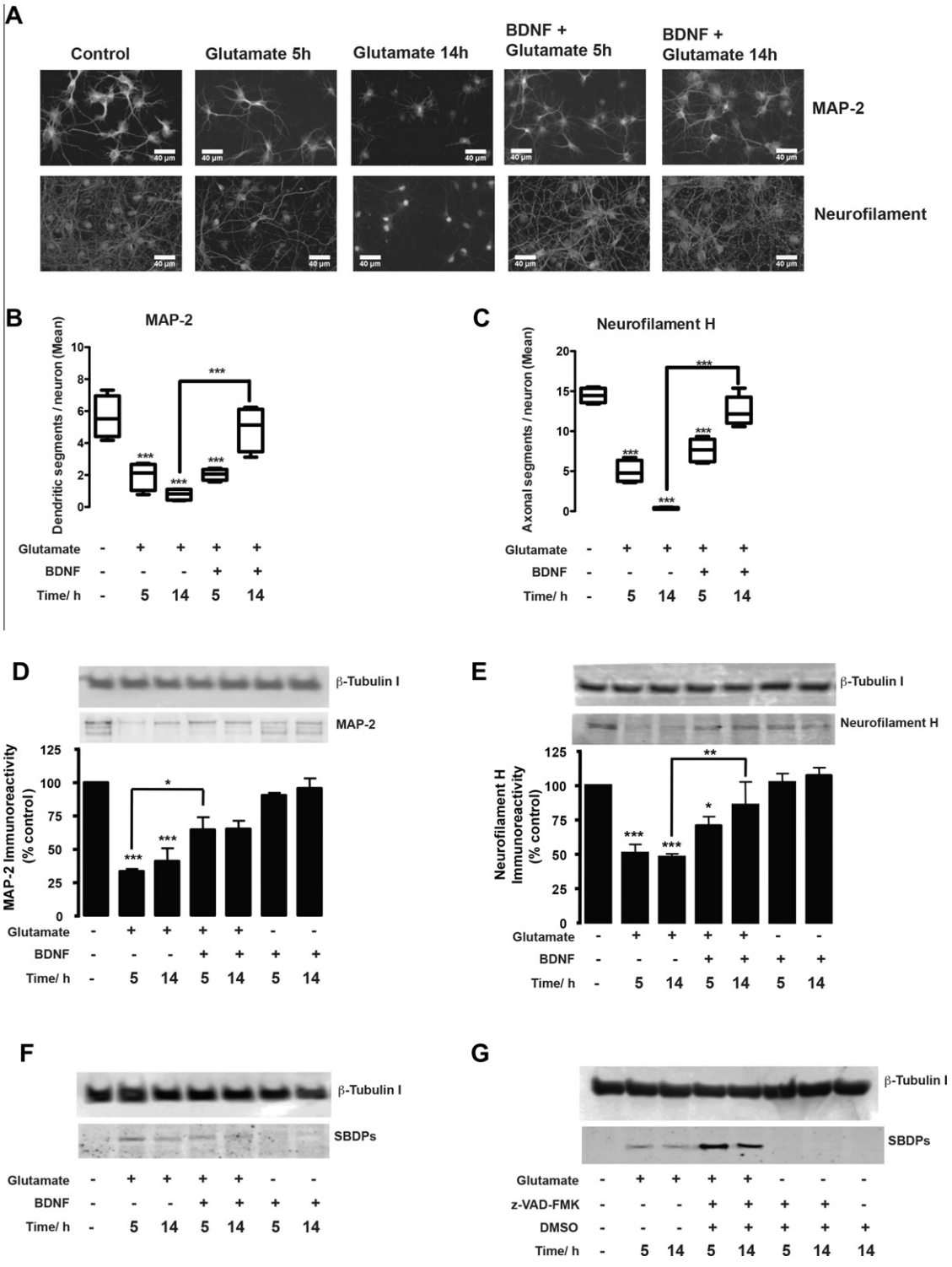


Fig. 5. BDNF protects axonal and dendritic neuronal markers in hippocampal neurons subjected to glutamate-induced excitotoxicity. Cultured hippocampal neurons, pre-incubated with BDNF (100 ng/ml), for 24 h (A, B, D–F), or z-VAD-FMK (50 μM), for 2 h (G), were then stimulated with 125 μM glutamate (in Neurobasal medium), for 20 min, and fixed (A) or lysed (D–G) after different incubation periods with conditioned medium. (A) The cellular distribution of proteins was visualized with mouse Alexa 594-conjugated secondary antibody (MAP-2 and Neurofilament H) using a Zeiss Axiovert fluorescence microscope (20× objective) coupled to a digital camera. The images are representative of four independent experiments, performed in distinct preparations. Mean dendritic (B) and axonal (C) segments per neuron were quantified using the Sholl analysis plugin on Image J. The control images may appear overexposed, but this was required to visualize the neurons subjected to glutamate excitotoxicity, with comparatively much weaker signal, due to the cellular damage, particularly the loss of the protein markers used for immunolabeling. The extracts were analyzed by immunoblotting with specific antibodies for MAP-2 (D), NF-H (E) and SBDPs (F, G). (B–E) Data are presented as mean ± SEM of at least four different experiments, performed in independent preparations. Statistical analysis was performed using the Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

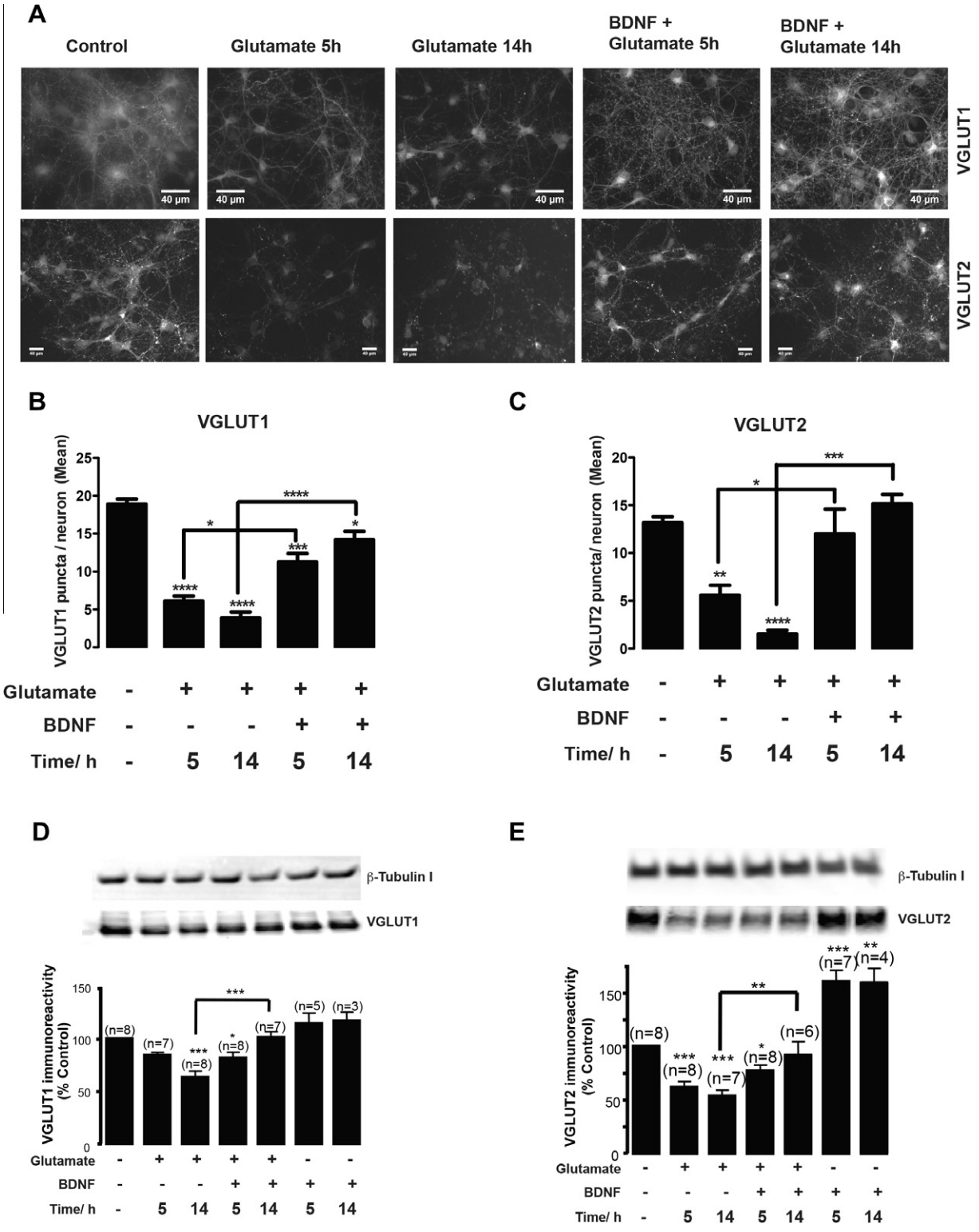


Fig. 6. BDNF protects VGLUT1 and VGLUT2 in hippocampal neurons subjected to excitotoxic stimulation. Cultured hippocampal neurons, pre-incubated or not with BDNF (100 ng/ml), for 24 h, were exposed to a toxic insult with 125 μM glutamate (in Neurobasal medium), for 20 min, and then fixed (A) or lysed (D, E) after different incubation periods in conditioned medium. (A) The cellular distribution of proteins was visualized with rabbit Alexa 488 (VGLUT1 and VGLUT2) using a Zeiss Axiovert fluorescence microscope (40× objective) coupled to a digital camera. The images are representative of four independent experiments, performed in distinct preparations. Mean VGLUT1 (B) and VGLUT2 (C) puncta per neuron were assessed using the cell counter and analyze particles plugins on Image J. The extracts were analyzed by immunoblotting with specific antibodies for VGLUT1 (D) and VGLUT2 (E). Data are presented as mean ± SEM of four independent experiments (B–E). Statistical analysis was performed using the Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

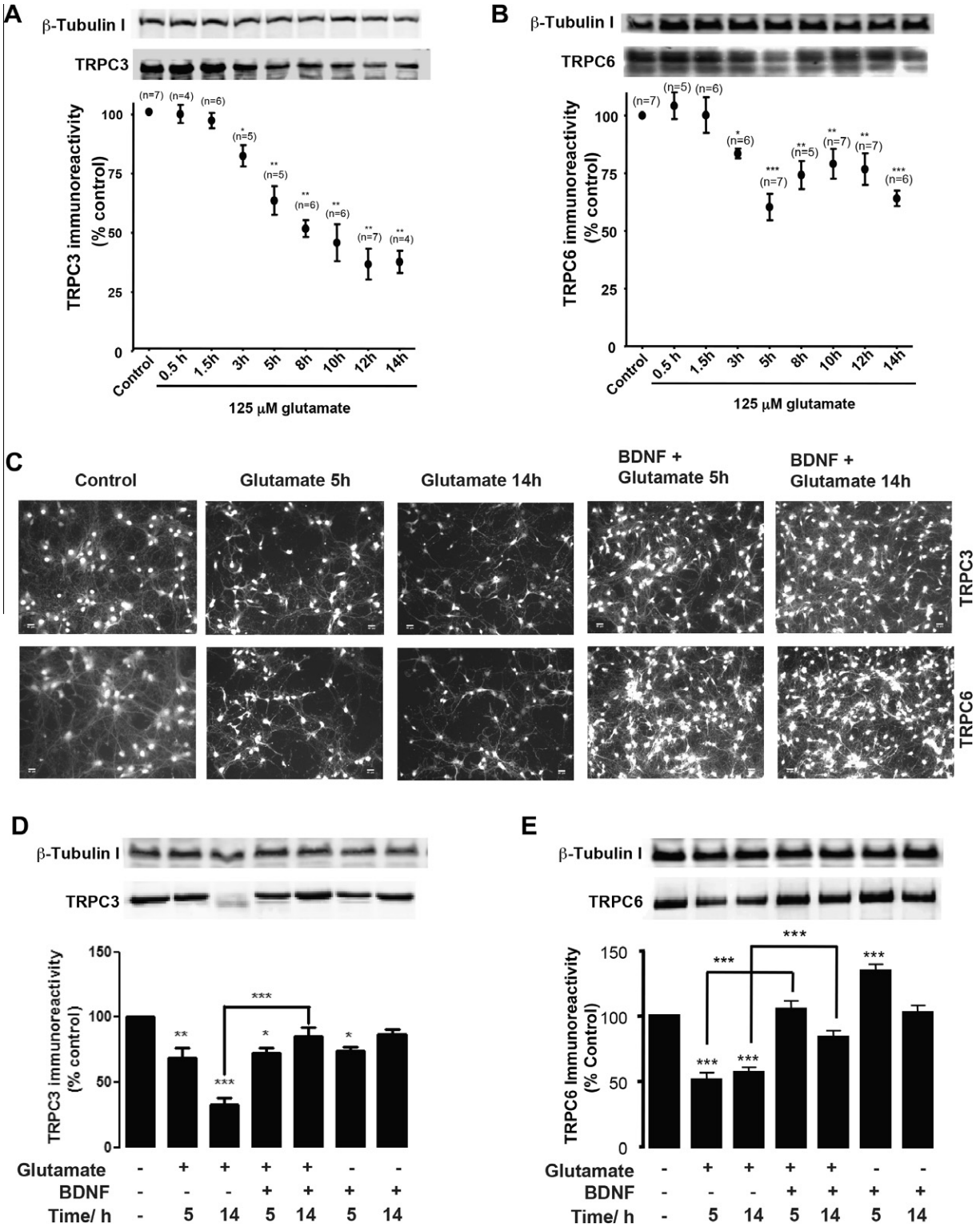


Fig. 7. BDNF protects TRPC3 and TRPC6 in hippocampal neurons subjected to excitotoxic stimulation. (A, B) Glutamate excitotoxicity induces a time-dependent decrease in the protein levels of TRPC3 (A) and TRPC6 (B). Cultured hippocampal neurons were challenged with 125 μM glutamate, for 20 min, and then lysed after different incubation periods. The extracts were analyzed by immunoblotting with specific antibodies raised against TRPC3 (A) and TRPC6 (B). (C-E) Cultured hippocampal neurons, pre-incubated or not with BDNF (100 ng/ml), for 24 h, were exposed to a toxic insult with 125 μM glutamate (in Neurobasal medium), for 20 min, and then fixed (C) or lysed (D, E) after different incubation periods in conditioned medium. (C) The cellular distribution of TRPC proteins was visualized with rabbit Alexa 488 using a Zeiss Axiovert fluorescence microscope (20× objectives) coupled to a digital camera. The images are representative of four independent experiments, performed in distinct preparations. (D, E) The extracts were analyzed by immunoblotting with specific antibodies for TRPC3 (D) and TRPC6 (E). Data are presented as mean ± SEM of four independent experiments (D, E) or the number indicated in the figure (A, B). Statistical analysis was performed using the Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

levels found upon stimulation with the neurotrophin for 24 + 5 h under control conditions. These results were confirmed by fluorescence microscopy imaging of cultured hippocampal neurons under the same conditions, labeled with TRPC3 and TRPC6 antibodies (Fig. 7C). While neurons subjected to glutamate excitotoxicity without pre-incubation with BDNF displayed an increasing loss of TRPC-labeled neurites from 5 to 14 h after the insult, we found a clear preservation of TRPC3 and TRPC6 positive neurites in hippocampal neurons pre-incubated with BDNF. Moreover, in some experiments we observed an increased TRPC6 immunoreactivity in BDNF-preincubated neurons and subjected to the toxic insult, when compared with the control.

BDNF protective effect on axons and dendrites requires the activation of both PI-3K and PLCγ signaling pathways

Taking into account the protective effect of BDNF on structural and functional markers of neurites and synapses, additional immunofluorescence experiments were performed to study the signaling mechanisms involved in the protection of axons and dendrites, using selective chemical inhibitors of the different pathways activated by TrkB receptors, at the concentrations used. The phosphatidylinositol 3-kinase (PI-3K) chemical inhibitors wortmannin and LY294002, as well as the phospholipase C inhibitor U73122 blocked the protective effect of BDNF against glutamate-induced dendritic and axonal loss, as determined 14 h after the insult (Fig. 8A–C). Similarly, inhibition of the PI-3K or PLCγ pathways prevented BDNF from precluding the loss of VGLUT2 puncta and TRPC3/6 immunoreactivity, but only the latter mechanism appears to be involved in the protection of VGLUT1 punctate labeling (Fig. 8D–F). Conversely, neither the MAPK/ERK (mitogen-activated protein kinase/ extracellular signal-regulated kinase) inhibitors, PD098,059 and U0126, nor the Src-family non-receptor protein tyrosine kinase inhibitor, SU6656, was effective in blocking the protective effect of BDNF when incubated before exogenous application of the neurotrophin (Fig. 8A–G). As expected, the protective effects of BDNF were inhibited in the presence of the Trk receptor inhibitor K252a, for all markers tested (Fig. 8A–G). The chemical inhibitors tested under control conditions had no effect on the TRPC 3 and 6, VGLUT1, MAP2 and NF-H immunoreactivity (data not shown). Therefore, whereas BDNF prevents the condensation of chromatin through activation of the ERK and PI3-K signaling pathways (Almeida et al., 2005), the neurotrophin also prevents degeneration of axons and dendrites through activation of the phosphatidylinositol 3-kinase (PI-3K) and phospholipase Cγ signaling pathways.

Hence, there is a spatiotemporal differentiation between the protective role of BDNF at the cell soma and neurites, not only on the proteolytic mechanisms restrained by this neurotrophin, but also on the intracellular signaling mechanisms it activates in order to do so.

BDNF partially prevents the excitotoxicity-induced downregulation of synaptic activity

In order to determine whether BDNF would also preserve the activity of the vesicular glutamate transporters, critical for glutamatergic synaptic function, we used a recombinantly expressed and purified glutamate FRET [Förster (Fluorescence) resonance energy transfer] nanosensor, added to the extracellular medium (Okumoto et al., 2005) with an inhibitor of glutamate transporters, TBOA. We found a time-dependent decrease in KCl-induced exocytotic glutamate release at 5 and 14 h after the toxic insult. At 14 h, synaptic activity measured by FRET was significantly lower compared to the control level but pre-incubation with BDNF for 24 h before the toxic insult partially restored the activity-dependent exocytotic glutamate release to ~90% of the control levels (Fig. 9). Incubation of hippocampal neurons with BDNF under control conditions significantly increased exocytotic glutamate release, as previously found for the VGLUT2 proteins levels upon 24 + 5 h or 24 + 14 h incubation with BDNF alone (Fig. 6E).

DISCUSSION

In the present work we show that BDNF neuroprotection extends beyond apoptotic cell soma death, significantly reducing the loss of neurites and synaptic activity induced by excitotoxicity, in cultured hippocampal neurons. While in the cell soma, BDNF inhibits caspase-3-like activity and reduces neuronal death by a protein synthesis-dependent mechanism, through the PI3-K and ERK signaling pathways (Almeida et al., 2005), in axons and dendrites BDNF reduces the calpain-mediated downregulation of protein markers and the early activation of calpains induced by a toxic glutamate insult, which precedes chromatin condensation. In between, there is a transient accumulation of ubiquitin-conjugated proteins and we found that specific inhibition of the proteasome protects the neuritic markers MAP-2 and NF-H. The effect of BDNF on neurites and glutamatergic synapses is mediated by the PLCγ and to some extent by the PI3-K signaling pathways.

Role of calpains and the UPS in neurite damage induced by excitotoxic stimulation

The degeneration of neurites has been consistently described as an active (Lunn et al., 1989; Glass et al., 1993; Raff et al., 2002; Saxena and Caroni, 2007), non-apoptotic (Finn et al., 2000; Ikegami and Koike, 2003) process, occurring independently of the cell soma demise (Koike et al., 2008), both during development (Deckwerth and Johnson, 1994) and following chemical (Ikegami and Koike, 2003) or toxic (Berliocchi et al., 2005) insults. In this study we have found that glutamate-induced degeneration of neurite markers is sensitive to proteasome and calpain inhibitors, lactacystin and PD150606, respectively, but unaffected by Z-VAD-fmk, a pan-caspase inhibitor. In contrast, Z-VAD-fmk abrogated glutamate-evoked chromatin condensation (Fig. 1A) similarly to the effect of caspase-

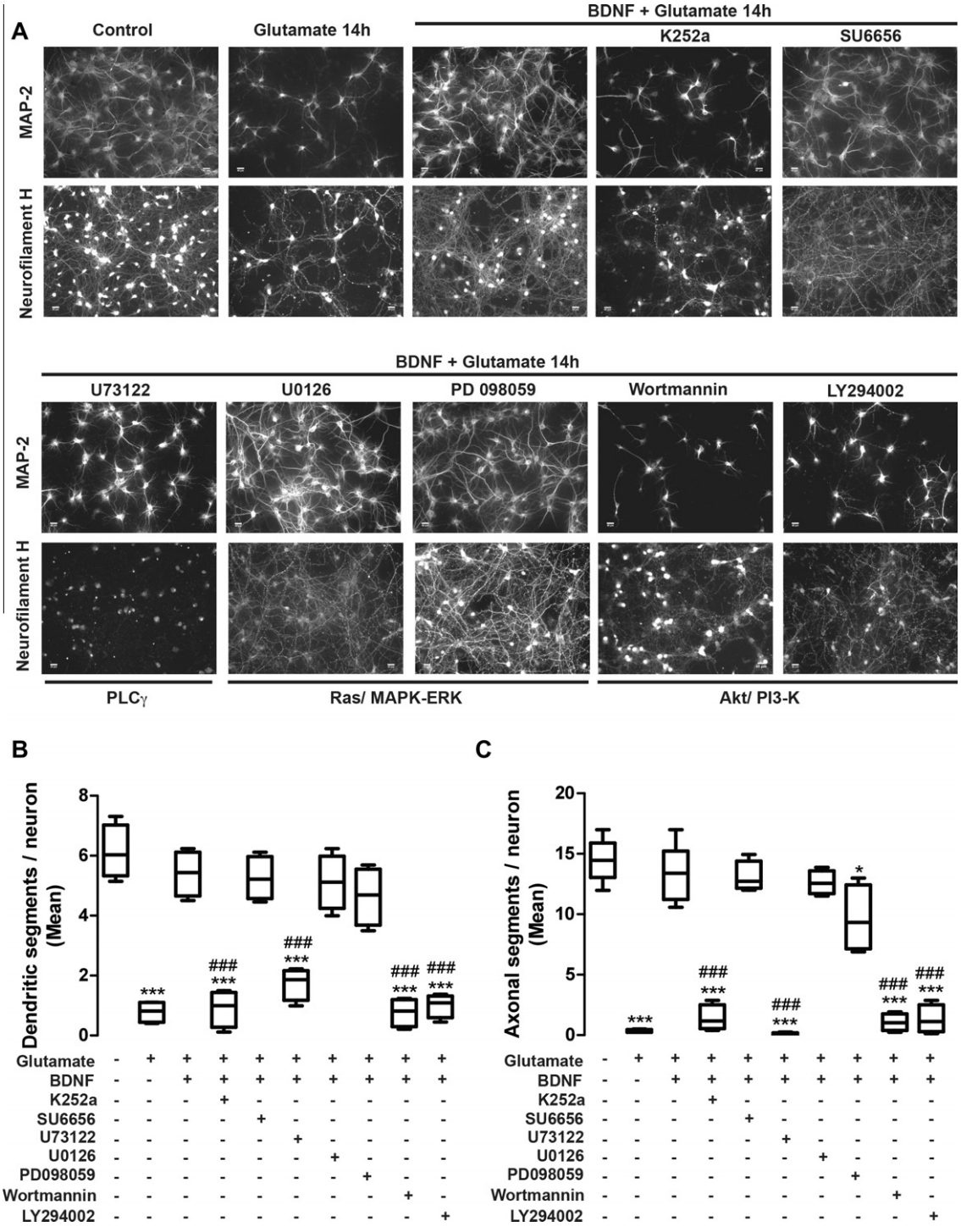


Fig. 8. The neuroprotective effect of BDNF against glutamate toxicity requires the activation of PI-3K and PLCγ signaling pathways. (A, D, G) Cultured hippocampal neurons were pre-incubated with specific chemical inhibitors of BDNF-induced intracellular signaling pathways: Phosphatidylinositol 3-kinase (PI-3K) inhibitors Wortmannin (100 nM) and LY294002 (30 μM), MAPK/ERK (Mitogen-activated protein kinase/ Extracellular signal-regulated kinase) inhibitors PD098,059 (20 μM) and U0126 (300 nM), Phospholipase C inhibitor U73122 (5 μM), or Src-family protein tyrosine kinases inhibitor SU6656 (10 μM), and then incubated with BDNF during 24 h. Afterward, the neurons were challenged, at DIV7, with 125 μM glutamate, for 20 min, and then incubated with the conditioned Neurobasal medium. Cells were fixed 14 h after the toxic insult and labeled with specific antibodies raised against the proteins indicated in the left side of the panel. The localization of the proteins was visualized with mouse Alexa 594-conjugated (MAP-2 and Neurofilament H) secondary antibody (A) or rabbit Alexa 488 [(VGLUT1 and VGLUT2) (D) and (TRPC3 and TRPC6) (G)], using a Zeiss Axiovert fluorescence microscope (20× and 40× objectives), coupled to a digital camera. (B, C) The images are representative of four independent experiments, performed in distinct preparations. Mean dendritic (B) and axonal (C) segments per neuron were quantified using the Sholl analysis plugin on Image J. (E, F) Mean VGLUT1 (E) and VGLUT2 (F) puncta per neuron were assessed using the cell counter and analyze particles plugins on Image J. Data are presented as mean ± SEM of four independent experiments (B, C, E, F). Statistical analysis was performed using the Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

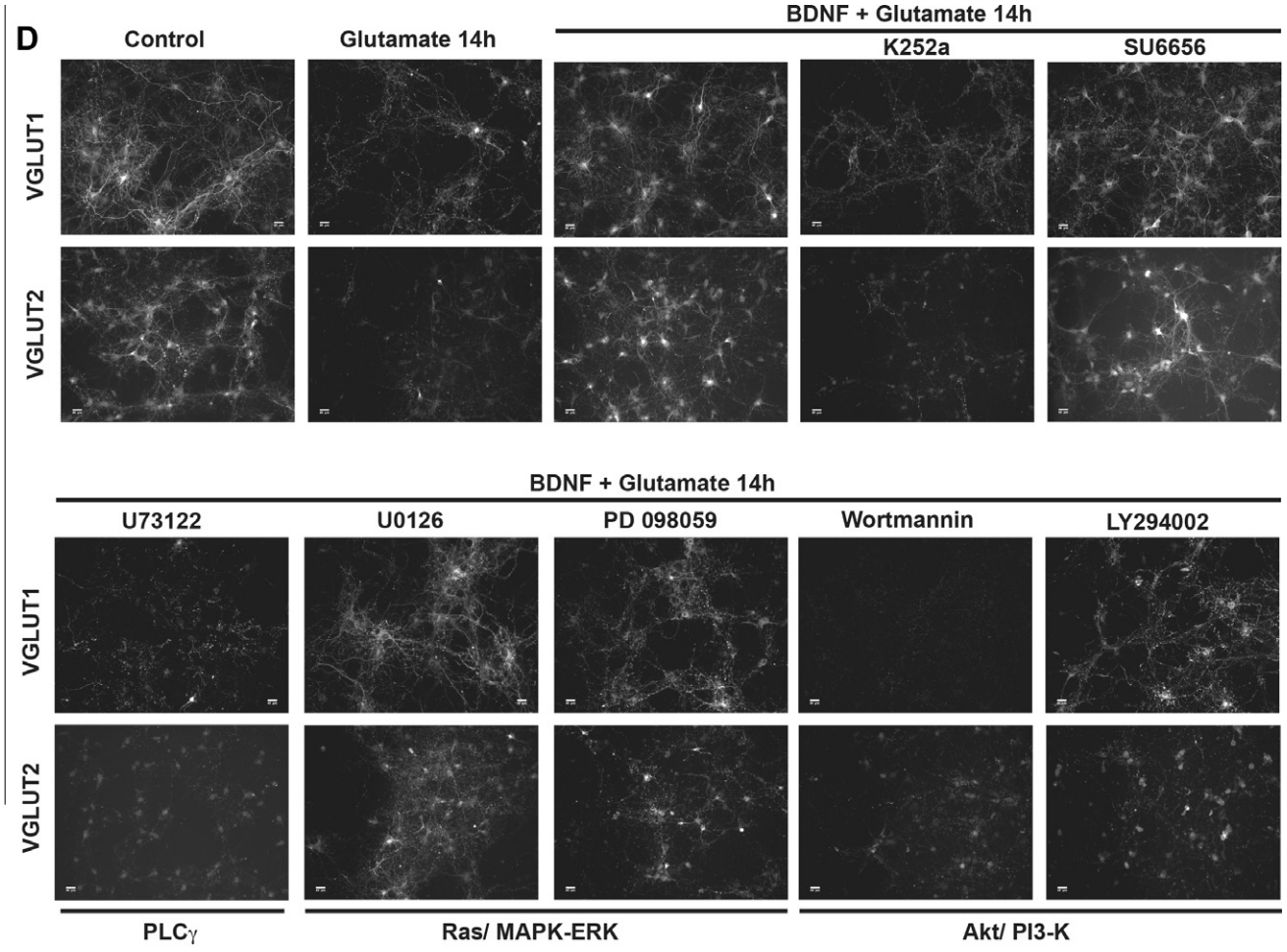


Fig. 8. (*continued*)

3 inhibition (Almeida et al., 2005). The results correlate with the activation of calpain and accumulation of ubiquitin conjugates found in neurites and, to a lesser extent, in the soma, following excitotoxic stimulation, in contrast to caspase-3 activation, limited to the soma. Together, these findings indicate a spatial segregation

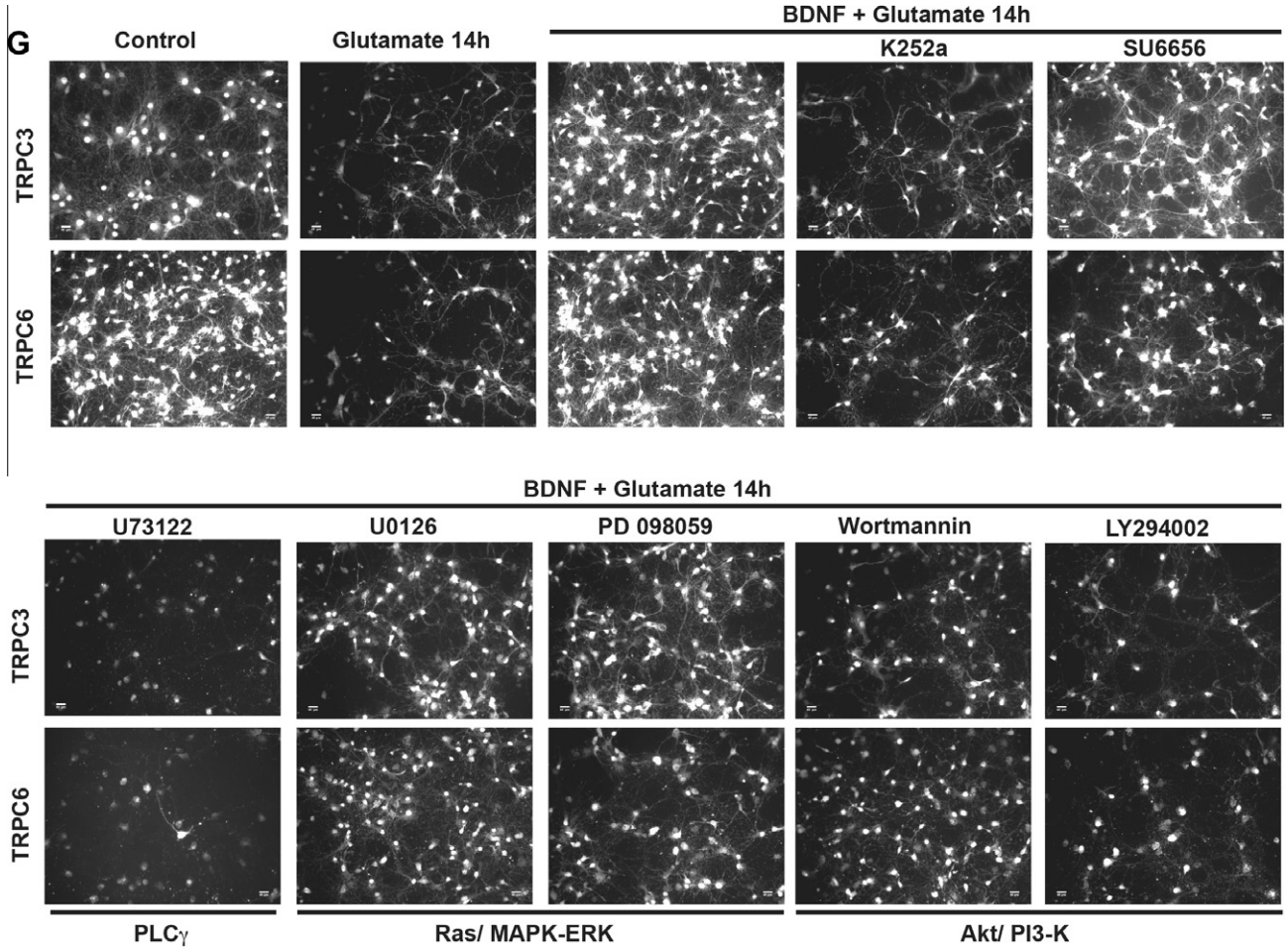


Fig. 8. (*continued*)

of proteolytic effects in neuronal damage, wherein calpain activation and proteasome deregulation play a predominant role in neurites, whereas caspases are further activated in the cell body. However, lactacystin was previously shown to partly inhibit a cathepsin A-like activity released from platelets (Ostrowska et al., 1997) and, therefore, we may not exclude a similar non-specific effect under the experimental conditions used herein. In contrast, PD150606 was shown not to inhibit cathepsin B and L at the range of concentrations required to target calpains (Edelstein et al., 1996; Wang et al., 1996).

BDNF activates different neuroprotective programs in the neuronal soma and neurites

In order to distinguish the different neuronal compartments, we studied the protein markers of dendrites (MAP-2) (Dotti et al., 1988; Garner et al., 1988), axons (NF-H) (Mori et al., 2012) and glutamatergic synapses (VGLUT1-2) (Fremeau et al., 2004), which encompass over 80% of total synapses in cultured hippocampal neurons (Baptista et al., 2010). Under physiological conditions, MAP-2 regulates microtubule-dependent transport (Lopez and Sheetz, 1993; Hagiwara et al., 1994; Maas et al., 2009) and modulates synaptic strength and long-term potentiation (Zhong et al., 2009). These critical roles of MAP-2 in neuronal function are all potentially disturbed under excitotoxicity. We have found that a toxic glutamate insult elicits a time-dependent decrease in MAP2 protein levels (Fig. 3A) and disrupts microtubule structure without significantly changing tubulin protein levels (Fig. 2A), similarly to other models of neurodegeneration (Guo et al., 2012). MAP-2 downregulation has also been detected in cerebrocortical (Irving et al., 1996) and hippocampal neurons (Miyamoto et al., 1998; Hoskison et al., 2007) subjected to a toxic insult. Likewise, there is a rapid breakdown and redistribution of MAP-2 to the soma of pyramidal neurons following oxygen-glucose deprivation (Buddle et al., 2003) or injections of the glutamate transport inhibitor dihydrokainate and kainate (Arias et al., 1997) into the rat hippocampus.

Since MAP-2 may be cleaved by calpain (Buddle et al., 2003) the protective effects of BDNF on MAP-2 downregulation (Fig. 5A, B, D) may result, at least in part, from its effect on calpain activity (Fig. 5F). Other proteolytic mechanisms, besides calpain activity, may also be indirectly involved in MAP-2 turnover, given that proteasome inhibition with lactacystin mirrored the neuroprotective effect of BDNF on MAP-2, 5 h after the toxic insult (Fig. 4). Still, proteasome-mediated

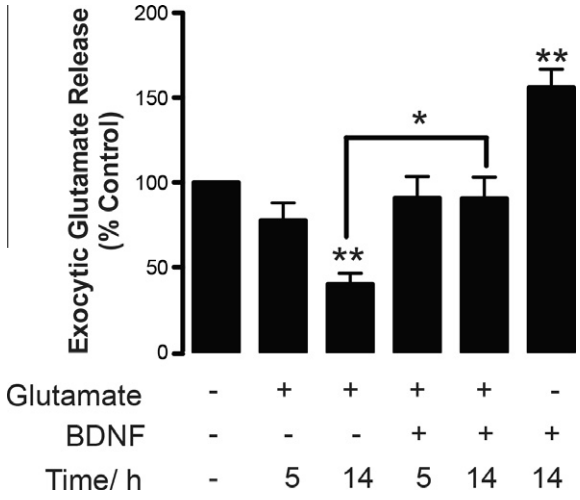


Fig. 9. BDNF protects exocytotic glutamate release in cultured hippocampal neurons subjected to excytotoxicity. Hippocampal neurons cultured in 12-well plates, pre-incubated or not with BDNF (100 ng/ml), for 24 h, were exposed to a toxic insult with 125 μM glutamate (in Neurobasal medium), for 20 min, and further incubated in the conditioned media for 5 or 14 h, as indicated. The cells were then washed with Tyrode's solution at 37 °C and depolarized with high-K^+ Tyrodes solution (90 mM KCl and 31.5 mM NaCl) for 60 s, which was immediately replaced with normal Tyrode's solution containing the recombinantly expressed and titered glutamate FRET sensor and 10 μM TBOA. Quantification of the exocytotic glutamate release was performed by a fluorescence plate reader at an excitation wavelength of 433/12 nm and emission wavelengths of 485/12 nm and 528/12 nm. The ratio between the fluorescence measured at the two wavelengths was calculated and the results were expressed as a percentage of the control. Data are presented as mean ± SEM of four independent experiments. Statistical analysis was performed using the Bonferroni's Multiple Comparison Test. $^*p < 0.05$; $^{**}p < 0.01$.

degradation of MAP-2 has only been linked to predominantly cytoplasmic 20S in cortical neurons of AIDS patients with neurological dysfunctions, albeit associated with deterioration of neuronal processes (Aprea et al., 2006). Furthermore, inhibition of lysosomal pathways with NH_4Cl and chloroquine showed no effect on MAP-2 under excitotoxicity (data not shown). BDNF may also protect MAP-2 and dendrites through redistribution of MAP-2-associated NMDA receptors (Buddle et al., 2003), suggesting that acute exogenous application of BDNF in hippocampal neurons may play a more general role in neuroprotection. This further suggests that neuroprotection of neurites mediated by BDNF is not limited to activation of protein synthesis-dependent mechanisms, as previously found in the cell soma (Almeida et al., 2005).

In this study we have also analyzed the neuroprotective effect of BDNF in axons by studying the protein marker NF-H, which is critical for the regulation of axonal transport (Ackerley et al., 2003) and diameter (Perrot et al., 2008). We have found that glutamate excitotoxicity triggers a progressive loss of total (phosphorylated and non-phosphorylated) NF-H protein levels (Fig. 3A), and the formation of NF-H-positive swellings and axonal beads (not shown), as previously found in the cortical neurons overlying the stereotaxic injection site of AMPA (Fowler et al., 2003), or following inhibition of plasma membrane calcium ATPase isoform 2 (PMCA2) in an animal model of Multiple Sclerosis (Kurnellas et al., 2005). Preincubation of hippocampal neurons with BDNF significantly reduced the loss of NF-H following excitotoxic stimulation with glutamate (Fig. 5C), precluding the disarray of the NF network, which would otherwise impair axoplasmic flow, leading to retrograde degeneration and loss of connectivity (Perrot et al., 2008). These results corroborate evidence on a similar model of primary septo-hippocampal cell cultures wherein BDNF lipotransfection either pre-injury or 24 h following depolarization injury promoted recovery from neurofilament loss (Hayes et al., 1995). This neuroprotective effect of BDNF was mimicked by the proteasome inhibitor lactacystin, and a calpain inhibitor, PD150606, although, to a lessened extent (Fig. 4A, C), indicating an indirect effect of calpain activity on the regulation of NF-H turnover, as previously suggested (Kampfl et al., 1996).

In addition to the changes in NF-H protein levels observed in the present work glutamate excitotoxicity also induces phosphorylation of NF-H in the cell body, slowing its axonal transport (Ackerley et al., 2000) and promoting the formation of phosphorylated neurofilamentous inclusions (Kesavapany et al., 2007). Since phosphorylation protects NF-H against calpain-mediated proteolysis (Goldstein et al., 1987; Pant, 1988; Greenwood et al., 1993) and the 20S proteasome only degrades non-phosphorylated NF-H (Kimura et al., 2007), the perikaryal accumulation of NF-H proteins may be an endogenous protective mechanism of neurons coping with such neurodegerative insult. However, despite these protective mechanisms, a significant fraction of NF-H protein is lost by a mechanism involving calpains and the proteasome in hippocampal neurons subjected to excitotoxic stimulation (Fig. 4). We have only found significant levels of NF-H-positive axonal beadings in the later time points analyzed, i.e., these structures were absent in the earlier time points (data not shown) that coincide with maximal calpain activation (Fig. 2B) and the decrease in NF-H protein levels was delayed compared to the decline of MAP-2 (Fig. 3A).

BDNF protects glutamatergic neuronal function

We have previously shown that excitotoxicity induces the cleavage and deregulation of both GABAergic and glutamatergic neuronal markers, respectively, glutamic acid decarboxylase isoforms GAD65 and GAD67 (Baptista et al., 2010), and the vesicular glutamate transporters VGLUT1 and VGLUT2 (Lobo et al., 2011). However, acute treatment with BDNF does not preclude the loss of GAD65 and GAD67 (authors' unpublished observations) whereas the protein levels and punctate labeling of VGLUT1 and VGLUT2 are significantly protected (Fig. 6). Furthermore, BDNF prevents the downregulation of exocytotic glutamate release (Fig. 9), suggesting a selective protection of glutamatergic function, in addition to the exocytotic machinery involved in neurotransmitter release. However, since BDNF

upregulates VGLUT protein levels and glutamate release under control conditions (Fig. 6E), the protective effects observed in cells pre-incubated with the neurotrophin may be due, at least in part, to an increase in the expression of the vesicular transporters with a concomitant upregulation of glutamate release in the cells that are resistant to the excitotoxic injury. Although TrkB receptors are equally associated with excitatory and inhibitory markers, BDNF is preferentially associated with VGLUT1 and GluN1 in cultured hippocampal neurons (Swanwick et al., 2004). Likewise, glutamatergic and GABAergic neurons react differently to postsynaptic BDNF, with an upregulation of glutamatergic synaptic inputs and downregulation of GABAergic synaptic terminal numbers in hippocampal neurons (Singh et al., 2006).

BDNF-induced neuroprotection via protein synthesis *vs.* inhibition of protein degradation

TRPC 3 and 6 protect cerebellar granule neurons from serum deprivation-induced cell death (Jia et al., 2007) and inhibition of calpain-mediated TRPC6 cleavage also provided neuroprotection in a rat model of stroke (Du et al., 2010). The TRPC6 agonist 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) significantly increases retinal ganglion cell survival in a rat model of retinal ischemia/reperfusion-induced cell death, through a mechanism mediated by BDNF (Wang et al., 2010). Likewise, we have found that BDNF significantly protects TRPC3 and TRPC6 protein levels (Fig. 7D, E) and TRPC3/6-immunopositive neurites (Fig. 7C) in cultured hippocampal neurons subjected to glutamate excitotoxicity.

Although the aforementioned evidence from other cell death models indicates inhibition of protein degradation, specifically calpain-mediated proteolysis, as the mechanism of neuroprotection, similarly to what we have found for several neuritic markers studied (Figs. 4 and 5F), BDNF alone significantly upregulates the protein levels of TRPC6 (Fig. 7E) under control conditions, and similar effects were observed for VGLUT2 (Fig. 6E). Therefore, we may not exclude the contribution of protein synthesis-dependent mechanisms for BDNF-induced maintenance of TRPC6 protein levels (as well as VGLUT2) in hippocampal neurons subjected to excitotoxic stimulation, which is likely to contribute to the neuroprotective effects of the neurotrophin. Furthermore, VGLUT2 and TRPC6 protein levels in hippocampal neurons pre-incubated with BDNF are still lower than the control levels 14 h after the glutamate toxic insult, despite the partial but significant recovery (Figs. 6E and 7E). This evidence suggests a change in the sub-cellular distribution of the proteins, with increased trafficking to the neurites (Figs. 6A and 7C), further suggesting that BDNF promotes the functional recovery of neurons subjected to glutamate excitotoxicity (Fig. 9), in addition to an attenuation of proteolytic mechanisms activated by the toxic glutamate insult (Fig. 4).

We have found that BDNF requires activation of both PI3-K and PLCγ signaling pathways in order to prevent the loss of immunoreactivity of all neuritic (Fig. 8A–C) and synaptic markers (Fig. 8D–F) tested, in addition to TRPC3 and TRPC6 (Fig. 8G). PI3-K has a pivotal role in promoting neuronal survival upon toxic insults (Almeida et al., 2005; Yao et al., 2009) via transcription-dependent and -independent mechanisms (Brunet et al., 2001), either by impairing the proteolytic machinery activation (Datta et al., 1997; del Peso et al., 1997) or upregulating the mammalian target of rapamycin (mTOR), the key regulator of protein synthesis (Sarbassov et al., 2005), arresting glycogen synthase kinase-3 (GSK-3) activity (Cross et al., 1995), and suppressing axonal (Cheng et al., 2011) and dendritic (Baki et al., 2008) retraction. In addition, the PI3-K/Akt signaling pathway promotes axon regeneration in peripheral (Namikawa et al., 2000) and CNS (Park et al., 2008) neurons, which may be correlated with its role in neuritic morphogenesis (Jaworski et al., 2011) and synaptic potentiation (Wang et al., 2003). Similarly, the PLCγ signaling pathway not only mediates neuroprotection in hippocampal neurons (Beazely et al., 2009) but also regulates intracellular calcium transients contributing to growth cone guidance and synaptic plasticity (Li et al., 2005; Amaral and Pozzo-Miller, 2007).

In summary, we have found several lines of evidence in this study suggesting a spatiotemporal differentiation of the neuroprotective effect of BDNF in different neuronal compartments: (1) the activation of the machinery responsible for the loss of synapses and degeneration of neurites precedes caspase-3 activation which leads to cell soma demise; (2) the proteolytic mechanisms activated in the neurites differ from the ones activated in the cell soma; (3) BDNF activates distinct signaling mechanisms in order to induce neuroprotection of different sub-cellular compartments. Some studies suggest that neurite pruning and injury-induced degeneration share a common disassembly pathway (Luo and O'Leary, 2005; Schoenmann et al., 2010) while others advocate different molecular mechanisms for the developmental and pathological degeneration of neurites (Hoopfer et al., 2006; Tao and Rolls, 2011). In either case, BDNF signaling has been implicated not only in the developmental regulation of pruning in axons (Cao et al., 2007; Singh et al., 2008) and dendrites (An et al., 2008), but also in the regenerative sprouting of neurotoxin-injured serotonergic axons (Mamounas et al., 2000). Moreover, exogenous BDNF increases axon growth and synaptogenesis after NMDA or kainate-induced excitotoxicity (Wang and Green, 2011). Although BDNF-mediated effects may differ between developmental and neurodegenerative contexts, understanding the key effectors of BDNF signaling mechanisms involved in neuronal development and synaptic maturation may enable to develop new therapeutic approaches aimed at reactivating the same developmental programs in order to promote functional recovery of neurons after a neurodegenerative input.

Acknowledgements—This work was funded by Fundação para a Ciência e a Tecnologia (FCT) and Fundo Europeu para o Desenvolvimento Regional (FEDER) (PTDC/SAU-FCF/72283/2006 and PTDC/SAU-NMC/120144/2010; to C.B.D.) and by the Calou-

ste Gulbenkian Foundation (Proc. 71949; to C.M.). S.O. and W.B.F. were supported by an NIH Grant (NIDDK; 1RO1DK079109). We are very thankful to Dr. Craig Garner and the Nancy Pritzker Laboratory, Stanford University, for their collaboration in the preparation of the hippocampal cultures used in the FRET experiments. We would also like to thank Elisabete Carvalho for assistance in the preparation of cell cultures at University of Coimbra. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Ackerley S, Grierson AJ, Brownlees J, Thornhill P, Anderton BH, Leigh PN, Shaw CE, Miller CC (2000) Glutamate slows axonal transport of neurofilaments in transfected neurons. J Cell Biol 150:165–176.

Ackerley S, Thornhill P, Grierson AJ, Brownlees J, Anderton BH, Leigh PN, Shaw CE, Miller CC (2003) Neurofilament heavy chain side arm phosphorylation regulates axonal transport of neurofilaments. J Cell Biol 161:489–495.

Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, Graos MM, Carvalho RF, Carvalho AP, Duarte CB (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 12:1329–1343.

Amaral MD, Pozzo-Miller L (2007) TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. J Neurosci 27:5179–5189.

An JJ, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B (2008) Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 134:175–187.

Aprea S, Del Valle L, Mameli G, Sawaya BE, Khalili K, Peruzzi F (2006) Tubulin-mediated binding of human immunodeficiency virus-1 Tat to the cytoskeleton causes proteasomal-dependent degradation of microtubule-associated protein 2 and neuronal damage. J Neurosci 26:4054–4062.

Arias C, Arrieta I, Massieu L, Tapia R (1997) Neuronal damage and MAP2 changes induced by the glutamate transport inhibitor dihydrokainate and by kainate in rat hippocampus in vivo. Exp Brain Res 116:467–476.

Bahr BA (2000) Integrin-type signaling has a distinct influence on NMDA-induced cytoskeletal disassembly. J Neurosci Res 59:827–832.

Baki L, Neve RL, Shao Z, Shioi J, Georgakopoulos A, Robakis NK (2008) Wild-type but not FAD mutant presenilin-1 prevents neuronal degeneration by promoting phosphatidylinositol 3-kinase neuroprotective signaling. J Neurosci 28:483–490.

Bano D, Young KW, Guerin CJ, Lefeuvre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P (2005) Cleavage of the plasma membrane Na^+/Ca^{2+} exchanger in excitotoxicity. Cell 120:275–285.

Baptista MS, Melo CV, Armelao M, Herrmann D, Pimentel DO, Leal G, Caldeira MV, Bahr BA, Bengtson M, Almeida RD, Duarte CB (2010) Role of the proteasome in excitotoxicity-induced cleavage of glutamic acid decarboxylase in cultured hippocampal neurons. PLoS One 5:e10139.

Beazely MA, Lim A, Li H, Trepanier C, Chen X, Sidhu B, Macdonald JF (2009) Platelet-derived growth factor selectively inhibits NR2B-containing N-methyl-D-aspartate receptors in CA1 hippocampal neurons. J Biol Chem 284:8054–8063.

Beck J, Lenart B, Kintner DB, Sun D (2003) Na–K–Cl cotransporter contributes to glutamate-mediated excitotoxicity. J Neurosci 23:5061–5068.

Berliocchi L, Fava E, Leist M, Horvat V, Dinsdale D, Read D, Nicotera P (2005) Botulinum neurotoxin C initiates two different programs for neurite degeneration and neuronal apoptosis. J Cell Biol 168:607–618.

Bindokas VP, Miller RJ (1995) Excitotoxic degeneration is initiated at non-random sites in cultured rat cerebellar neurons. J Neurosci 15:6999–7011.

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297–305.

Buddle M, Eberhardt E, Ciminello LH, Levin T, Wing R, DiPasquale K, Raley-Susman KM (2003) Microtubule-associated protein 2 (MAP2) associates with the NMDA receptor and is spatially redistributed within rat hippocampal neurons after oxygen–glucose deprivation. Brain Res 978:38–50.

Caldeira MV, Curcio M, Leal G, Salazar IL, Mele M, Santos AR, Melo CV, Pereira P, Canzoniero LM, Duarte CB (2013) Excitotoxic stimulation downregulates the ubiquitin–proteasome system through activation of NMDA receptors in cultured hippocampal neurons. Biochim Biophys Acta 1832:263–274.

Cao L, Dhilla A, Mukai J, Blazeski R, Lodovichi C, Mason CA, Gogos JA (2007) Genetic modulation of BDNF signaling affects the outcome of axonal competition in vivo. Curr Biol 17:911–921.

Carlsson Y, Schwendimann L, Vontell R, Rousset CI, Wang X, Lebon S, Charriaut-Marlangue C, Supramaniam V, Hagberg H, Gressens P, Jacotot E (2011) Genetic inhibition of caspase-2 reduces hypoxic-ischemic and excitotoxic neonatal brain injury. Ann Neurol 70:781–789.

Cheng HC, Kim SR, Oo TF, Kareva T, Yarygina O, Rzhetskaya M, Wang C, During M, Talloczy Z, Tanaka K, Komatsu M, Kobayashi K, Okano H, Kholodilov N, Burke RE (2011) Akt suppresses retrograde degeneration of dopaminergic axons by inhibition of macroautophagy. J Neurosci 31:2125–2135.

Choi DW (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci 11:465–469.

Chung RS, McCormack GH, King AE, West AK, Vickers JC (2005) Glutamate induces rapid loss of axonal neurofilament proteins from cortical neurons in vitro. Exp Neurol 193:481–488.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785–789.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231–241.

Dawson VL, Dawson TM (2004) Deadly conversations: nuclear-mitochondrial cross-talk. J Bioenerg Biomembr 36:287–294.

Deckwerth TL, Johnson Jr EM (1994) Neurites can remain viable after destruction of the neuronal soma by programmed cell death (apoptosis). Dev Biol 165:63–72.

del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278:687–689.

Di Napoli M, McLaughlin B (2005) The ubiquitin–proteasome system as a drug target in cerebrovascular disease: therapeutic potential of proteasome inhibitors. Curr Opin Investig Drugs 6:686–699.

Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. J Neurosci 8:1454–1468.

Du W, Huang J, Yao H, Zhou K, Duan B, Wang Y (2010) Inhibition of TRPC6 degradation suppresses ischemic brain damage in rats. J Clin Invest 120:3480–3492.

Edelstein CL, Yaqoob MM, Alkhunaizi AM, Gengaro PE, Nemenoff RA, Wang KK, Schrier RW (1996) Modulation of hypoxia-induced calpain activity in rat renal proximal tubules. Kidney Int 50:1150–1157.

Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC (2000) Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. J Neurosci 20:1333–1341.

Fowler JH, McCracken E, Dewar D, McCulloch J (2003) Intracerebral injection of AMPA causes axonal damage in vivo. Brain Res 991:104–112.

Fremeau Jr RT, Kam K, Qureshi T, Johnson J, Copenhagen DR, Storm-Mathisen J, Chaudhry FA, Nicoll RA, Edwards RH (2004) Vesicular glutamate transporters 1 and 2 target to functionally distinct synaptic release sites. Science 304:1815–1819.

Garner CC, Tucker RP, Matus A (1988) Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. Nature 336:674–677.

Ge P, Luo Y, Liu CL, Hu B (2007) Protein aggregation and proteasome dysfunction after brain ischemia. Stroke 38:3230–3236.

Glass JD, Brushart TM, George EB, Griffin JW (1993) Prolonged survival of transected nerve fibres in C57BL/Ola mice is an intrinsic characteristic of the axon. J Neurocytol 22:311–321.

Glazner GW, Chan SL, Lu C, Mattson MP (2000) Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. J Neurosci 20:3641–3649.

Goldstein ME, Sternberger NH, Sternberger LA (1987) Phosphorylation protects neurofilaments against proteolysis. J Neuroimmunol 14:149–160.

Gomes JR, Costa JT, Melo CV, Felizzi F, Monteiro P, Pinto MJ, Inacio AR, Wieloch T, Almeida RD, Graos M, Duarte CB (2012) Excitotoxicity downregulates TrkB.FL signaling and upregulates the neuroprotective truncated TrkB receptors in cultured hippocampal and striatal neurons. J Neurosci 32:4610–4622.

Gomes JR, Lobo AC, Melo CV, Inacio AR, Takano J, Iwata N, Saido TC, de Almeida LP, Wieloch T, Duarte CB (2011) Cleavage of the vesicular GABA transporter under excitotoxic conditions is followed by accumulation of the truncated transporter in nonsynaptic sites. J Neurosci 31:4622–4635.

Greenwood JA, Troncoso JC, Costello AC, Johnson GV (1993) Phosphorylation modulates calpain-mediated proteolysis and calmodulin binding of the 200-kDa and 160-kDa neurofilament proteins. J Neurochem 61:191–199.

Guo Y, Gong HS, Zhang J, Xie WL, Tian C, Chen C, Shi Q, Wang SB, Xu Y, Zhang BY, Dong XP (2012) Remarkable reduction of MAP2 in the brains of scrapie-infected rodents and human prion disease possibly correlated with the increase of calpain. PLoS One 7:e30163.

Hagiwara H, Yorifuji H, Sato-Yoshitake R, Hirokawa N (1994) Competition between motor molecules (kinesin and cytoplasmic dynein) and fibrous microtubule-associated proteins in binding to microtubules. J Biol Chem 269:3581–3589.

Hayes RL, Yang K, Whitson JS, Xue JJ, Kampfl A, Mu XS, Zhao X, Faustinella F, Clifton GL (1995) Rescue of injury-induced neurofilament loss by BDNF gene transfection in primary septo-hippocampal cell cultures. Neurosci Lett 191:121–125.

Higuchi M, Tomioka M, Takano J, Shirotani K, Iwata N, Masumoto H, Maki M, Itohara S, Saido TC (2005) Distinct mechanistic roles of calpain and caspase activation in neurodegeneration as revealed in mice overexpressing their specific inhibitors. J Biol Chem 280:15229–15237.

Hoopfer ED, McLaughlin T, Watts RJ, Schuldiner O, O'Leary DD, Luo L (2006) Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron 50:883–895.

Hoskison MM, Yanagawa Y, Obata K, Shuttleworth CW (2007) Calcium-dependent NMDA-induced dendritic injury and MAP2 loss in acute hippocampal slices. Neuroscience 145:66–79.

Ikegami K, Koike T (2003) Non-apoptotic neurite degeneration in apoptotic neuronal death: pivotal role of mitochondrial function in neurites. Neuroscience 122:617–626.

Irving EA, McCulloch J, Dewar D (1996) Intracortical perfusion of glutamate in vivo induces alterations of tau and microtubule-associated protein 2 immunoreactivity in the rat. Acta Neuropathol 92:186–196.

Jaworski T, Lechat B, Demedts D, Gielis L, Devijver H, Borghgraef P, Duimel H, Verheyen F, Kugler S, Van Leuven F (2011) Dendritic degeneration, neurovascular defects, and inflammation precede neuronal loss in a mouse model for tau-mediated neurodegeneration. Am J Pathol 179:2001–2015.

Jia Y, Zhou J, Tai Y, Wang Y (2007) TRPC channels promote cerebellar granule neuron survival. Nat Neurosci 10:559–567.

Kampfl A, Zhao X, Whitson JS, Posmantur R, Dixon CE, Yang K, Clifton GL, Hayes RL (1996) Calpain inhibitors protect against depolarization-induced neurofilament protein loss of septo-hippocampal neurons in culture. Eur J Neurosci 8:344–352.

Kesavapany S, Patel V, Zheng YL, Pareek TK, Bjelogrlic M, Albers W, Amin N, Jaffe H, Gutkind JS, Strong MJ, Grant P, Pant HC (2007) Inhibition of Pin1 reduces glutamate-induced perikaryal accumulation of phosphorylated neurofilament-H in neurons. Mol Biol Cell 18:3645–3655.

Kimura N, Kumamoto T, Ueyama H, Horinouchi H, Ohama E (2007) Role of proteasomes in the formation of neurofilamentous inclusions in spinal motor neurons of aluminum-treated rabbits. Neuropathology 27:522–530.

Koike T, Yang Y, Suzuki K, Zheng X (2008) Axon & dendrite degeneration: its mechanisms and protective experimental paradigms. Neurochem Int 52:751–760.

Kurnellas MP, Nicot A, Shull GE, Elkabes S (2005) Plasma membrane calcium ATPase deficiency causes neuronal pathology in the spinal cord: a potential mechanism for neurodegeneration in multiple sclerosis and spinal cord injury. FASEB J 19:298–300.

Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ, Yuan XB (2005) Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 434:894–898.

Lobo AC, Gomes JR, Catarino T, Mele M, Fernandez P, Inacio AR, Bahr BA, Santos AE, Wieloch T, Carvalho AL, Duarte CB (2011) Cleavage of the vesicular glutamate transporters under excitotoxic conditions. Neurobiol Dis 44:292–303.

Lopez LA, Sheetz MP (1993) Steric inhibition of cytoplasmic dynein and kinesin motility by MAP2. Cell Motil Cytoskeleton 24:1–16.

Lu C, Fu W, Salvesen GS, Mattson MP (2002) Direct cleavage of AMPA receptor subunit GluR1 and suppression of AMPA currents by caspase-3: implications for synaptic plasticity and excitotoxic neuronal death. Neuromolecular Med 1:69–79.

Luetjens CM, Bui NT, Sengpiel B, Munstermann G, Poppe M, Krohn AJ, Bauerbach E, Krieglstein J, Prehn JH (2000) Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. J Neurosci 20:5715–5723.

Lunn ER, Perry VH, Brown MC, Rosen H, Gordon S (1989) Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. Eur J Neurosci 1:27–33.

Luo L, O'Leary DD (2005) Axon retraction and degeneration in development and disease. Annu Rev Neurosci 28:127–156.

Maas C, Belgardt D, Lee HK, Heisler FF, Lappe-Siefke C, Magiera MM, van Dijk J, Hausrat TJ, Janke C, Kneussel M (2009) Synaptic activation modifies microtubules underlying transport of postsynaptic cargo. Proc Natl Acad Sci U S A 106:8731–8736.

Mamounas LA, Altar CA, Blue ME, Kaplan DR, Tessarollo L, Lyons WE (2000) BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. J Neurosci 20:771–782.

Miyamoto O, Minami J, Toyoshima T, Nakamura T, Masada T, Nagao S, Negi T, Itano T, Okabe A (1998) Neurotoxicity of *Clostridium perfringens* epsilon-toxin for the rat hippocampus via the glutamatergic system. Infect Immun 66:2501–2508.

Mori Y, Matsui T, Furutani Y, Yoshihara Y, Fukuda M (2012) Small GTPase Rab17 regulates dendritic morphogenesis and postsynaptic development of hippocampal neurons. J Biol Chem 287:8963–8973.

Munirathinam S, Rogers G, Bahr BA (2002) Positive modulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. Toxicol Appl Pharmacol 185:111–118.

Namikawa K, Honma M, Abe K, Takeda M, Mansur K, Obata T, Miwa A, Okado H, Kiyama H (2000) Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. J Neurosci 20:2875–2886.

Okumoto S, Looger LL, Micheva KD, Reimer RJ, Smith SJ, Frommer WB (2005) Detection of glutamate release from neurons by genetically encoded surface-displayed FRET nanosensors. Proc Natl Acad Sci U S A 102:8740–8745.

Ostrowska H, Wojcik C, Omura S, Worowski K (1997) Lactacystin, a specific inhibitor of the proteasome, inhibits human platelet lysosomal cathepsin A-like enzyme. Biochem Biophys Res Commun 234:729–732.

Pant HC (1988) Dephosphorylation of neurofilament proteins enhances their susceptibility to degradation by calpain. Biochem J 256:665–668.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322:963–966.

Perrot R, Berges R, Bocquet A, Eyer J (2008) Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration. Mol Neurobiol 38:27–65.

Raff MC, Whitmore AV, Finn JT (2002) Axonal self-destruction and neurodegeneration. Science 296:868–871.

Ray SK, Karmakar S, Nowak MW, Banik NL (2006) Inhibition of calpain and caspase-3 prevented apoptosis and preserved electrophysiological properties of voltage-gated and ligand-gated ion channels in rat primary cortical neurons exposed to glutamate. Neuroscience 139:577–595.

Salinska E, Danysz W, Lazarewicz JW (2005) The role of excitotoxicity in neurodegeneration. Folia Neuropathol 43:322–339.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor–mTOR complex. Science 307:1098–1101.

Saxena S, Caroni P (2007) Mechanisms of axon degeneration: from development to disease. Prog Neurobiol 83:174–191.

Schoenmann Z, Assa-Kunik E, Tiomny S, Minis A, Haklai-Topper L, Arama E, Yaron A (2010) Axonal degeneration is regulated by the apoptotic machinery or a NAD^+-sensitive pathway in insects and mammals. J Neurosci 30:6375–6386.

Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int 51:333–355.

Simpkins KL, Guttmann RP, Dong Y, Chen Z, Sokol S, Neumar RW, Lynch DR (2003) Selective activation induced cleavage of the NR2B subunit by calpain. J Neurosci 23:11322–11331.

Singh B, Henneberger C, Betances D, Arevalo MA, Rodriguez-Tebar A, Meier JC, Grantyn R (2006) Altered balance of glutamatergic/GABAergic synaptic input and associated changes in dendrite morphology after BDNF expression in BDNF-deficient hippocampal neurons. J Neurosci 26:7189–7200.

Singh KK, Park KJ, Hong EJ, Kramer BM, Greenberg ME, Kaplan DR, Miller FD (2008) Developmental axon pruning mediated by BDNF-p75NTR-dependent axon degeneration. Nat Neurosci 11:649–658.

Swanwick CC, Harrison MB, Kapur J (2004) Synaptic and extrasynaptic localization of brain-derived neurotrophic factor and the tyrosine kinase B receptor in cultured hippocampal neurons. J Comp Neurol 478:405–417.

Takeuchi H, Mizuno T, Zhang G, Wang J, Kawanokuchi J, Kuno R, Suzumura A (2005) Neuritic beading induced by activated microglia is an early feature of neuronal dysfunction toward neuronal death by inhibition of mitochondrial respiration and axonal transport. J Biol Chem 280:10444–10454.

Tao J, Rolls MM (2011) Dendrites have a rapid program of injury-induced degeneration that is molecularly distinct from developmental pruning. J Neurosci 31:5398–5405.

Thompson RJ, Zhou N, MacVicar BA (2006) Ischemia opens neuronal gap junction hemichannels. Science 312:924–927.

Vanderklish PW, Bahr BA (2000) The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 81:323–339.

Wang KK, Nath R, Posner A, Raser KJ, Buroker-Kilgore M, Hajimohammadreza I, Probert Jr AW, Marcoux FW, Ye Q, Takano E, Hatanaka M, Maki M, Caner H, Collins JL, Fergus A, Lee KS, Lunney EA, Hays SJ, Yuen P (1996) An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. Proc Natl Acad Sci U S A 93:6687–6692.

Wang Q, Green SH (2011) Functional role of neurotrophin-3 in synapse regeneration by spiral ganglion neurons on inner hair cells after excitotoxic trauma in vitro. J Neurosci 31:7938–7949.

Wang X, Teng L, Li A, Ge J, Laties AM, Zhang X (2010) TRPC6 channel protects retinal ganglion cells in a rat model of retinal ischemia/reperfusion-induced cell death. Invest Ophthalmol Vis Sci 51:5751–5758.

Wang Z, Xu NL, Wu CP, Duan S, Poo MM (2003) Bidirectional changes in spatial dendritic integration accompanying long-term synaptic modifications. Neuron 37:463–472.

Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, Green DR (1999) Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood 94:1683–1692.

Xu J, Kurup P, Zhang Y, Goebel-Goody SM, Wu PH, Hawasli AH, Baum ML, Bibb JA, Lombroso PJ (2009) Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP. J Neurosci 29:9330–9343.

Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M (2007) Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. Neuron 53:399–412.

Yao H, Peng F, Fan Y, Zhu X, Hu G, Buch SJ (2009) TRPC channel-mediated neuroprotection by PDGF involves Pyk2/ERK/CREB pathway. Cell Death Differ 16:1681–1693.

Zhong H, Sia GM, Sato TR, Gray NW, Mao T, Khuchua Z, Huganir RL, Svoboda K (2009) Subcellular dynamics of type II PKA in neurons. Neuron 62:363–374.

Zhou M, Baudry M (2006) Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. J Neurosci 26:2956–2963.

(Accepted 28 January 2013)
(Available online 4 February 2013)

Chapter 3

BDNF regulates the expression of VGLUTs in cultured hippocampal neurons

The control conditions of experiments assessing BDNF neuroprotection of vesicular glutamate transporters 1 and 2 showed that incubation with BDNF alone significantly upregulated the protein levels of VGLUT2, considerably above the control levels. Therefore, we examined whether or not BDNF could regulate the expression of vesicular glutamate transporters, which would correlate its neuroprotective effect to a protein synthesis-dependent mechanism, while concomitantly suggesting a likely link between BDNF-induced mechanisms of synaptic plasticity, with VGLUTs as molecular effectors of its mechanism of action.

Therefore, the present study aimed to examine whether and how BDNF would regulate the expression of VGLUT levels in hippocampal neuronal cultures and showed that exogenous BDNF increased VGLUT expression through transcription and protein-synthesis dependent mechanisms. Exogenous application of BDNF to cultured hippocampal neurons at DIV7 rapidly increases VGLUT2 mRNA and protein levels, in a dose-dependent manner. VGLUT1 expression also increased but only transiently. However, at DIV14, BDNF stably increased VGLUT1 expression, whilst VGLUT2 levels remained low. Transcription inhibition with actinomycin-D or α-amantine, and translation inhibition with emetine or anisomycin, fully blocked BDNF-induced VGLUT upregulation.

The results further demonstrated thatinhibition of TrkB receptors with K252a and PLCγ signaling with U-73122 precludes the BDNF-induced VGLUT upregulation. BDNF activates different signaling pathways to up-regulate the expression of VGLUT1 and VGLUT2 at DIV7, which rely on CAMKII and PKC activation, respectively. Hippocampal neurons express both VGLUT isoforms during embryonic and neonatal development in contrast to adult tissue expressing only VGLUT1. Furthermore, fluorescence microscopy imaging 30 min after BDNF incubation showed a transient upregulation of VGLUT1 axonal trafficking and redistribution of VGLUT2-positive vesicles, indicating that BDNF may also affect VGLUT subcellular distribution during development. Increased VGLUT1 and VGLUT2 somatic signals were respectivelyfound 3 and 6 h later, further suggesting increased de novo transcription and translation.

Overall, these results suggest that BDNF regulates VGLUT expression during development and its effect on VGLUT1 may contribute to enhance glutamate release in LTP.Moreover, these results indicate that BDNF actives overlapping mechanisms under physiological conditions, during development, and in pathological conditions, induced by glutamate excitotoxicity, concomitantly promoting connectivity between neurons and neuroprotection.

OPEN ACCESS Freely available online

PLOS ONE

BDNF Regulates the Expression and Distribution of Vesicular Glutamate Transporters in Cultured Hippocampal Neurons

Carlos V. Melo[1], Miranda Mele[1☯], Michele Curcio[1,2☯], Diogo Comprido[1☯], Carla G. Silva[1], Carlos B. Duarte[1,3]*

1 CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal, **2** Department of Biological and Environmental Science, University of Sannio, Benevento, Italy, **3** Department of Life Sciences, University of Coimbra, Coimbra, Portugal

Abstract

BDNF is a pro-survival protein involved in neuronal development and synaptic plasticity. BDNF strengthens excitatory synapses and contributes to LTP, presynaptically, through enhancement of glutamate release, and postsynaptically, via phosphorylation of neurotransmitter receptors, modulation of receptor traffic and activation of the translation machinery. We examined whether BDNF upregulated vesicular glutamate receptor (VGLUT) 1 and 2 expression, which would partly account for the increased glutamate release in LTP. Cultured rat hippocampal neurons were incubated with 100 ng/ml BDNF, for different periods of time, and VGLUT gene and protein expression were assessed by real-time PCR and immunoblotting, respectively. At DIV7, exogenous application of BDNF rapidly increased VGLUT2 mRNA and protein levels, in a dose-dependent manner. VGLUT1 expression also increased but only transiently. However, at DIV14, BDNF stably increased VGLUT1 expression, whilst VGLUT2 levels remained low. Transcription inhibition with actinomycin-D or α-amanitine, and translation inhibition with emetine or anisomycin, fully blocked BDNF-induced VGLUT upregulation. Fluorescence microscopy imaging showed that BDNF stimulation upregulates the number, integrated density and intensity of VGLUT1 and VGLUT2 puncta in neurites of cultured hippocampal neurons (DIV7), indicating that the neurotrophin also affects the subcellular distribution of the transporter in developing neurons. Increased VGLUT1 somatic signals were also found 3 h after stimulation with BDNF, further suggesting an increased de novo transcription and translation. BDNF regulation of VGLUT expression was specifically mediated by BDNF, as no effect was found upon application of IGF-1 or bFGF, which activate other receptor tyrosine kinases. Moreover, inhibition of TrkB receptors with K252a and PLCγ signaling with U-73122 precluded BDNF-induced VGLUT upregulation. Hippocampal neurons express both isoforms during embryonic and neonatal development in contrast to adult tissue expressing only VGLUT1. These results suggest that BDNF regulates VGLUT expression during development and its effect on VGLUT1 may contribute to enhance glutamate release in LTP.

Citation: Melo CV, Mele M, Curcio M, Comprido D, Silva CG, et al. (2013) BDNF Regulates the Expression and Distribution of Vesicular Glutamate Transporters in Cultured Hippocampal Neurons. PLoS ONE 8(1): e53793. doi:10.1371/journal.pone.0053793

Editor: Xiang Yang Zhang, Baylor College of Medicine, United States of America

Received June 5, 2012; **Accepted** December 5, 2012; **Published** January 11, 2013

Funding: FCT (Fundação para a Ciência e a Tecnologia) and FEDER (Fundo Europeu de Desenvolvimento Regional, Portugal) (grants PTDC/SAU-NMC/120144/2010 and PTDC/SAU-NEU/104297/2008) funded this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: cbduarte@ci.uc.pt

☯ These authors contributed equally to this work.

Introduction

BDNF (brain-derived neurotrophic factor) is a pro-survival protein that promotes neuronal differentiation and synaptic plasticity [1,2], in addition to neuroprotection [3,4]. During development, BDNF stimulates the formation of appropriate synaptic connections, controlling the direction and rate of axon growth [5,6], as well as the shape of dendritic arbors and spines [7–9]. In the adult hippocampus, BDNF is also involved in learning [10,11] and memory formation [12,13], and is essential for long-term potentiation (LTP) [14–18].

The effects of BDNF are mainly mediated through activation of the TrkB (tropomyosin-related kinase B receptor) receptor tyrosine kinase as well as the p75 neutrotrophin receptor ($p75^{NTR}$) [19]. Activation of TrkB receptors by BDNF leads to receptor dimerization and trans-autophosphorylation of several tyrosine residues in the intracellular domain, including Y490 and Y816, which allow recruiting proteins containing PTB and SH2 (Src homology-type 2) domains, activating in parallel the Ras-ERK (extracellular signal-regulated kinase), PI3-K (phosphatidylinositol 3-kinase)/Akt and phospholipase C-γ (PLCγ) signaling pathways [20]. Trans-autophosphorylation of Y816 recruits and activates cytoplasmic PLCγ, which hydrolyzes PIP2 (phosphatidylinositol 4,5-bisphosphate) into IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP3 promotes Ca^{2+} release from internal stores, activating $[Ca^{2+}]_i$-regulated enzymes, including Ca^{2+}- and calmodulin-dependent protein kinases (CAMKs), and protein kinase C (PKC) isoforms [21]. Concomitantly, DAG stimulates DAG-regulated PKC isoforms, such as PKCδ [20]. The PLCγ pathway

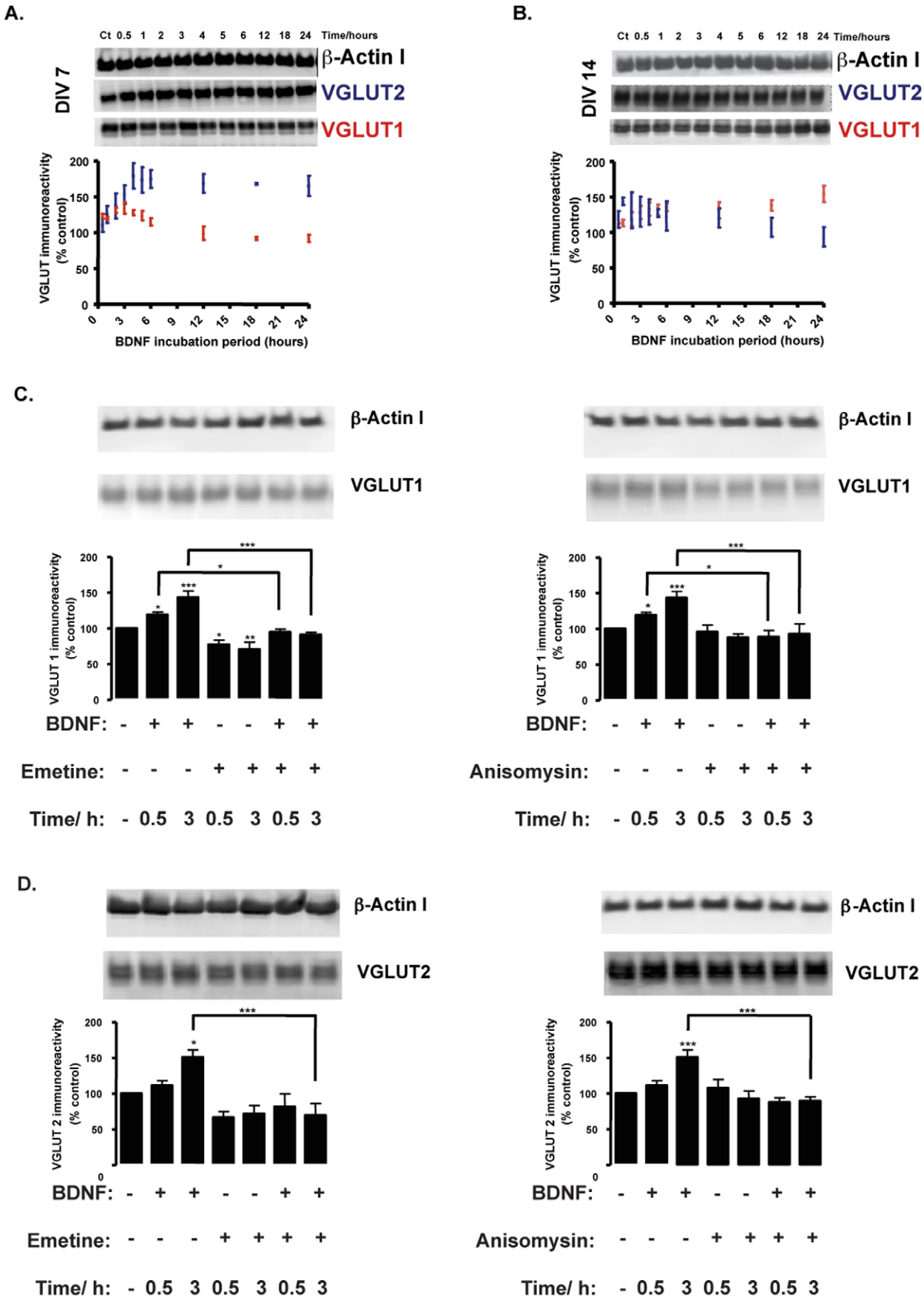

A.
Ct 0.5 1 2 3 4 5 6 12 18 24 Time/hours
DIV 7
β-Actin I
VGLUT2
VGLUT1
VGLUT immunoreactivity (% control)
BDNF incubation period (hours)
B.
Ct 0.5 1 2 3 4 5 6 12 18 24 Time/hours
DIV 14
β-Actin I
VGLUT2
VGLUT1
VGLUT immunoreactivity (% control)
BDNF incubation period (hours)
C.
β-Actin I
VGLUT1
VGLUT 1 immunoreactivity (% control)
BDNF:
Emetine:
Time/ h:
β-Actin I
VGLUT1
VGLUT 1 immunoreactivity (% control)
BDNF:
Anisomysin:
Time/ h:
D.
β-Actin I
VGLUT2
VGLUT 2 immunoreactivity (% control)
BDNF:
Emetine:
Time/ h:
β-Actin I
VGLUT2
VGLUT 2 immunoreactivity (% control)
BDNF:
Anisomysin:
Time/ h:

Figure 1. BDNF upregulates VGLUT1 and VGLUT2 protein expression through a translation-dependent mechanism. (A–B) Cultured hippocampal neurons at DIV7 (A) and DIV14 (B) were incubated with 100 ng/ml BDNF for different time periods and total VLGUT1 and VGLUT2 protein levels were compared to control (without BDNF) expression, upon normalization with β-actin I levels. (C–D) Cultured hippocampal neurons at DIV7 were pre-incubated or not with the translation inhibitors emetine or anisomycin (2 μM) for 30 min before BDNF stimulation during 30 min or 3 h and VGLUT1 (C) and VGLUT2 (D) protein levels were compared to control expression. When the effect of translation inhibitors was tested, the cells were incubated with the compounds during stimulation with BDNF. (A–D) Quantification of 3–5 different experiments, performed in independent preparations, is presented as mean percentage ± SEM compared to the control (unstimulated neurons). Statistical significance was determined by One Way ANOVA followed by Bonferronis multiple comparison test with a confidence interval of 99% (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
doi:10.1371/journal.pone.0053793.g001

is central in LTP [18,22,23] and growth cone guidance [6], retrograde synaptic modification [24] and dendritic spine morphology [25] regulated by activation of TRPC (transient receptor potential canonical) channels. Trans-autophosphorylation of Y490 enables recruitment of Shc (Src homology 2-containing protein), IRS1 (insulin receptor substrate 1) and IRS2 linker proteins, thereby activating the Ras-ERK and PI3K/Akt cascades [26]. ERK translocates to the nucleus upon phosphorylation, regulating gene expression through isoform-specific activation of transcription factors, including cAMP-response element binding protein (CREB) (through ERK1/2/5), MEF2 (downstream of ERK5) or Elk1 (following activation of ERK1/2) [27]. The Ras-ERK signaling pathway is crucial for neurogenesis [28], inhibition of proapoptotic proteins [29], stimulation of pro-survival gene expression [30] and protein synthesis-dependent plasticity [31]. The PI3K/Akt pathway has a pivotal role in cell survival [32], neuroprotection [3], trafficking of synaptic proteins [33] and can also directly control protein synthesis through mTOR (mammalian target of rapamycin) activation and 4EBP phosphorylation [34].

The TrkB-activated signaling pathways account for nearly all BDNF synaptic effects but their biological responses likely reflect BDNF or TrkB receptor levels and the spatiotemporal pattern of BDNF stimulation, especially when activated pre- and/or postsynaptically [35]. Nevertheless, the molecular mechanisms underlying BDNF signaling in short-term plasticity and long-term potentiation are not fully understood. We have previously reported that BDNF induces significant proteome changes [36], including the regulation of AMPA and NMDA receptors involved in molecular mechanisms of synaptic plasticity [37,38]. BDNF promotes phosphorylation of synapsin I [39] and beta-catenin [40] increasing synaptic vesicle docking at the active zone and quantal glutamate release [39,41]. However, direct presynaptic effectors of protein synthesis-dependent BDNF signaling on glutamatergic function, which also contributes to LTP and memory formation [22,23], have not been identified thus far. The vesicular glutamate transporters (VGLUT) are such target candidates because they mediate L-glutamate uptake into synaptic vesicles and are required for exocytic glutamate release at presynaptic terminals [42]. Moreover, VGLUT1 and VGLUT2 expression is developmentally regulated in order to match vesicle cycling and quantal amplitude [43,44]. In addition, VGLUT isoforms have similar substrate specificity, transport activity and kinetics but complementary expression, which correlates with release probability and potential for plasticity [45]. Therefore, the current study aimed at examining the effect of BDNF on the expression of VGLUT, given their relevance in LTP, learning and memory function [46,47]. We report that BDNF regulates VGLUT gene and protein expression during development of cultured hippocampal neurons, through activation of the PLCγ signaling pathway, and also affects VGLUT subcellular distribution, further suggesting a role in BDNF-induced LTP.

Materials and Methods

Ethics Statement

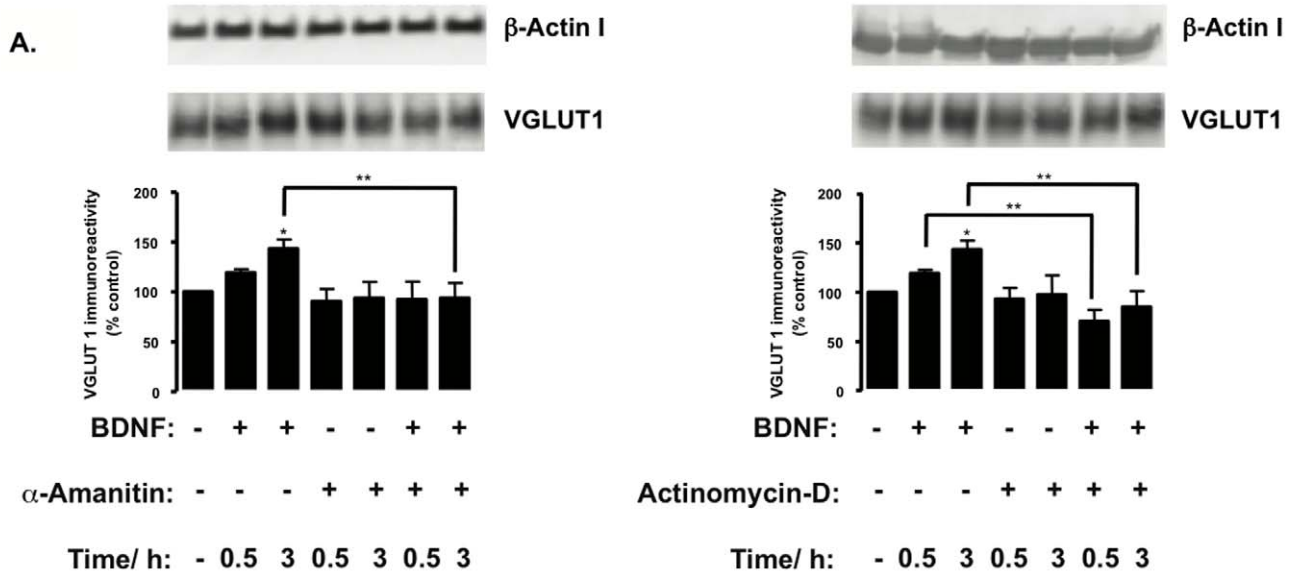
Experiments were performed according to the European Union Directive 86/609/EEC and the legislation Portaria n. 1005/92, issued by the Portuguese Government for the protection of animals used for experimental and other scientific purposes. Dams were sacrificed by cervical dislocation. Embryos were then surgically removed and sacrificed by decapitation.

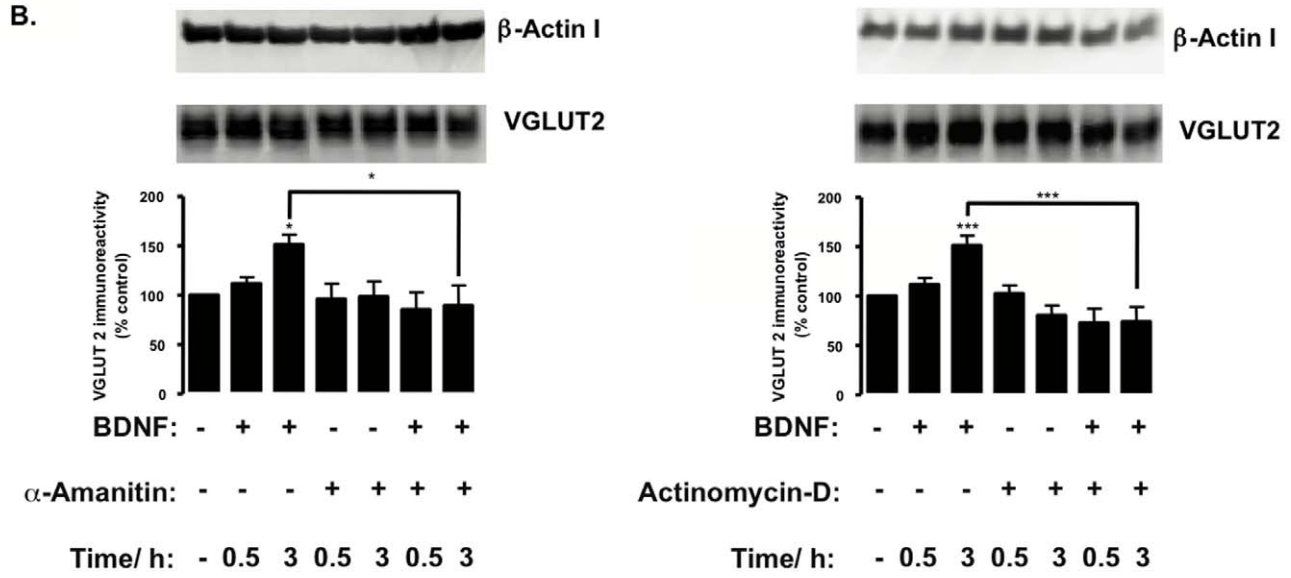
Hippocampal Cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18–E19 Wistar rat embryos, after treatment with trypsin (0.06%, for 15 min at 37°C; GIBCO-Invitrogen) and deoxyribonuclease I (5.36 mg/ml), in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO-Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO-Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO-Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6-well plates (870,000 cells/well) coated with poly-D-lysine (0.1 mg/ml), or on poly-D-lysine coated glass coverslips, at a density of 80,000 cells/well (12-well plates). The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37°C, for 7 or 14 days. BDNF stimulation was carried out by adding BDNF (Regeneron or PeproTech) in Neurobasal medium to a final concentration of 100 ng/ml, for the indicated period of time. When appropriate, 1.5 μM α-amanitin or actinomycin D (transcription inhibitors), 2.0 μM emetine or anisomycin (translation inhibitors) (Calbiochem), 200 nM K252a (TrkB inhibitor), 5 μM U73122 (PLCγ pathway inhibitor), 5 μM chelerythrine (PKC inhibitor) or 1 μM KN-93 (CAMKII inhibitor), 20 μM PD098059 or 10 μM U0126 (Ras-ERK pathway inhibitors), 30 μM LY294002 or 300 nM Wortmannin (PI3K/Akt pathway inhibitors) (Sigma-Aldrich Química) were added 30 min before BDNF stimulation, as indicated. The cells were further incubated with the signaling inhibitors for 3 h or 5 h, during BDNF stimulation. When appropriate, 100 ng/ml IGF-1 (insulin-like growth factor 1) and bFGF (basic fibroblast growth factor) (Sigma-Aldrich Química) were added in lieu of BDNF.

Preparation of Extracts

Hippocampal neurons (DIV7/DIV14) were washed twice with ice-cold PBS and once more with PBS supplemented with 1 mM DTT and a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma-Aldrich Química). The cells were then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5), supplemented with 50 mM NaF,


A.
β-Actin I
VGLUT1
VGLUT 1 immunoreactivity (% control)
BDNF:
α-Amanitin:
Time/ h:
Actinomycin-D:


B.
β-Actin I
VGLUT2
VGLUT 2 immunoreactivity (% control)
BDNF:
α-Amanitin:
Time/ h:
Actinomycin-D:

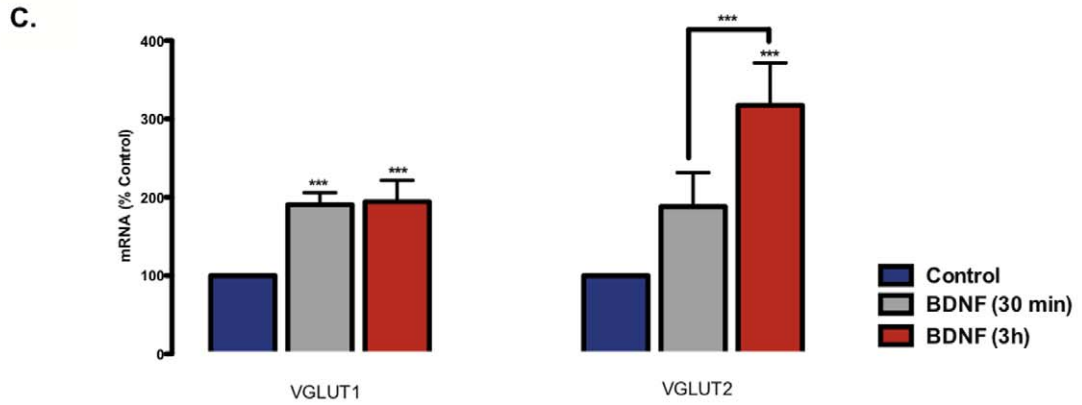

C.
mRNA (% Control)
VGLUT1
VGLUT2
Control
BDNF (30 min)
BDNF (3h)

Figure 2. The effect of BDNF on VGLUT1 and VGLUT2 protein levels is dependent on gene expression. (A–B) Hippocampal neurons were stimulated with BDNF (100 ng/ml) for the indicated periods of time, in the presence or in the absence of the transcription inhibitors α-amanitin (1.5 μM) or actinomycin-D (1.5 μM), and VGLUT1 (A) and VGLUT2 (B) protein levels were determined by western blot. (C) The variation of *Slc17a7* (VGLUT1) and *Slc17a6* (VGLUT2) mRNA levels was assayed by real-time PCR, as described in the methods section. The neurons were stimulated with 100 ng/ml BDNF during 30 minutes (grey) or 3 hours (red). (A–C) Quantification of 4–5 experiments, performed in independent preparations, is presented as mean percentage ± SEM compared to the control (unstimulated neurons), and normalized to *18S* reference gene. Statistical significance was determined by One Way ANOVA followed by Bonferronis multiple comparison test with a confidence interval of 99% (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
doi:10.1371/journal.pone.0053793.g002

1.5 mM sodium orthovanadate and a cocktail of protease inhibitors, and sonicated, on ice, using an ultrasonic cell disrupter microtip (VibraCell, Sonics & Materials, Inc.), with 2 cycles of 10 consecutive 1 s, low-intensity pulses interspaced by 30 s, in order to fully disrupt membrane structure. After centrifugation at 16,100 g for 10 min, protein in the supernatants was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific), and the samples were denaturated with 2x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue), without denaturation at 95°C for 5 min, which would otherwise cause loss of vesicular proteins to the insoluble fraction.

Total RNA Isolation and Reverse Transcription for Real-time PCR

Total RNA from cultured hippocampal neurons was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The full content of a 6-well cell cluster plate, with 870,000 cells/well (DIV7), was collected for each experimental condition. For first strand cDNA synthesis, 3 μg of total RNA was reverse-transcribed with avian myeloblastosis (AMV) reverse transcriptase (Roche Applied Science) using random primers $p(dN)_6$ (3.2 μg), dNTPs (1 mM each), $MgCl_2$ (25 mM), RNase inhibitor (50 units) and gelatin (0.01 μg/μl) in reaction buffer (10 mM Tris, 50 mM KCl, pH 8.3), in a total volume of 40 μl. The reaction was performed at 25°C for 10 min, followed by 60 min at 42°C, for primer annealing to the RNA template and cDNA synthesis, respectively. The reverse transcriptase was then denatured during 5 min at 99°C, and the sample was cooled to 4°C for 5 min and finally stored at -80°C until further use.

Real-time PCR

Real-Time PCR analysis of gene expression was performed using the LightCycler System II (Roche Applied Science). The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I (Roche et al., 1996) in 20 μl capillaries. The primers used for amplification of genes encoding VGLUT1 and VGLUT2 were, respectively, VGLUT1, forward: 5′ TGG AGT TCC GGC AGG AGG AGT T; VGLUT1, reverse: 5′ GTG TGT GTG GTG ACT GGG CGC; VGLUT2, forward: 5′ GAA GAA ACG GGG GAC ATC ACT GAG A; VGLUT2, reverse: 5′ GTC TTG CGC ACT TTC TTG CAC AAA T. The primers used for the amplification of endogenous control gene 18S ribosomal RNA were those included in the Applied Biosystems TaqMan Ribosomal RNA Control Reagents Kit. Each primer of a pair was added to the reaction mixture (10 μl) at a final concentration of 0.8 μM, with 3 mM $MgCl_2$, in addition to the "Hot Start" LightCycler Fast Start DNA Master SYBR Green I mix (1x) and 2.0 μl of cDNA sample. Thermal cycling was initiated with activation of the FastStart TaqDNA polymerase by denaturation during 10 min at 95°C followed by 45 cycles of a 30 s melting step at 95°C, a 5 s annealing step at 60°C, and a 25 s elongation step at 72°C. All temperature transition rates used were at 20°C/s. After amplification for 45 cycles, at least 10 cycles beyond the beginning of the linear phase of amplification, samples were subjected to a melting curve analysis according to the manufacturer's instructions in order to confirm the absence of unspecific amplification products and primer-dimers. Samples containing no template were included as negative controls in all experiments.

mRNA Quantitative Analysis

The mRNA levels of the constitutively expressed reference gene encoding 18S ribosomal RNA were used as a control, in all experiments. The relative changes in the mRNA levels of glutamate receptor subunits in cultured hippocampal neurons were determined using the $\Delta\Delta C_p$ method. Accordingly, for each experimental condition (unstimulated neurons and neurons treated with 100 ng/ml BDNF for 30 min or 3 h) the "crossing point" (C_p) values given by the LightCycler system II software, for each target gene, were subtracted by the respective C_p value determined for the 18S gene from the same sample and condition (ΔC_p). This allows normalizing changes in target gene expression. Afterward, the ΔC_p values were subtracted by the respective values of the control for the target gene giving $\Delta\Delta C_p$. The derivation to the value of $2^{-(\Delta\Delta C_p)}$ sets each control at the unity (or 100%), because $\Delta\Delta C_p$ (control) = 0, and the stimuli conditions used were set at percentage relative to control.

Immunoblotting

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to polyvinylidene (PVDF) membranes (Millipore Corp.), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4°C), washed and exposed to alkaline phosphatase (ECF)-conjugated secondary antibody (1 h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Storm 860 Gel and Blot Imaging System (GE Healthcare). The following primary antibodies were used: anti-VGLUT1 and anti-VGLUT2 (1:1000, Synaptic Systems); anti-β-Tubulin I (1:10000, Sigma-Aldrich Química), anti-β-actin I (1:20000, Sigma-Aldrich Química), anti-pERK1/2 (1:1000, Cell Signaling), anti-BDNF (1:1000, Santa Cruz Biotechnology). Anti-rabbit or anti-mouse IgG alkaline phosphatase-conjugated secondary antibodies (respectively, 1:20000 and 1:10000, GE Healthcare) were used for detection.

Immunocytochemistry

For immunocytochemistry, cultured hippocampal neurons were grown on poly-D-lysine coated glass coverslips, at a density of 80000 cells/well (12-well plates), and were then fixed in PBS supplemented with 4% paraformaldehyde/4% sucrose, for 15 min at room temperature. After fixation the cells were washed and permeabilized with 0.25% Triton X-100 in PBS, for 5 min at 4°C, washed once in PBS for 5 min, and then blocked with 10% BSA, for 1 h at room temperature, and stained with specific primary

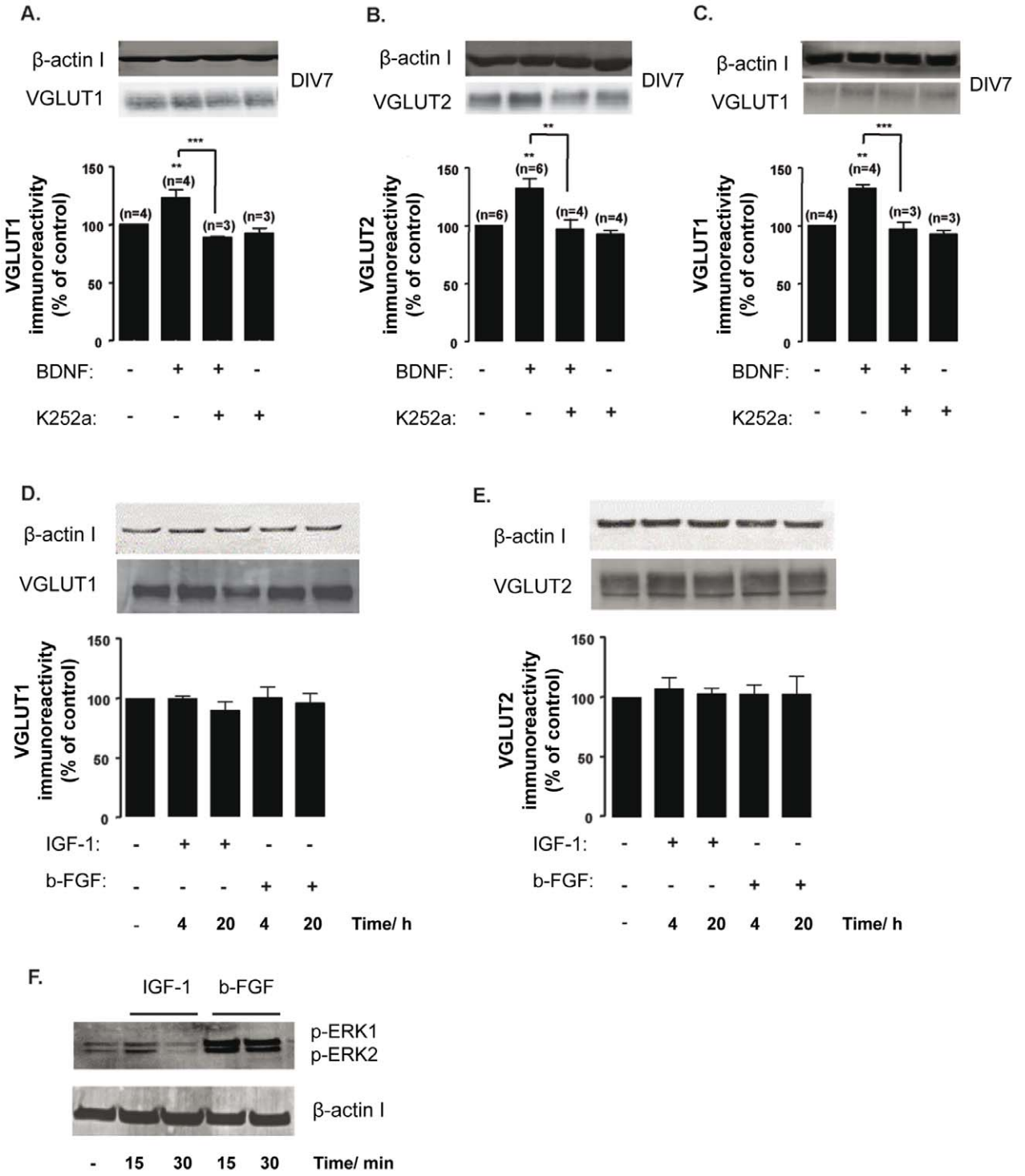


Figure 3. TrkB receptor inhibition blocks BDNF upregulation of VGLUT1 and VGLUT2. (A–C) Cultured hippocampal neurons at DIV7 (A, B) and DIV14 (C) were stimulated with BDNF (100 ng/ml), for the indicated periods of time, in the presence or absence of a selective inhibitor of tyrosine kinase activity, K252a (200 nM), and VGLUT1 (A, C) and VGLUT2 (B) protein levels were determined by western blot. Quantification of the indicated number of experiments, performed in independent preparations, is presented as mean percentage ± SEM compared to the control (unstimulated neurons). (D–F) DIV7 hippocampal neurons were stimulated with IGF-1 or bFGF, for 4 or 20 h, and VGLUT1 (D) and VGLUT2 (E) protein levels were determined by western blot. Quantification of 4 different experiments, performed in independent preparations, is presented as mean percentage ± SEM compared to the control. Statistical significance was determined by One Way ANOVA followed by Bonferronis multiple comparison test with a confidence interval of 99% (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (F) DIV7 hippocampal neurons were stimulated with IGF-1 or bFGF, for 15 or 30 min, and the levels of ERK1/2 phosphorylation were determined by western blot. The antibody used specifically recognizes the phosphorylated isoforms 1 and 2 of ERK, but not the nonphosphorylated (presumably inactive) proteins.
doi:10.1371/journal.pone.0053793.g003

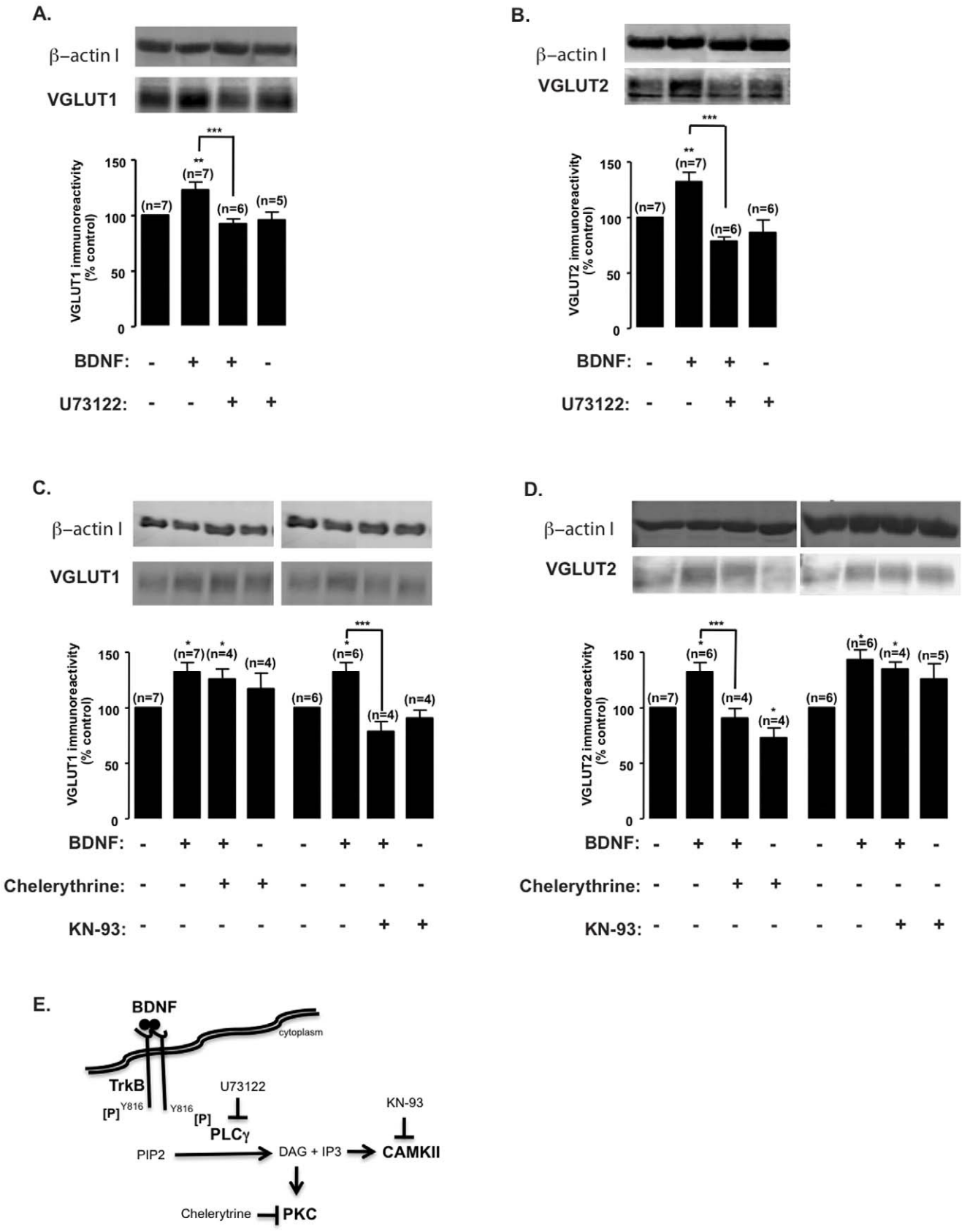


Figure 4. Inhibition of the PLCγ signaling pathway blocks BDNF-induced upregulation of VGLUT1 and VGLUT2 protein levels. (A–D) DIV7 cultured hippocampal neurons were stimulated with BDNF (100 ng/ml) for the indicated periods of time, in the presence or absence of U73122 (PLCγ inhibitor; 5 μM) (A, B), chelerythrine (PKC inhibitor; 5 μM) or KN-93 (CAMKII inhibitor; 1 μM) (C, D), and VGLUT1 (A, C) and VGLUT2 (B, D) protein

levels were determined by western blot. Quantification of the indicated number of experiments, performed in independent preparations, is presented as mean percentage ± SEM compared to the control (unstimulated neurons). Statistical significance was determined by One Way ANOVA followed by Bonferronis multiple comparison test with a confidence interval of 99% (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (E) Schematic representation of BDNF-induced TrkB receptor trans-activation and downstream PLCγ signaling pathway effectors and inhibitors.
doi:10.1371/journal.pone.0053793.g004

antibodies overnight at 4°C. The following primary antibodies were used: rabbit anti-VGLUT1 or anti-VGLUT2 (1:1000 and 1:500, respectively; Synaptic Systems) and mouse anti-β-Tubulin I (1:1000; Sigma-Aldrich Química). Subsequently, cells were washed six times and incubated for 1 h at 37°C with the secondary antibodies (Alexa Fluor® 488 goat anti-rabbit and Alexa Fluor® 568 goat anti-mouse, 1:500; Invitrogen). The cells were washed six times, mounted on glass slides with the Dako mounting medium and viewed on an Axio Observer 2.1 fluorescence microscope coupled to an Axiocam HRm digital camera. For each set of experiments the cell images were acquired using identical exposure settings. The regions of interest for the quantification were blindly chosen using the tubulin channel. The images were analyzed for the number, the integrated density (mean intensity× puncta area), and the intensity of VGLUT puncta along neurites, as well as the total immunoreactivity in the soma, using the ImageJ software (NIH). The quantification was performed after determination of the threshold and subtraction of the background. The results of the quantification were normalized for the length of the region of interest in the case of neurites or for the area in the case of the soma. At least 12 cells per condition were analyzed for each preparation.

Statistical Analysis

Data are presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Dunnett's or Bonferroni post-tests, at a 99% confidence interval, or using the Student's *t* test, as indicated in the figure captions.

Results

BDNF Upregulates VGLUT1 and VGLUT2 Total Protein Levels

7 and 14 DIV cultured hippocampal neurons were incubated with or without 100 ng/ml BDNF, for different time periods (30 min to 24 h), in order to determine whether acute stimulation with BDNF affects the protein expression of vesicular glutamate transporters. VGLUT1 and VGLUT2 protein levels were determined by Western blotting (Fig. 1A). At DIV7, BDNF rapidly and significantly upregulated VGLUT2 protein levels, while VGLUT1 protein levels were only transiently upregulated at the initial time points, subsequently returning to levels similar to the control condition (unstimulated neurons). In contrast, at DIV14, BDNF did not significantly change VGLUT2 protein levels but instead upregulated VGLUT1 throughout time (Fig. 1B). The increase in VGLUT2 protein levels at DIV7 and in VGLUT1 at DIV14 had distinct kinetics, and the maximal effects were found after incubation with BDNF for 3 h and 24 h, respectively. At DIV7, after a rapid increase, VGLUT2 protein levels remained high and relatively similar to the maximal value (3 h), even 24 h after incubation, while the abundance of VGLUT1 showed a slow and gradual increase, in comparison to the control. The sustained increase in VGLUT1 and VGLUT2 protein levels observed in hippocampal neurons (DIV14 and DIV7, respectively) subjected to a chronic stimulation with BDNF was not observed when the incubation was limited to 4 h, and followed by 14 h incubation in culture conditioned medium ($p>0.05$) (Fig. S1). The effect of BDNF on VGLUT2 expression was not further examined at DIV14 because it was not significant and the endogenous expression levels of this isoform are rather low and variable, in developed neurons. These results mimic the developmental switch, from VGLUT2 to VGLUT1 expression, observed in postnatal hippocampal neurons [42,43,48].

In order to test whether the effect of BDNF resulted from an increase in protein synthesis, we used two translation inhibitors, anisomycin and emetine. Hippocampal neurons were stimulated with BDNF for 30 min or 3 h, in the presence or absence of translation inhibitors, which were added to the cultured media 30 min prior to BDNF stimulation and kept in the media during incubation with BDNF. Emetine (2 μM) or anisomycin (2 μM) fully abrogated the effect of BDNF on VGLUT1 (Fig. 1C) and VGLUT2 (Fig. 1D) isoforms at DIV7. None of the protein synthesis inhibitors reduced VGLUT1 or VGLUT2 protein levels under control conditions ($p>0.05$), in agreement with the relatively long half-life suggested for VGLUT2 [49]. However, translation inhibition was not tested at DIV14 because BDNF only upregulates VGLUT1 protein levels for long incubation periods, above the cellular toxicity threshold of emetine and anisomycin [50]. Treatment with anisomycin or emetine alone did not alter VGLUT1 or VGLUT2 protein levels in the time periods tested ($p>0.05$). Taken together, these results indicate that BDNF upregulates VGLUT isoforms 1 and 2 through a protein-synthesis dependent mechanism, and rule out the hypothesis of a reduction in protein degradation.

BDNF Upregulates VGLUT1 and VGLUT2 by Enhancing Transcriptional Activity

BDNF signaling may stimulate gene transcription [51] and/or protein synthesis [52,53]. Hence, we used two different transcription inhibitors, α-amanitin (1.5 μM) and actinomycin D (1.5 μM) to test the role of transcription in the upregulation of vesicular glutamate transporters by BDNF. Both transcription inhibitors blocked the effect of BDNF on VGLUT1 (Fig. 2A) and VGLUT2 (Fig. 2B) protein levels and had no effect on the abundance of VGLUT variants in the absence of this neurotrophin, relative to the control condition. In agreement with these findings, real-time PCR experiments showed that BDNF stimulation for 30 min caused an approximately 2-fold increased in VGLUT1 mRNA levels ($p<0.001$). A significant increase in VGLUT2 mRNA (approximately 3-fold) was also found when cells were incubated with the neurotrophin for 3 h ($p<0.001$), with VGLUT1 mRNA levels remaining at a similar level to that of stimulation for only 30 min (Fig. 2C). Overall, the results suggest that BDNF regulates VGLUT1 and VGLUT2 gene expression, likely through activation of a BDNF signaling-modulated transcription mechanism and/or transcription factor(s).

VGLUT Upregulation Depends on TrkB Receptor Activation Specifically Induced by BDNF

BDNF signaling may stimulate gene transcription [51] and/or protein synthesis [52,53], essentially through activation of TrkB receptors [33]. K252a is a potent inhibitor of tyrosine protein kinase activity of TrkA, TrkB and TrkC receptors, blocking receptor autophosphorylation and, consequently, the biological

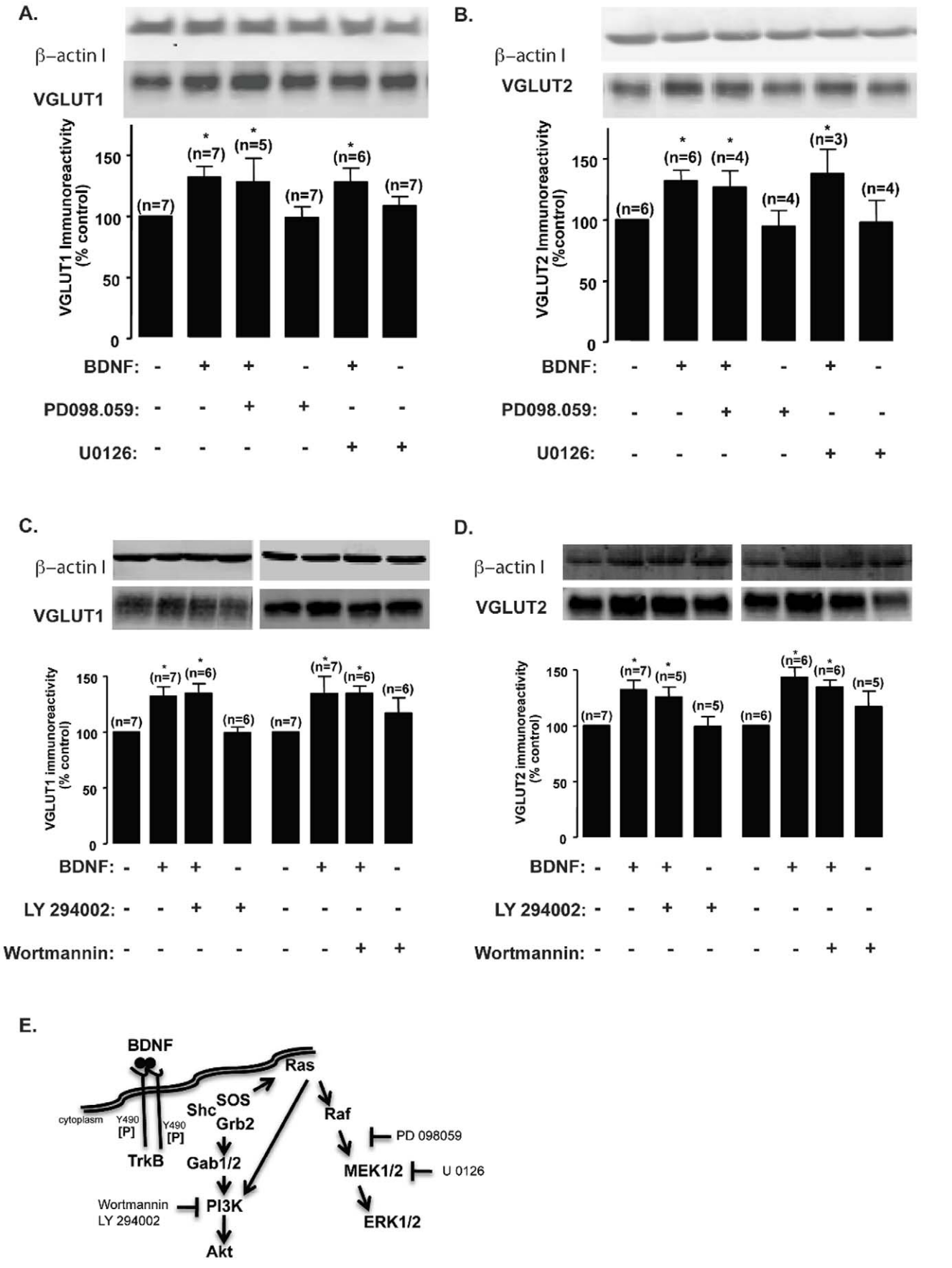

A.
β–actin I
VGLUT1
VGLUT1 Immunoreactivity (% control)
150
100
50
0
(n=7)
(n=7)
(n=5)
(n=7)
(n=6)
(n=7)
BDNF: - + + - + -
PD098.059: - - + + - -
U0126: - - - - + +
B.
β–actin I
VGLUT2
VGLUT2 Immunoreactivity (%control)
150
100
50
0
(n=6)
(n=6)
(n=4)
(n=4)
(n=3)
(n=4)
BDNF: - + + - + -
PD098.059: - - + + - -
U0126: - - - - + +
C.
β–actin I
VGLUT1
VGLUT1 immunoreactivity (% control)
150
100
50
0
(n=7)
(n=7)
(n=6)
(n=6)
(n=7)
(n=7)
(n=6)
(n=6)
BDNF: - + + - - + + -
LY 294002: - - + + - - - -
Wortmannin: - - - - - - + +
D.
β–actin I
VGLUT2
VGLUT2 immunoreactivity (% control)
150
100
50
0
(n=7)
(n=7)
(n=5)
(n=5)
(n=6)
(n=6)
(n=6)
(n=5)
BDNF: - + + - - + + -
LY 294002: - - + + - - - -
Wortmannin: - - - - - - + +
E.
BDNF
Ras
cytoplasm
Y490 [P]
Y490 [P]
Shc
SOS
Grb2
Raf
PD 098059
TrkB
Gab1/2
MEK1/2
U 0126
Wortmannin
LY 294002
PI3K
ERK1/2
Akt

Figure 5. Inhibition of the PI3-K/Akt and Ras-ERK signaling pathways has no significant effect on BDNF-induced upregulation of VGLUT1 and VGLUT2 protein levels. (A–D) DIV7 cultured hippocampal neurons were stimulated with BDNF (100 ng/ml), for the indicated periods of time, in the presence or absence of Ras-ERK pathway inhibitors PD098059 (20 μM) or U0126 (10 μM) (A, B), or PI3K/Akt inhibitors LY294002 (30 μM) or Wortmannin (300 nM) (C, D), and VGLUT1 (A, C) and VGLUT2 (B, D) protein levels were determined by western blot. Quantification of the indicated number of experiments, performed in independent preparations, is presented as mean percentage ± SEM compared to the control (unstimulated neurons). Statistical significance was determined by One Way ANOVA followed by Bonferronis multiple comparison test with a confidence interval of 99% (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (E) Schematic representation of BDNF-induced TrkB receptor trans-activation and downstream effectors and inhibitors of the PI3-K/Akt and Ras-ERK signaling pathways.
doi:10.1371/journal.pone.0053793.g005

functions of their neurotrophin ligands [54]. In addition to TrkB, cultures of embryonic day 18 (E18) hippocampal neurons express TrkC, but not TrkA receptors [55] and BDNF does not bind to TrkC receptors [56,57]. The results found show that TrkB receptor activation is required for upregulation of VGLUT1 (DIV7 and DIV14) and VGLUT2 (DIV7) protein levels because no effect of BDNF was found when the stimulation with the neurotrophin was performed in the presence of 200 nM K252a ($p>0.05$) (Fig. 3A–C). K252a alone did not significantly alter either VGLUT1 or VGLUT2 protein levels, when compared to the control condition (without treatment) ($p>0.05$), which demonstrates a specific action of BDNF in the upregulation of VGLUT expression (Fig. 3A–C). In addition, the lack of effect of K252a on VGLUT2 expression at DIV14 did not result from the endogenous release of saturating amounts of BDNF, which would prevent any additional effect by its exogenous application, because incubation with the Trk receptor inhibitor alone did not decrease VGLUT2 protein expression below the control levels (Fig. S2). This further suggests that BDNF signaling may regulate the developmental switch from VGLUT2 to VGLUT1 expression in hippocampal neurons.

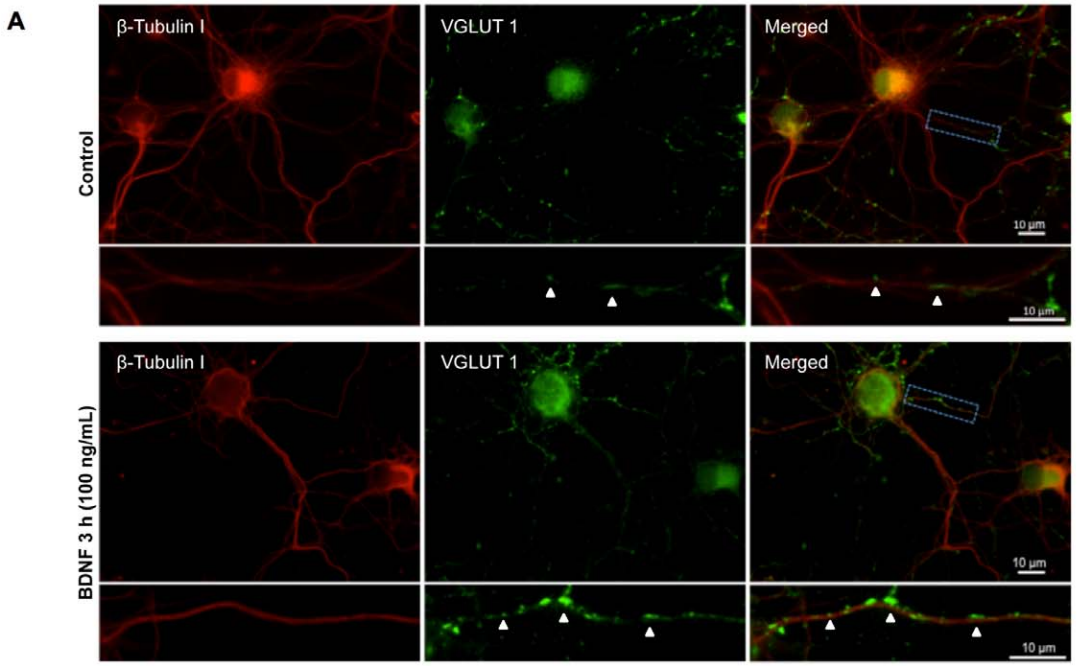
Cultured hippocampal neurons express receptors for other trophic factors, including IGF-1 [58,59] and bFGF [60,61], which activate the same BDNF-induced signaling pathways in cultured hippocampal neurons. Moreover, IGF-I enhances the expression of TrkB receptors and the ability of BDNF to induce ERK1/2 phosphorylation in cerebrocortical neurons [62] while bFGF rapidly stimulates BDNF expression in the hippocampal cell line HiB5 [63]. In this context, we tested whether acute stimulation with IGF-1 or bFGF in lieu of BDNF would affect the expression levels of VGLUT isoforms, at two different time points (4 h and 20 h). Either brief or prolonged incubation with 100 ng/ml IGF-1 or bFGF had no effect on VGLUT1 (Fig. 3D) and VGLUT2 (Fig. 3E) protein levels, at DIV7, when compared to the control condition ($p>0.05$). Since exogenously applied neurotrophic factors are only effective when their receptors are expressed at the cell surface and free to bind their ligands, the absence of any effect on VGLUT expression could have resulted from ligand or receptor inactivity. In order to exclude this possibility, we tested the levels of ERK1/2 phosphorylation, upon 15 or 30 min of stimulation with IGF-1 or bFGF. The antibody used specifically recognizes the phosphorylated isoforms 1 and 2 of ERK, but not the non-phosphorylated (presumably inactive) proteins. The results show ERK1/2 phosphorylation after 15 min incubation with both IGF-1 and bFGF, and the effect of bFGF was still observed after 30 min of stimulation. These results confirm that the lack of effect of IGF-1 and bFGF on VGLUT expression was not due to inactivity of trophic factors or their receptors (Fig. 3F). Therefore, we may conclude that BDNF upregulates VGLUT1 and VGLUT2 specifically through activation of TrkB receptors as K252a fully abrogated the effect in cultured hippocampal neurons at DIV7 (Fig. 3A, B) and DIV14 for VGLUT1 (Fig. 3C).

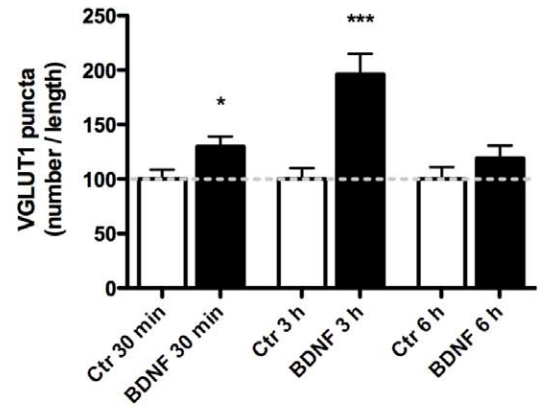
BDNF Regulates VGLUT Expression through PLCγ Signaling Pathway Activation

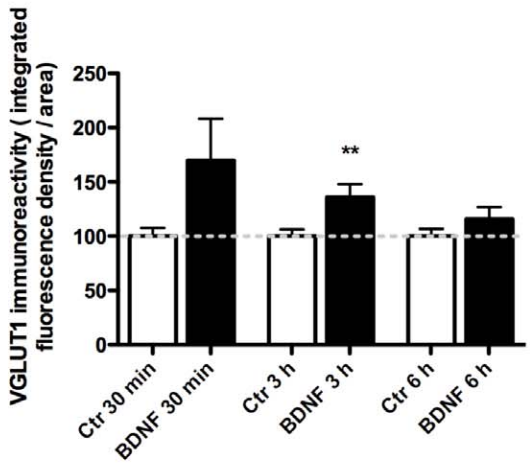
The specificity of BDNF signaling through TrkB activation prompted us to further assess which pathway(s) triggered by TrkB trans-autophosphorylation was (were) involved in BDNF-mediated VGLUT upregulation. For this purpose, we used the chemical inhibitors U73122 (PLCγ pathway), PD098059 or U0126 (Ras-ERK pathway), and LY294002 or Wortmannin (PI3K/Akt pathway). At DIV7, U73122 fully abrogated BDNF-induced VGLUT1 (Fig. 4A) and VGLUT2 (Fig. 4B) upregulation ($p>0.05$), indicating that this pathway plays a key role in response to BDNF. Chemical inhibitors chelerythrine and KN-93 selectively and potently block the activation of PKC and CAMKII, respectively, two kinases that act downstream of PLCγ. We have found that incubation with KN-93 (1 μM) prevented BDNF-induced VGLUT1 ($p>0.05$), but not VGLUT2 upregulation ($p<0.05$), while chelerythrin (5 μM) blocked VGLUT2 upregulation ($p>0.05$), but was without effect on VGLUT1 ($p<0.05$) (Fig. 4C, D). These results indicate that BDNF regulates VGLUT 1 and 2 expression through signaling mechanisms acting downstream of PLCγ. VGLUT1 transient upregulation at DIV7 is dependent on CAMKII activation whereas VGLUT2 long lasting upregulation, at the same developmental stage, requires PKC activation. Blocking the Ras-ERK (Fig. 5A, B) or PI3-K/Akt (Fig. 5C, D) signaling pathways with PD098059 or U0126 and LY294002 or Wortmannin, respectively, showed no significant effect ($p>0.05$).

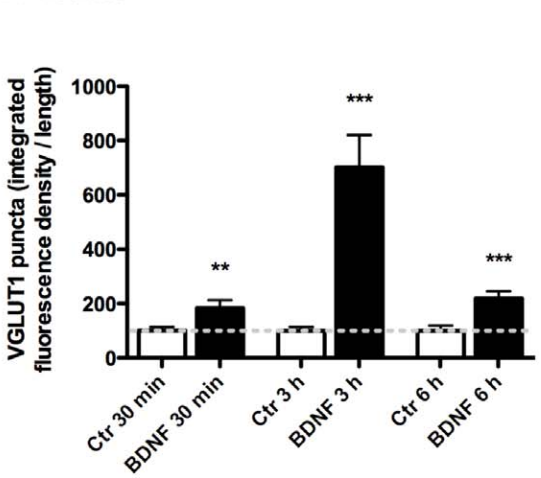
BDNF Affects VGLUT Subcellular Distribution

The subcellular distribution of VGLUT1 and VGLUT2 was assessed by immunocytochemistry in cultured hippocampal neurons (DIV7) stimulated or not with 100 ng/ml BDNF, for different time periods, and imaged by fluorescence microscopy. VGLUT1-positive neuritic (presumably axonal) labeling transiently increased after 30 min - 3 h of incubation with BDNF when compared with the control (Fig. 6A). Quantification of the immunofluorescence images showed a BDNF-induced increase in the number (Fig. 6B), integrated density (mean intensity× puncta area) (Fig. 6D) and intensity (Fig. 6E) of VGLUT1 puncta along neurites, as well as an upregulation in the total immunoreactivity in the soma (Fig. 6C). A small but non-significant upregulation of VGLUT1 protein levels was found in the soma, at an early time point (30 min), before the maximal increase in VGLUT1 puncta intensity (3 h) ($p<0.001$). VGLUT2 punctate labeling was also increased in the neurites of hippocampal neurons following stimulation with BDNF for 30 min or 6 h (Fig. 7). In this case, BDNF was also found to increase the number (Fig. 7B), integrated density (Fig. 7D) and intensity (Fig. 7E) of VGLUT2 puncta in neurites, with maximal effects at 30 min of incubation with the neurotrophin. However, BDNF was without effect on total VGLUT2 immunoreactivity in the soma ($p>0.05$) (Fig. 7C), in contrast with the results obtained for VGLUT1. Overall, these imaging results not only provide further support to the biochemistry results previously presented but also show that BDNF affects


A
β-Tubulin I
VGLUT 1
Merged
Control
BDNF 3 h (100 ng/mL)
10 µm


B. Neurites
VGLUT1 puncta (number / length)
250
200
150
100
50
0
*
***
Ctr 30 min
BDNF 30 min
Ctr 3 h
BDNF 3 h
Ctr 6 h
BDNF 6 h


C. Soma
VGLUT1 immunoreactivity (integrated fluorescence density / area)
250
200
150
100
50
0
**
Ctr 30 min
BDNF 30 min
Ctr 3 h
BDNF 3 h
Ctr 6 h
BDNF 6 h


D. Neurites
VGLUT1 puncta (integrated fluorescence density / length)
1000
800
600
400
200
0
**
***
***
Ctr 30 min
BDNF 30 min
Ctr 3 h
BDNF 3 h
Ctr 6 h
BDNF 6 h

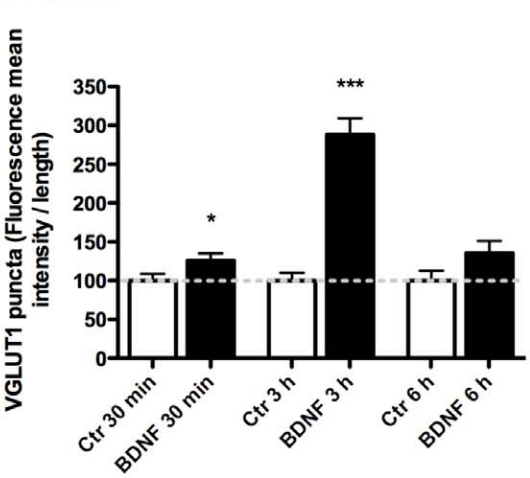

E. Neurites
VGLUT1 puncta (Fluorescence mean intensity / length)
350
300
250
200
150
100
50
0
*
***
Ctr 30 min
BDNF 30 min
Ctr 3 h
BDNF 3 h
Ctr 6 h
BDNF 6 h

Figure 6. Effect of BDNF on the subcellular distribution of VGLUT1 in cultured rat hippocampal neurons. Hippocampal neurons were stimulated at 7 DIV with BDNF (100 ng/ml) for 30 min, 3 h or 6 h. Neurons were then stained for total VGLUT1 (green) and β-tubulin I (red) (A) (Scale bar: 10 μm). Arrowheads show the location of VGLUT1 puncta. The acquired fluorescence images were analysed to assess the number (B), integrated density (mean intensity×puncta area) (D) and intensity (E) of VGLUT1 puncta in neurites, as well as for VGLUT1 immunoreactivity in the soma (C). Results were normalized for neuritic length (B, D and E) or for soma area (C). The protein localization was visualized using a Zeiss Axio Observer 2.1 fluorescence microscope (63x Objective). Quantitative particle analysis was performed using ImageJ software. Results are shown as mean percentage of control of at least three independent experiments (n ≥30 cells per condition). *p<0.05; **p<0.01; ***p<0.001, significantly different in comparison to the respective control (unpaired Student's *t*-test).
doi:10.1371/journal.pone.0053793.g006

the subcellular distribution and trafficking of VGLUT1 and VGLUT2 in hippocampal neurons.

Discussion

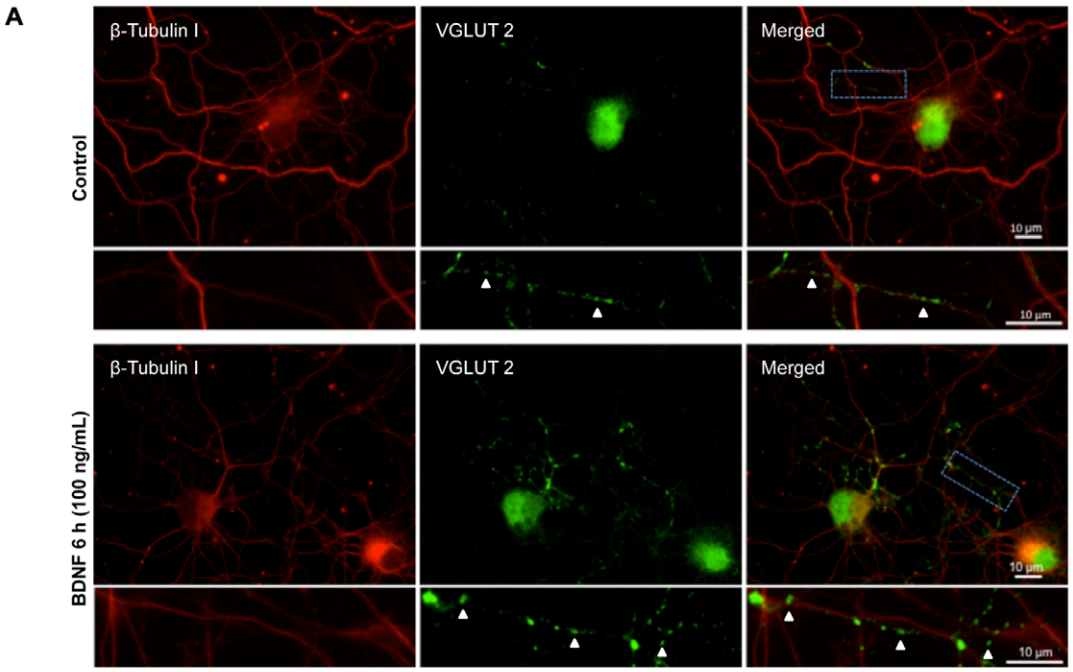
We and others have previously shown direct presynaptic effects of BDNF, which upregulates K^+-evoked [^{3}H] glutamate release from hippocampal synaptosomes, in a subset of glutamatergic synapses expressing TrkB receptors on the plasma membrane [39,64]. In the current study, we have shown that BDNF regulates VGLUT1 and VGLUT2 gene and protein expression, during development of cultured hippocampal neurons, specifically through activation of TrkB receptors and the PLCγ signaling pathway. At DIV7, BDNF-induced transient VGLUT1 upregulation requires the activation of the PLCγ downstream effector CAMKII, whereas VGLUT2 sustained upregulation, at the same developmental stage, depends on PKC activation. At DIV14, BDNF upregulates VGLUT1 expression with no significant effect on VGLUT2 expression, which was endogenously downregulated during this period, approximately corresponding to the developmental switch from VGLUT2 to VGLUT1 neurons in postnatal hippocampus [42,43]. The results also indicate that BDNF affects VGLUT1 and VGLUT2 subcellular distribution, further suggesting a role in BDNF-induced short-term plasticity and LTP. These findings correlate with the BDNF-induced increase in the number of docked vesicles at the active zone and quantal glutamate release observed at hippocampal excitatory synapses [39,41,65].

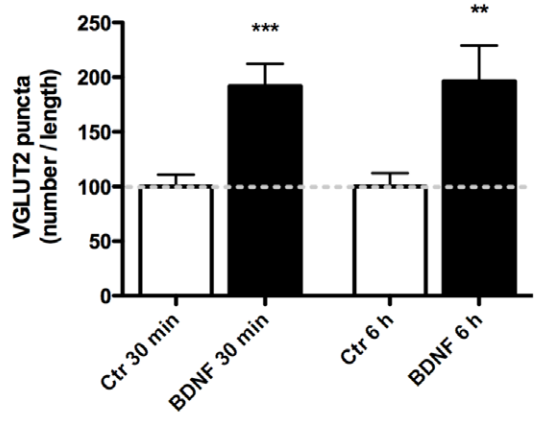
Effect of BDNF on VGLUT1 and VGLUT2 Gene and Protein Expression

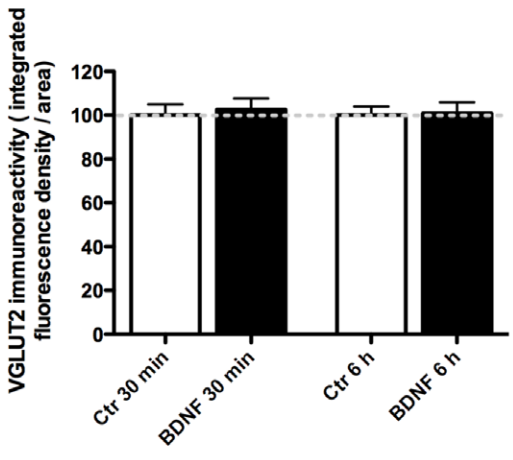
In this study, we show that BDNF differentially upregulates VGLUT isoforms 1 and 2 during the development of hippocampal neurons in a time-dependent manner (Fig. 1A), by a mechanism sensitive to inhibition of transcription (Fig. 2A–D) and translation (Fig. 1C, D). The BDNF-induced sustained increase in VGLUT2 protein levels contrasts with the transient upregulation of VGLUT1 protein levels induced by the neurotrophin. The latter effects correlate with the transient increase in TrkB signaling activity observed in hippocampal neurons incubated with BDNF, which reached a maximum after about 10 min of exposure to the neurotrophin and decreased to control levels after 24 h of incubation [3]. These results also indicate that VGLUT1 synthesized in response to BDNF stimulation is degraded within less than 24 h. The more sustained BDNF-induced increase in VGLUT2 protein levels suggests that this transporter has a longer half-life in hippocampal neurons than VGLUT1. Alternatively, the results may indicate that the TrkB signaling pathway coupled to the regulation of VGLUT2 expression (which is distinct from the pathway responsible for VGLUT1 upregulation) may undergo a slower inactivation after desensitization of the TrkB receptors. In addition to the effect on VGLUT protein levels reported here, BDNF was previously shown to upregulate the expression of the synaptic vesicle proteins synaptophysin, synaptobrevin and synaptotagmin, but showed no effect on the presynaptic membrane proteins syntaxin and SNAP-25, or the vesicle-binding protein synapsin-I, in organotypical cultures of hippocampal neurons [66]. BDNF overexpression in a Huntington's disease mouse model was previously shown to prevent the decrease of striatal VGLUT1, but that effect most likely resulted from a neuroprotective mechanism of BDNF, which may have precluded the loss of glutamatergic synapses [67].

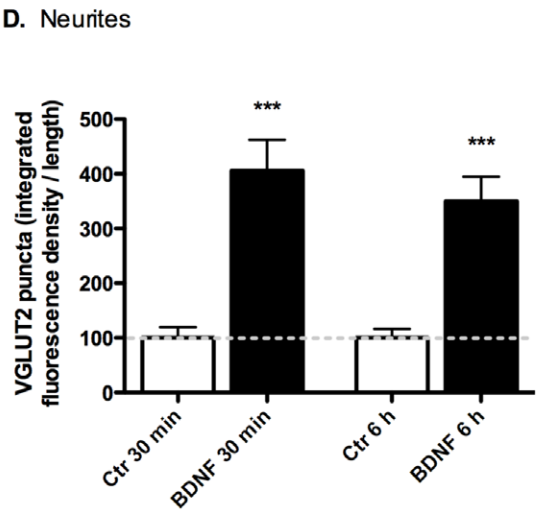
Immunocytochemistry experiments also showed a rapid effect of BDNF on VGLUT1 protein levels in the soma, which was followed by an increase in the expression of this protein in puncta along neurites (presumably axons). These results suggest that newly synthesized vesicular glutamate transporters are delivered to neurites within 3 h, being clustered in both new and pre-existing puncta. BDNF also induced a sustained increase in the intensity of VGLUT2 puncta in neurites, but no changes were found in the somatic abundance of the transporter. This suggests that VGLUT2 synthesized in the soma following BDNF stimulation may be rapidly delivered to neurites or, alternatively, VGLUT2 may be produced locally at the neurites in response to stimulation with the neurotrophin. Furthermore, the increase noted in the number of VGLUT1/2 puncta and in the number of transporters clustered in these regions, in BDNF-stimulated hippocampal neurons may result, at least partly, from a redistribution of vesicles containing the vesicular transporters already available in neurites.

VGLUT1 and VGLUT2 were initially identified as Na^+-dependent inorganic phosphate transporters BNPI and DNPI [68–70] in screenings of cDNAs upregulated by NMDA and growth factors, respectively [53]. However, the characterization of VGLUT1 [71,72] and VGLUT2 promoters [73] has only recently been performed, and no transcription factors or signaling pathways directly modulating the expression of these genes have been identified thus far, to our knowledge. Nonetheless, VGLUT1 protein levels show strong diurnal cycling, which is lost in mice lacking the period gene *Period 2* [74]. CAMKII is maximally active during the subjective day, in contrast to Erk [75], and the CAMKII inhibitor KN-93 was shown to block *Period 2* expression while the MEK inhibitors PD98059 and U0126 were without effect [76]. In agreement with these results, we have found that CAMKII inhibition blocks BDNF-induced upregulation of VGLUT1 (Fig. 4C), whereas MEK/ERK inhibition had no significant effect (Fig. 5A). VGLUT1 is also upregulated in cerebrocortical and hippocampal regions of rat brains upon antidepressant treatment with fluoxetine, paroxetine or desipramine [77], or in striatal neurons due to intraperitoneal injection of methanphetamine [78]. In turn, VGLUT2 is upregulated in vasopressin and oxytocin neurons after osmotic stimulation [79] or the thalamus of schizophrenic patients [80] although, in all cases, the underlying transcriptional mechanisms are still unknown. Nevertheless, these results demonstrate that glutamatergic neurons regulate glutamate release through modulation of VGLUT expression, which is endogenously regulated in developing and mature neurons enabling synaptic refinement and plasticity [44].


A
β-Tubulin I
VGLUT 2
Merged
Control
BDNF 6 h (100 ng/mL)
10 µm


B. Neurites
VGLUT2 puncta (number / length)
250
200
150
100
50
0
***
**
Ctr 30 min
BDNF 30 min
Ctr 6 h
BDNF 6 h


C. Soma
VGLUT2 immunoreactivity (integrated fluorescence density / area)
120
100
80
60
40
20
0
Ctr 30 min
BDNF 30 min
Ctr 6 h
BDNF 6 h


D. Neurites
VGLUT2 puncta (integrated fluorescence density / length)
500
400
300
200
100
0
***
***
Ctr 30 min
BDNF 30 min
Ctr 6 h
BDNF 6 h

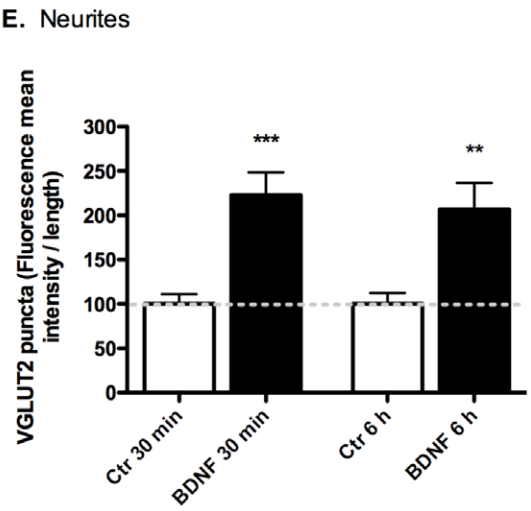

E. Neurites
VGLUT2 puncta (Fluorescence mean intensity / length)
300
250
200
150
100
50
0
***
**
Ctr 30 min
BDNF 30 min
Ctr 6 h
BDNF 6 h

Figure 7. Effect of BDNF on the subcellular distribution of VGLUT2 in cultured rat hippocampal neurons. Hippocampal neurons at 7 DIV were stimulated with BDNF (100 ng/ml) for 30 min or 6 h. Neurons were immunolabeled with specific antibodies for total VGLUT2 (green) and β-tubulin I (red) (A) (Scale bar: 10 μm). Arrowheads show the location of VGLUT2 puncta. The acquired fluorescence images were analysed to assess the number (B), integrated density (mean intensity×puncta area) (D) and intensity (E) of VGLUT2 puncta in neurites, as well as for VGLUT1 immunoreactivity in the soma (C). Results were normalized for neuritic length (B, D and E) or soma area (C). The protein localization was visualized using a Zeiss Axio Observer 2.1 fluorescence microscope (63x Objective). Quantitative particle analysis was performed using ImageJ software. Results are shown as mean percentage of control of at least three independent experiments (n ≥30 cells per condition). **p<0.01; ***p<0.001, significantly different in comparison to the respective control (unpaired Student's *t*-test).
doi:10.1371/journal.pone.0053793.g007

TrkB Activation and PLCγ Signaling in BDNF-mediated Regulation of Glutamatergic Function

We have found that BDNF regulation of VGLUT expression in developing hippocampal neurons depends specifically on the activation of TrkB receptors and PLCγ signaling (Fig. 4), and although stimulation with BDNF is also coupled to the activation of the Ras/ERK and PI3-K signaling pathways in cultured hippocampal neurons [3] these pathways do not participate in the regulation of VGLUT1 and VGLUT2 expression. Interestingly, the effects of BDNF are specific since stimulation of cultured hippocampal neurons with IGF-1 and bFGF, which also activate receptor tyrosine kinases, did not affect VGLUT protein levels. This difference may be due to a distinct location of the receptors in the cells and/or to a differential coupling to intracellular signaling mechanisms. In cultured cerebrocortical neurons, TrkB receptors were found in all major compartments of each neuron (cell bodies, dendrites, and axons) both before (DIV4) and during the peak of (DIV10) synapse formation [81].

The role of PLCγ signaling in BDNF-induced upregulation of VGLUT protein levels in cultured hippocampal neurons correlates with its role in the modulation of other components of glutamatergic synapses by BDNF. BDNF-induced glutamate release depends on the PLCγ pathway [82,83] and ceases following treatment with a synthetic glucocorticoid (DEX) that decreases glucocorticoid receptor-TrkB interaction thereby attenuating PLCγ activation [84]. Likewise, in cultured hippocampal neurons, BDNF enhances glutamatergic synaptic transmission by raising the presynaptic intracellular calcium concentration, due to Ca^{2+} release from IP3-sensitive stores [85]. Furthermore, in hippocampal synaptosomes, the effect of BDNF on K^+-evoked [^{3}H] glutamate release correlates with increased PLCγ phosphorylation but not ERK or Akt phosphorylation [64]. Additionally, both the early and late phases of long-term potentiation are impaired in the CA1 hippocampus region of homozygous mice with mutant PLCγ docking sites at TrkB receptors, as a result of impaired CAMKIV and CREB phosphorylation, whilst mutation of Shc docking site, upstream of Ras-Erk and PI3K/Akt, had no effect on LTP [18]. The effects of BDNF on LTP are likely mediated by activation of pre- and post-synaptic TrkB receptors since selectively blocking of pre- or postsynaptic signaling showed no significant reduction in LTP [22,23]. The current results showing BDNF-induced differential upregulation of VGLUT1 and VGLUT2, via CAMKII and PKC activation, respectively, identify VGLUT as potential presynaptic molecular targets of BDNF contribution to protein-synthesis dependent mechanisms of synaptic plasticity. This is supported by evidences showing that inhibition of BDNF signaling impairs LTM [86,87], VGLUT1 deletion results in impaired LTP, learning and memory function [46,47], and both isoforms are crucial effectors of synaptic plasticity [42,88].

In addition to the effects on VGLUT protein levels, activation of TrkB receptors by BDNF has also been shown to enhance glutamate release in cultured hippocampal neurons by increasing the frequency of miniature excitatory postsynaptic currents (mEPSCs) [85,89]. Other authors have also reported that BDNF enhances presynaptic function by increasing the number of docked vesicles at the active zone and quantal glutamate release [39,41] when the postsynaptic neuron is glutamatergic or excitatory but not when GABAergic or inhibitory [90]. At the postsynaptic level, BDNF may potentiate excitatory synaptic transmission by regulating the expression and synaptic delivery of AMPA receptor subunit GluA1, through activation of PKC and CAMKII [37], and upregulating the expression of GluN1, GluN2A and GluN2B NMDA subunits in a transcription-dependent mechanism [38]. CAMKII and PKC, activated downstream of BDNF binding to TrkB receptors and PLCγ stimulation, have a key role in the potentiation of NMDA receptors by BDNF [91,92]. These findings support the model whereby BDNF induces LTP through targeting of both pre- and postsynaptic mechanisms, critical for synaptic function. We have also found that incubation with BDNF has no effect on protein markers of GABAergic neurons, glutamate decarboxylase 65 and 67 (data not shown), further demonstrating the correlation between BDNF signaling-dependent regulation of neuronal protein levels and function.

In addition to TrkB receptors, BDNF may also bind to $p75^{NTR}$, abundantly expressed in the hippocampus during the late embryonic and early postnatal [93,94] period of developmental cell death [95,96], although with low affinity [19]. Furthermore, BDNF binds to truncated TrkB receptors, but their endogenous expression does not peak until postnatal days 10–15 (P10–15), in contrast with the full-length (FL) TrkB mRNA, which reaches adult levels at birth (P0) [97]. Hence, BDNF signaling in developing hippocampal neurons is essentially dependent on TrkB.FL receptor activation. This is in accordance with the results reported here showing that BDNF-induced upregulation of VGLUT was inhibited by the Trk receptor inhibitor K252a.

VGLUT1 and VGLUT2 may Mediate BDNF-induced Mechanisms of Synaptic Plasticity

The current study showing a BDNF-induced upregulation of total VGLUT protein levels in hippocampal neurons, in addition to an increase in the punctate distribution of the transporters along neurites, provides further evidence indicating a role of this neurotrophin on presynaptic potentiation of glutamatergic transmission. The following evidences suggest that BDNF-induced upregulation in VGLUT clustering in neurites may significantly potentiate excitatory neurotransmission: 1) VGLUT expression directly correlates with synaptic strength [43,44] and biogenesis or recycling of synaptic vesicles [42,98]; 2) VGLUT1 deficient mice exhibit decreased spontaneous glutamate release and quantal synaptic transmission due to exocytosis of partially filled vesicles in hippocampal synapses [43]; 3) VGLUT1 overexpression not only rescues this phenotype but also enhances AMPA receptor-mediated evoked EPSCs by increasing glutamate release per vesicle [44]; 4) loss of VGLUT 1 and 2 causes changes in synaptic vesicle shape and leads to decreased number of vesicles [42,98]; 5) VGLUT2 deficiency decreases evoked glutamate release probability and reduces LTD at hippocampal CA3-CA1 synapses of

young postnatal (P11–P14) mice [99]; 6) even though one transporter apparently suffices to fill a vesicle [100], enhanced VGLUT expression may increase the number of transporters per vesicle, thus, accelerating the rate of vesicle filling or its volume [101]. Conversely, decreased VGLUT1 expression causes depressive behavior and impaired memory in mice [47], while VGLUT2 heterozygotes show decreased neuropathic pain and defense responses [98,102]. Hence, differences between transcription and translation rates or synaptic delivery of VGLUT isoforms, otherwise quite similar in function, further explain presynaptic regulation of quantal size.

In conclusion, the results presented herein suggest that BDNF signaling regulates differentially the gene and protein expression of VGLUT1 and VGLUT2 in developing and mature hippocampal neurons. Nevertheless, future in vivo studies as required for verifying the potential role of BDNF-mediated regulation of VGLUT expression in hippocampal synaptic mechanisms of short-term plasticity and long-term potentiation.

Supporting Information

Figure S1 Acute BDNF stimulation does not induce a sustained increase in VGLUT protein levels after removal of the neurotrophin. (A–B) Cultured hippocampal neurons at DIV14 (A) and DIV7 (B) were incubated with 100 ng/ml BDNF for 4 hours in Neurobasal medium followed by a 14 h recovery period in culture conditioned medium. Total VGLUT1 (A) and VGLUT2 (B) protein levels were compared to control (without BDNF) expression, upon normalization with β-Tubulin levels. Quantification of ten different experiments, performed in independent preparations, is presented as mean percentage ±SEM compared to the control (unstimulated neurons). The differences obtained are not statistically significant, as determined by paired Student's *t*-test with a confidence interval of 95%.
(TIF)

Figure S2 TrkB receptor inhibition has no effect on VGLUT2 expression. Cultured hippocampal neurons at DIV14 were incubated with a selective inhibitor of tyrosine protein kinase activity K252a (200 nM) and VGLUT2 protein levels were determined by western blot. Quantification of the indicated number of experiments, performed in independent preparations, is presented as mean percentage ±SEM compared to the control (unstimulated neurons). Statistical significance was determined by paired Student's *t*-test with a confidence interval of 95%.
(TIF)

Acknowledgments

The authors thank Elisabete Lopes for technical assistance in the preparation of rat hippocampal cultures and Regeneron for the kind gift of BDNF.

Author Contributions

Conceived and designed the experiments: CVM MM MC DC CBD. Performed the experiments: CVM MM MC DC CGS. Analyzed the data: CVM MM MC DC CBD. Wrote the paper: CVM.

References

1. Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2: 24–32.
2. Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S (2010) Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol 70: 271–288.
3. Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, et al. (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 12: 1329–1343.
4. Murray PS, Holmes PV (2011) An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus. Int J Pept 2011: 654085.
5. Wang GX, Poo MM (2005) Requirement of TRPC channels in netrin-1-induced chemotropic turning of nerve growth cones. Nature 434: 898–904.
6. Li Y, Jia YC, Cui K, Li N, Zheng ZY, et al. (2005) Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 434: 894–898.
7. Ji Y, Pang PT, Feng L, Lu B (2005) Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat Neurosci 8: 164–172.
8. An JJ, Gharami K, Liao GY, Woo NH, Lau AG, et al. (2008) Distinct role of long 3′ UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 134: 175–187.
9. Kwon M, Fernandez JR, Zegarek GF, Lo SB, Firestein BL (2011) BDNF-promoted increases in proximal dendrites occur via CREB-dependent transcriptional regulation of cypin. J Neurosci 31: 9735–9745.
10. Linnarsson S, Bjorklund A, Ernfors P (1997) Learning deficit in BDNF mutant mice. Eur J Neurosci 9: 2581–2587.
11. Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, et al. (1999) Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24: 401–414.
12. Liu IY, Lyons WE, Mamounas LA, Thompson RF (2004) Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. J Neurosci 24: 7958–7963.
13. Bekinschtein P, Cammarota M, Katche C, Slipczuk L, Rossato JI, et al. (2008) BDNF is essential to promote persistence of long-term memory storage. Proc Natl Acad Sci U S A 105: 2711–2716.
14. Korte M, Carroll P, Wolf E, Brem G, Thoenen H, et al. (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 92: 8856–8860.
15. Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, et al. (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16: 1137–1145.
16. Chen G, Kolbeck R, Barde YA, Bonhoeffer T, Kossel A (1999) Relative contribution of endogenous neurotrophins in hippocampal long-term potentiation. J Neurosci 19: 7983–7990.
17. Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, et al. (2000) The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J Neurosci 20: 6888–6897.
18. Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, et al. (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36: 121–137.
19. Rodriguez-Tebar A, Dechant G, Barde YA (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4: 487–492.
20. Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72: 609–642.
21. Ouyang Y, Kantor D, Harris KM, Schuman EM, Kennedy MB (1997) Visualization of the distribution of autophosphorylated calcium/calmodulin-dependent protein kinase II after tetanic stimulation in the CA1 area of the hippocampus. J Neurosci 17: 5416–5427.
22. Gartner A, Polnau DG, Staiger V, Sciarretta C, Minichiello L, et al. (2006) Hippocampal long-term potentiation is supported by presynaptic and postsynaptic tyrosine receptor kinase B-mediated phospholipase Cgamma signaling. J Neurosci 26: 3496–3504.
23. Gruart A, Sciarretta C, Valenzuela-Harrington M, Delgado-Garcia JM, Minichiello L (2007) Mutation at the TrkB PLC{gamma}-docking site affects hippocampal LTP and associative learning in conscious mice. Learn Mem 14: 54–62.
24. Du JL, Poo MM (2004) Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. Nature 429: 878–883.
25. Amaral MD, Pozzo-Miller L (2007) TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. J Neurosci 27: 5179–5189.
26. Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361: 1545–1564.
27. Grewal SS, York RD, Stork PJ (1999) Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol 9: 544–553.
28. Barnabe-Heider F, Miller FD (2003) Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci 23: 5149–5160.
29. Datta R, Kojima H, Yoshida K, Kufe D (1997) Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis. J Biol Chem 272: 20317–20320.

30. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, et al. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286: 1358–1362.
31. Kelleher RJ, 3rd, Govindarajan A, Jung HY, Kang H, Tonegawa S (2004) Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell 116: 467–479.
32. Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11: 297–305.
33. Yoshii A, Constantine-Paton M (2007) BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. Nat Neurosci 10: 702–711.
34. Takei N, Inamura N, Kawamura M, Namba H, Hara K, et al. (2004) Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. J Neurosci 24: 9760–9769.
35. Cunha C, Brambilla R, Thomas KL (2010) A simple role for BDNF in learning and memory? Front Mol Neurosci 3: 1.
36. Manadas B, Santos AR, Szabadfi K, Gomes JR, Garbis SD, et al. (2009) BDNF-induced changes in the expression of the translation machinery in hippocampal neurons: protein levels and dendritic mRNA. J Proteome Res 8: 4536–4552.
37. Caldeira MV, Melo CV, Pereira DB, Carvalho R, Correia SS, et al. (2007) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. J Biol Chem 282: 12619–12628.
38. Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, et al. (2007) BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol Cell Neurosci 35: 208–219.
39. Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat Neurosci 3: 323–329.
40. Bamji SX, Rico B, Kimes N, Reichardt LF (2006) BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions. J Cell Biol 174: 289–299.
41. Tyler WJ, Pozzo-Miller LD (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. J Neurosci 21: 4249–4258.
42. Fremeau RT, Jr., Kam K, Qureshi T, Johnson J, Copenhagen DR, et al. (2004) Vesicular glutamate transporters 1 and 2 target to functionally distinct synaptic release sites. Science 304: 1815–1819.
43. Wojcik SM, Rhee JS, Herzog E, Sigler A, Jahn R, et al. (2004) An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. Proc Natl Acad Sci U S A 101: 7158–7163.
44. Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, et al. (2005) Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1. J Neurosci 25: 6221–6234.
45. Santos MS, Li H, Voglmaier SM (2009) Synaptic vesicle protein trafficking at the glutamate synapse. Neuroscience 158: 189–203.
46. Balschun D, Moechars D, Callaerts-Vegh Z, Vermaercke B, Van Acker N, et al. (2010) Vesicular glutamate transporter VGLUT1 has a role in hippocampal long-term potentiation and spatial reversal learning. Cereb Cortex 20: 684–693.
47. Tordera RM, Totterdell S, Wojcik SM, Brose N, Elizalde N, et al. (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). Eur J Neurosci 25: 281–290.
48. Miyazaki T, Fukaya M, Shimizu H, Watanabe M (2003) Subtype switching of vesicular glutamate transporters at parallel fibre-Purkinje cell synapses in developing mouse cerebellum. Eur J Neurosci 17: 2563–2572.
49. Dal Bo G, Berube-Carriere N, Mendez JA, Leo D, Riad M, et al. (2008) Enhanced glutamatergic phenotype of mesencephalic dopamine neurons after neonatal 6-hydroxydopamine lesion. Neuroscience 156: 59–70.
50. Butts BD, Hudson HR, Linseman DA, Le SS, Ryan KR, et al. (2005) Proteasome inhibition elicits a biphasic effect on neuronal apoptosis via differential regulation of pro-survival and pro-apoptotic transcription factors. Mol Cell Neurosci 30: 279–289.
51. Messaoudi E, Ying SW, Kanhema T, Croll SD, Bramham CR (2002) Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation in vivo. J Neurosci 22: 7453–7461.
52. Takei N, Kawamura M, Hara K, Yonezawa K, Nawa H (2001) Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes: comparison with the effects of insulin. J Biol Chem 276: 42818–42825.
53. Santos AR, Comprido D, Duarte CB (2010) Regulation of local translation at the synapse by BDNF. Prog Neurobiol 92: 505–516.
54. Nye SH, Squinto SP, Glass DJ, Stitt TN, Hantzopoulos P, et al. (1992) K-252a and staurosporine selectively block autophosphorylation of neurotrophin receptors and neurotrophin-mediated responses. Mol Biol Cell 3: 677–686.
55. Ip NY, Li Y, Yancopoulos GD, Lindsay RM (1993) Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J Neurosci 13: 3394–3405.
56. Lamballe F, Klein R, Barbacid M (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66: 967–979.
57. Dorsey SG, Bambrick LL, Balice-Gordon RJ, Krueger BK (2002) Failure of brain-derived neurotrophic factor-dependent neuron survival in mouse trisomy 16. J Neurosci 22: 2571–2578.
58. Zheng WH, Quirion R (2004) Comparative signaling pathways of insulin-like growth factor-1 and brain-derived neurotrophic factor in hippocampal neurons and the role of the PI3 kinase pathway in cell survival. J Neurochem 89: 844–852.
59. Nelson TJ, Sun MK, Hongpaisan J, Alkon DL (2008) Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. Eur J Pharmacol 585: 76–87.
60. Katsuki H, Itsukaichi Y, Matsuki N (2000) Distinct signaling pathways involved in multiple effects of basic fibroblast growth factor on cultured rat hippocampal neurons. Brain Res 885: 240–250.
61. Hong J, Yoshida K, Rosner MR (2002) Characterization of a cysteine proteinase inhibitor induced during neuronal cell differentiation. J Neurochem 81: 922–934.
62. McCusker RH, McCrea K, Zunich S, Dantzer R, Broussard SR, et al. (2006) Insulin-like growth factor-I enhances the biological activity of brain-derived neurotrophic factor on cerebrocortical neurons. J Neuroimmunol 179: 186–190.
63. Kwon YK (1997) Expression of brain-derived neurotrophic factor mRNA stimulated by basic fibroblast growth factor and platelet-derived growth factor in rat hippocampal cell line. Mol Cells 7: 320–325.
64. Pereira DB, Rebola N, Rodrigues RJ, Cunha RA, Carvalho AP, et al. (2006) Trkb receptors modulation of glutamate release is limited to a subset of nerve terminals in the adult rat hippocampus. J Neurosci Res 83: 832–844.
65. Amaral MD, Pozzo-Miller L (2012) Intracellular Ca2+ stores and Ca2+ influx are both required for BDNF to rapidly increase quantal vesicular transmitter release. Neural Plast 2012: 203536.
66. Tartaglia N, Du J, Tyler WJ, Neale E, Pozzo-Miller L, et al. (2001) Protein synthesis-dependent and -independent regulation of hippocampal synapses by brain-derived neurotrophic factor. J Biol Chem 276: 37585–37593.
67. Giralt A, Carreton O, Lao-Peregrin C, Martin ED, Alberch J (2011) Conditional BDNF release under pathological conditions improves Huntington's disease pathology by delaying neuronal dysfunction. Mol Neurodegener 6: 71.
68. Ni B, Rosteck PR, Jr., Nadi NS, Paul SM (1994) Cloning and expression of a cDNA encoding a brain-specific Na(+)-dependent inorganic phosphate cotransporter. Proc Natl Acad Sci U S A 91: 5607–5611.
69. Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, et al. (2000) Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter. J Neurochem 74: 2622–2625.
70. Takamori S, Rhee JS, Rosenmund C, Jahn R (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 407: 189–194.
71. Zhang GR, Geller AI (2010) A helper virus-free HSV-1 vector containing the vesicular glutamate transporter-1 promoter supports expression preferentially in VGLUT1-containing glutamatergic neurons. Brain Res 1331: 12–19.
72. Zhang GR, Li X, Cao H, Zhao H, Geller AI (2011) The vesicular glutamate transporter-1 upstream promoter and first intron each support glutamatergic-specific expression in rat postrhinal cortex. Brain Res 1377: 1–12.
73. Li T, Bai L, Li J, Igarashi S, Ghishan FK (2008) Sp1 is required for glucose-induced transcriptional regulation of mouse vesicular glutamate transporter 2 gene. Gastroenterology 134: 1994–2003.
74. Yelamanchili SV, Pendyala G, Brunk I, Darna M, Albrecht U, et al. (2006) Differential sorting of the vesicular glutamate transporter 1 into a defined vesicular pool is regulated by light signaling involving the clock gene Period2. J Biol Chem 281: 15671–15679.
75. Ko GY, Ko ML, Dryer SE (2001) Circadian regulation of cGMP-gated cationic channels of chick retinal cones. Erk MAP Kinase and Ca2+/calmodulin-dependent protein kinase II. Neuron 29: 255–266.
76. Yokota S, Yamamoto M, Moriya T, Akiyama M, Fukunaga K, et al. (2001) Involvement of calcium-calmodulin protein kinase but not mitogen-activated protein kinase in light-induced phase delays and Per gene expression in the suprachiasmatic nucleus of the hamster. J Neurochem 77: 618–627.
77. Tordera RM, Pei Q, Sharp T (2005) Evidence for increased expression of the vesicular glutamate transporter, VGLUT1, by a course of antidepressant treatment. J Neurochem 94: 875–883.
78. Mark KA, Quinton MS, Russek SJ, Yamamoto BK (2007) Dynamic changes in vesicular glutamate transporter 1 function and expression related to methamphetamine-induced glutamate release. J Neurosci 27: 6823–6831.
79. Kawasaki A, Shutoh F, Nogami H, Hisano S (2006) VGLUT2 expression is up-regulated in neurohypophysial vasopressin neurons of the rat after osmotic stimulation. Neurosci Res 56: 124–127.
80. Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH (2001) Vesicular glutamate transporter transcript expression in the thalamus in schizophrenia. Neuroreport 12: 2885–2887.
81. Gomes RA, Hampton C, El-Sabeawy F, Sabo SL, McAllister AK (2006) The dynamic distribution of TrkB receptors before, during, and after synapse formation between cortical neurons. J Neurosci 26: 11487–11500.
82. Numakawa T, Yamagishi S, Adachi N, Matsumoto T, Yokomaku D, et al. (2002) Brain-derived neurotrophic factor-induced potentiation of Ca(2+) oscillations in developing cortical neurons. J Biol Chem 277: 6520–6529.

83. Matsumoto T, Numakawa T, Yokomaku D, Adachi N, Yamagishi S, et al. (2006) Brain-derived neurotrophic factor-induced potentiation of glutamate and GABA release: different dependency on signaling pathways and neuronal activity. Mol Cell Neurosci 31: 70–84.
84. Numakawa T, Kumamaru E, Adachi N, Yagasaki Y, Izumi A, et al. (2009) Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-gamma signaling for glutamate release via a glutamate transporter. Proc Natl Acad Sci U S A 106: 647–652.
85. Li YX, Xu Y, Ju D, Lester HA, Davidson N, et al. (1998) Expression of a dominant negative TrkB receptor, T1, reveals a requirement for presynaptic signaling in BDNF-induced synaptic potentiation in cultured hippocampal neurons. Proc Natl Acad Sci U S A 95: 10884–10889.
86. Monteggia LM, Barrot M, Powell CM, Berton O, Galanis V, et al. (2004) Essential role of brain-derived neurotrophic factor in adult hippocampal function. Proc Natl Acad Sci U S A 101: 10827–10832.
87. Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 12: 656–670.
88. Weston MC, Nehring RB, Wojcik SM, Rosenmund C (2011) Interplay between VGLUT isoforms and endophilin A1 regulates neurotransmitter release and short-term plasticity. Neuron 69: 1147–1159.
89. Lessmann V, Gottmann K, Heumann R (1994) BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. Neuroreport 6: 21–25.
90. Schinder AF, Berninger B, Poo M (2000) Postsynaptic target specificity of neurotrophin-induced presynaptic potentiation. Neuron 25: 151–163.
91. Crozier RA, Black IB, Plummer MR (1999) Blockade of NR2B-containing NMDA receptors prevents BDNF enhancement of glutamatergic transmission in hippocampal neurons. Learn Mem 6: 257–266.
92. Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, et al. (2001) Protein kinase C modulates NMDA receptor trafficking and gating. Nat Neurosci 4: 382–390.
93. Buck CR, Martinez HJ, Chao MV, Black IB (1988) Differential expression of the nerve growth factor receptor gene in multiple brain areas. Brain Res Dev Brain Res 44: 259–268.
94. Lu B, Buck CR, Dreyfus CF, Black IB (1989) Expression of NGF and NGF receptor mRNAs in the developing brain: evidence for local delivery and action of NGF. Exp Neurol 104: 191–199.
95. Ferrer I, Bernet E, Soriano E, del Rio T, Fonseca M (1990) Naturally occurring cell death in the cerebral cortex of the rat and removal of dead cells by transitory phagocytes. Neuroscience 39: 451–458.
96. Gould E, Woolley CS, McEwen BS (1991) Naturally occurring cell death in the developing dentate gyrus of the rat. J Comp Neurol 304: 408–418.
97. Fryer RH, Kaplan DR, Feinstein SC, Radeke MJ, Grayson DR, et al. (1996) Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. J Comp Neurol 374: 21–40.
98. Wallen-Mackenzie A, Gezelius H, Thoby-Brisson M, Nygard A, Enjin A, et al. (2006) Vesicular glutamate transporter 2 is required for central respiratory rhythm generation but not for locomotor central pattern generation. J Neurosci 26: 12294–12307.
99. He H, Mahnke AH, Doyle S, Fan N, Wang C-C, et al. (2012) Neurodevelopmental role for VGLUT2 in pyramidal neuron plasticity, dendritic refinement, and in spatial learning. J Neurosci 32: 15886–15901.
100. Daniels RW, Collins CA, Chen K, Gelfand MV, Featherstone DE, et al. (2006) A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. Neuron 49: 11–16.
101. Edwards RH (2007) The neurotransmitter cycle and quantal size. Neuron 55: 835–858.
102. Moechars D, Weston MC, Leo S, Callaerts-Vegh Z, Goris I, et al. (2006) Vesicular glutamate transporter VGLUT2 expression levels control quantal size and neuropathic pain. J Neurosci 26: 12055–12066.

VI. Conclusions

The results shown in the previous sections (Chapters 1-3), regarding the mechanisms of neuroprotection and plasticity induced by BDNF, in cultured hippocampal neurons, lead to the following conclusions:

(i) Glutamate excitotoxicity triggers a time-dependent activation of spatially segregated proteolytic mechanisms, which likely reflect different pathogenic pathways;

(ii) The onset of excitotoxicity-induced neurodegeneration coincides with calpain activation in axons and dendrites, gradually progressing to caspase activation in the cell soma, with significant proteasome dysregulation in the transition phase between calpain and caspase activation;

(iii) The UPS regulates GAD67 cleavage under excitotoxic conditions, possibly through modulation of an unknown GAD binding partner. GAD cleavage has significant functional consequences as enzyme activity decreases and the characteristic punctate distribution of GAD65 along neurites is also affected.

(iv) BDNF protects the neurites and cell bodies of hippocampal neurons through the activation of different signaling pathways, further suggesting that glutamate excitotoxicity triggers different neurodegenerative mechanisms;

(v) BDNF-induced neuroprotection is correlated with an attenuation of calpain activation upon a toxic glutamate insult;

(vi) BDNF protects key functional markers of glutamatergic neurons, including the vesicular glutamate transporters, and activity-dependent neurotransmitter release, indicating that BDNF protects the components of the exocytotic machinery and/or enables the functional recovery of synaptic activity upon a toxic insult;

(vii) BDNF regulates the expression of vesicular glutamate transporters 1 and 2 in developing and mature hippocampal neurons, through the PLCγ signaling pathway;

(viii) The overlap of signaling pathways (PLCγ) and protein markers (VGLUTs and TRPCs) involved in BDNF-induced mechanisms of synaptic plasticity and neuroprotection, and the ability of BDNF to attenuate excitotoxicity-triggered proteolytic activity, suggest

that BDNF may simultaneously diminish the damage caused to neurons and enable the functional recovery. The results indicate that the hypothesis ofBDNF inducing regeneration through activity-dependent mechanisms should not be excluded;

(xix) The reactivation of BDNF-induced developmental programs may be used to promote the regeneration of neurons, based on *de novo* protein synthesis mechanisms, involving molecular targets with a key role in neuronal connectivity and plasticity;

(xx) The spatial and temporal segregation of BDNF-induced mechanisms of neuroprotection and its ability to attenuate damage and/or promote the functional recovery of neurons through different signaling cascades demonstrate the potential use of BDNF mimetics as therapeutic agents in the treatment of neurodegenerative diseases.

VII. Future Perspectives

From the functional point of view it is critical to clarify whether BDNF acts by preventing the loss of neurites and/or by inducing the reorganization of the neuronal cytoskeleton and functional recovery of neuronal processes. In order to address this issue, morphological measurements could be made using time-lapse fluorescence microscopy. The changes in neuritic morphology after an excitotoxic insult with glutamate would be assessed in cultured hippocampal neurons transfected with EGFP and mounted into a heat-controlled perfusion system. The fluorescence would be followed immediately after the insult, during 14 hours, in cells pre-incubated in the absence or presence of BDNF. The role of calpains and of the proteasome in the loss of neurites following the excitotoxic insult would be evaluated by performing similar experiments in the presence or absence of chemical calpain inhibitors (e.g. calpain inhibitor I) and proteasome inhibitors (e.g. lactacystin). The role of calpain could also be further addressed by transfecting the neurons with calpastatin cDNA under control of a CMV promotor.

Excitotoxicity decreases the TRPC3 and TRPC6 protein levels and their mRNA. The transient calcium channels are essential for the refilling of calcium stores and guidance of nerve growth cones by BDNF in developing neurons, and pre-incubation of hippocampal neurons with BDNF reverts this effect, as previously shown (Chapter 2), most likely due to de novo protein systhesis. Fura-2 imaging experiments could be performed to determine the functional consequences of TRPC cleavage. Since these channels are permeable to Sr^{2+}, and directly activated by oleoyl-2-acetyl-sn-glycerol (OAG) (Jung et al., 2002; Venkatachalam et al., 2003), we could study the effect of glutamate toxicity on OAG-induced Sr^{2+} entry, in cultured hippocampal neurons. The effect of BDNF on the activity of TRPCs under normal conditions and after an excitotoxic insult would also be determined. Considering that BDNF upregulates TRPC3 and TRPC6 protein levels in hippocampal neurons and the role of these channels in growth cone guidance (Li et al., 2005), we would determine the role of TRPCs in neurite regeneration after a toxic insult with glutamate. Neurite regeneration after the toxic insult would be compared in the presence or in the absence of the TRPC inhibitor BTP2. Studies should also be performed in cells transfected with specific siRNA for TRPCs in order to determine their relative role in the effects of BDNF. According to the results, further assessment of the possible regenerative effect of overexpressing TRPC channels in mature hippocampal neurons (DIV21) submitted to excitotoxic insults (glutamate and NMDA) would enable the comparison to its overexpression in developing neurons (DIV7).

In this study, the putative neuroprotective effect of BDNF on dendritic spines was not assessed although previous studies have shown that sublethal activation of glutamate

receptors induces the collapse of dendritic spines, by a mechanism involving the activity of cathepsin B-like proteases. Therefore, it would be of interest to determine whether the neurotrophin prevents the collapse of the spines and/or promotes the formation of new spines that would contribute to maintain synaptic activity. The latter hypothesis is particularly appealing, given the known effect of BDNF on spine formation in mature hippocampal neurons (Ji et al., 2005). Studies using the fluorogenic cathepsin B substrate (z-RR)2-MR-Cathepsin would allow determining whether a subtoxic stimulation with glutamate increases enzyme activity in the spines of mature hippocampal neurons in culture. The enzyme activity could then be compared in glutamate-stimulated cells, pre-incubated or not with BDNF. The relative role of Ca^{2+} entry through VGCCs and reversal of the Na^{+}/Ca^{2+} exchanger to the activation of cathepsin B-like activity would be determined using appropriate chemical inhibitors. The collapse of dendritic spines is likely to involve a time-dependent change in actin dynamics and a reduction in F-actin content. Phalloidin staining of F- actin should, therefore, be performed in control cells and cells subjected to subtoxic stimulation with glutamate, and the relative role of different Ca^{2+} influx pathways to spine collapse may be investigated using chemical inhibitors of VGCCs and the Na^{+}/Ca^{2+} exchanger. Experiments conducted in the presence and in the absence of BDNF would, thereby, show whether the neurotrophin prevents dendritic spine collapse or induces the formation of new spines after their collapse.

Furthermore, studies using $trkB^{PLC/PLC}$ and $trkB^{SHC/SHC}$ mutant mice (Minichiello et al., 1998; Minichiello et al., 1999; He et al., 2004;Zhai et al., 2011), expressing TrkB receptors unable to activate PLCγ and the Ras/ERK pathways, respectively, would also allow determining the in vivo role of these pathways in the regulation of VGLUT expression in the early phase of hippocampal development.

VIII. References

Aakalu G, Smith WB, Nguyen N, Jiang C, Schuman EM (2001) Dynamic visualization of local protein synthesis in hippocampal neurons. Neuron 30:489-502.

Aarts MM, Tymianski M (2004) Molecular mechanisms underlying specificity of excitotoxic signaling in neurons. Curr Mol Med 4:137-147.

Aarts MM, Arundine M, Tymianski M (2003) Novel concepts in excitotoxic neurodegeneration after stroke. Expert Rev Mol Med 5:1-22.

Abe K, Horiuchi M, Yoshimura K (1997) Potentiation by DSP-4 of EEG slowing and memory impairment in basal forebrain-lesioned rats. Eur J Pharmacol 321:149-155.

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88:615-626.

Abraham WC (2003) How long will long-term potentiation last? Philos Trans R Soc Lond B Biol Sci 358:735-744.

Abramov AY, Duchen MR (2008) Mechanisms underlying the loss of mitochondrial membrane potential in glutamate excitotoxicity. Biochim Biophys Acta 1777:953-964.

Ackerley S, Thornhill P, Grierson AJ, Brownlees J, Anderton BH, Leigh PN, Shaw CE, Miller CC (2003) Neurofilament heavy chain side arm phosphorylation regulates axonal transport of neurofilaments. J Cell Biol 161:489-495.

Adachi N, Kohara K, Tsumoto T (2005) Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. BMC Neurosci 6:42.

Ahmed Z, Mazibrada G, Seabright RJ, Dent RG, Berry M, Logan A (2006) TACE-induced cleavage of NgR and p75NTR in dorsal root ganglion cultures disinhibits outgrowth and promotes branching of neurites in the presence of inhibitory CNS myelin. FASEB J 20:1939-1941.

Ahmed Z, Dent RG, Suggate EL, Barrett LB, Seabright RJ, Berry M, Logan A (2005) Disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on CNS myelin by siRNA-mediated knockdown of NgR, p75NTR and Rho-A. Mol Cell Neurosci 28:509-523.

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85:525-535.

Akaneya Y, Takahashi M, Hatanaka H (1995) Selective acid vulnerability of dopaminergic neurons and its recovery by brain-derived neurotrophic factor. Brain Res 704:175-183.

Akaneya Y, Tsumoto T, Hatanaka H (1996) Brain-derived neurotrophic factor blocks long-term depression in rat visual cortex. J Neurophysiol 76:4198-4201.

Al-Bassam J, Ozer RS, Safer D, Halpain S, Milligan RA (2002) MAP2 and tau bind longitudinally along the outer ridges of microtubule protofilaments. J Cell Biol 157:1187-1196.

Aliaga EE, Mendoza I, Tapia-Arancibia L (2009) Distinct subcellular localization of BDNF transcripts in cultured hypothalamic neurons and modification by neuronal activation. J Neural Transm 116:23-32.

Allen SJ, Dawbarn D (2006) Clinical relevance of the neurotrophins and their receptors. Clin Sci (Lond) 110:175-191.

Allendoerfer KL, Cabelli RJ, Escandon E, Kaplan DR, Nikolics K, Shatz CJ (1994) Regulation of neurotrophin receptors during the maturation of the mammalian visual system. J Neurosci 14:1795-1811.

Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, Graos MM, Carvalho RF, Carvalho AP, Duarte CB (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 12:1329-1343.

Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 389:856-860.

Alzheimer C (1994) A novel voltage-dependent cation current in rat neocortical neurones. J Physiol 479 (Pt 2):199-205.

Amaral MD, Pozzo-Miller L (2007a) BDNF induces calcium elevations associated with IBDNF, a nonselective cationic current mediated by TRPC channels. J Neurophysiol 98:2476-2482.

Amaral MD, Pozzo-Miller L (2007b) TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. J Neurosci 27:5179-5189.

Amaral MD, Pozzo-Miller L (2012) Intracellular Ca2+ stores and Ca2+ influx are both required for BDNF to rapidly increase quantal vesicular transmitter release. Neural Plast 2012:203536.

An JJ, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B (2008) Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 134:175-187.

Arakawa T, Haniu M, Narhi LO, Miller JA, Talvenheimo J, Philo JS, Chute HT, Matheson C, Carnahan J, Louis JC, et al. (1994) Formation of heterodimers from three neurotrophins, nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor. J Biol Chem 269:27833-27839.

Arancibia S, Silhol M, Mouliere F, Meffre J, Hollinger I, Maurice T, Tapia-Arancibia L (2008) Protective effect of BDNF against beta-amyloid induced neurotoxicity in vitro and in vivo in rats. Neurobiol Dis 31:316-326.

Asai A, Tanahashi N, Qiu JH, Saito N, Chi S, Kawahara N, Tanaka K, Kirino T (2002) Selective proteasomal dysfunction in the hippocampal CA1 region after transient forebrain ischemia. J Cereb Blood Flow Metab 22:705-710.

Audesirk G, Cabell L, Kern M (1997) Modulation of neurite branching by protein phosphorylation in cultured rat hippocampal neurons. Brain Res Dev Brain Res 102:247-260.

Bai Y, Li Q, Yang J, Zhou X, Yin X, Zhao D (2008) p75(NTR) activation of NF-kappaB is involved in PrP106-126-induced apoptosis in mouse neuroblastoma cells. Neurosci Res 62:9-14.

Bai Y, Xu J, Brahimi F, Zhuo Y, Sarunic MV, Saragovi HU (2010) An agonistic TrkB mAb causes sustained TrkB activation, delays RGC death, and protects the retinal structure in optic nerve axotomy and in glaucoma. Invest Ophthalmol Vis Sci 51:4722-4731.

Baj G, Leone E, Chao MV, Tongiorgi E (2011) Spatial segregation of BDNF transcripts enables BDNF to differentially shape distinct dendritic compartments. Proc Natl Acad Sci U S A 108:16813-16818.

Balkowiec A, Katz DM (2002) Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. J Neurosci 22:10399-10407.

Banfield MJ, Naylor RL, Robertson AG, Allen SJ, Dawbarn D, Brady RL (2001) Specificity in Trk receptor:neurotrophin interactions: the crystal structure of TrkB-d5 in complex with neurotrophin-4/5. Structure 9:1191-1199.

Bano D, Young KW, Guerin CJ, Lefeuvre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P (2005) Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. Cell 120:275-285.

Barbacid M (1993) Nerve growth factor: a tale of two receptors. Oncogene 8:2033-2042.

Barbacid M (1995) Structural and functional properties of the TRK family of neurotrophin receptors. Ann N Y Acad Sci 766:442-458.

Barco A, Patterson SL, Alarcon JM, Gromova P, Mata-Roig M, Morozov A, Kandel ER (2005) Gene expression profiling of facilitated L-LTP in VP16-CREB mice reveals that BDNF is critical for the maintenance of LTP and its synaptic capture. Neuron 48:123-137.

Barde YA (1988) What, if anything, is a neurotrophic factor? Trends Neurosci 11:343-346.

Barde YA (1990) The nerve growth factor family. Prog Growth Factor Res 2:237-248.

Barde YA, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1:549-553.

Barker PA, Barbee G, Misko TP, Shooter EM (1994) The low affinity neurotrophin receptor, p75LNTR, is palmitoylated by thioester formation through cysteine 279. J Biol Chem 269:30645-30650.

Barnabe-Heider F, Miller FD (2003) Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci 23:5149-5160.

Baumeister FA, Gsell W, Shin YS, Egger J (1994) Glutamate in pyridoxine-dependent epilepsy: neurotoxic glutamate concentration in the cerebrospinal fluid and its normalization by pyridoxine. Pediatrics 94:318-321.

Baumgartner BJ, Shine HD (1997) Targeted transduction of CNS neurons with adenoviral vectors carrying neurotrophic factor genes confers neuroprotection that exceeds the transduced population. J Neurosci 17:6504-6511.

Bazan-Peregrino M, Gutierrez-Kobeh L, Moran J (2007) Role of brain-derived neurotrophic factor in the protective action of N-methyl-D-aspartate in the apoptotic death of cerebellar granule neurons induced by low potassium. J Neurosci Res 85:332-341.

Bazenet CE, Mota MA, Rubin LL (1998) The small GTP-binding protein Cdc42 is required for nerve growth factor withdrawal-induced neuronal death. Proc Natl Acad Sci U S A 95:3984-3989.

Beck J, Lenart B, Kintner DB, Sun D (2003) Na-K-Cl cotransporter contributes to glutamate-mediated excitotoxicity. J Neurosci 23:5061-5068.

Beck T, Lindholm D, Castren E, Wree A (1994) Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus. J Cereb Blood Flow Metab 14:689-692.

Behar TN, Dugich-Djordjevic MM, Li YX, Ma W, Somogyi R, Wen X, Brown E, Scott C, McKay RD, Barker JL (1997) Neurotrophins stimulate chemotaxis of embryonic cortical neurons. Eur J Neurosci 9:2561-2570.

Bekinschtein P, Cammarota M, Izquierdo I, Medina JH (2008a) BDNF and memory formation and storage. Neuroscientist 14:147-156.

Bekinschtein P, Cammarota M, Katche C, Slipczuk L, Rossato JI, Goldin A, Izquierdo I, Medina JH (2008b) BDNF is essential to promote persistence of long-term memory storage. Proc Natl Acad Sci U S A 105:2711-2716.

Ben-Ari Y (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14:375-403.

Benson MD, Romero MI, Lush ME, Lu QR, Henkemeyer M, Parada LF (2005) Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth. Proc Natl Acad Sci U S A 102:10694-10699.

Bentley CA, Lee KF (2000) p75 is important for axon growth and schwann cell migration during development. J Neurosci 20:7706-7715.

Berchtold NC, Oliff HS, Isackson P, Cotman CW (1999) Hippocampal BDNF mRNA shows a diurnal regulation, primarily in the exon III transcript. Brain Res Mol Brain Res 71:11-22.

Berchtold NC, Kesslak JP, Pike CJ, Adlard PA, Cotman CW (2001) Estrogen and exercise interact to regulate brain-derived neurotrophic factor mRNA and protein expression in the hippocampus. Eur J Neurosci 14:1992-2002.

Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A (1991) Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7:857-866.

Berninger B, Garcia DE, Inagaki N, Hahnel C, Lindholm D (1993) BDNF and NT-3 induce intracellular Ca2+ elevation in hippocampal neurones. Neuroreport 4:1303-1306.

Berninger B, Marty S, Zafra F, da Penha Berzaghi M, Thoenen H, Lindholm D (1995) GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro. Development 121:2327-2335.

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21:13-26.

Beste C, Kolev V, Yordanova J, Domschke K, Falkenstein M, Baune BT, Konrad C (2010) The role of the BDNF Val66Met polymorphism for the synchronization of error-specific neural networks. J Neurosci 30:10727-10733.

Bibel M, Barde YA (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 14:2919-2937.

Biffo S, Offenhauser N, Carter BD, Barde YA (1995) Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. Development 121:2461-2470.

Bilimoria PM, Bonni A (2013) Molecular control of axon branching. Neuroscientist 19:16-24.

Bindokas VP, Miller RJ (1995) Excitotoxic degeneration is initiated at non-random sites in cultured rat cerebellar neurons. J Neurosci 15:6999-7011.

Birnbaumer L, Zhu X, Jiang M, Boulay G, Peyton M, Vannier B, Brown D, Platano D, Sadeghi H, Stefani E, Birnbaumer M (1996) On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. Proc Natl Acad Sci U S A 93:15195-15202.

Black DL (2003) Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 72:291-336.

Blaylock RL, Strunecka A (2009) Immune-glutamatergic dysfunction as a central mechanism of the autism spectrum disorders. Curr Med Chem 16:157-170.

Blochl A, Blochl R (2007) A cell-biological model of p75NTR signaling. J Neurochem 102:289-305.

Blochl A, Blumenstein L, Ahmadian MR (2004) Inactivation and activation of Ras by the neurotrophin receptor p75. Eur J Neurosci 20:2321-2335.

Blum R, Kafitz KW, Konnerth A (2002) Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature 419:687-693.

Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM (2009) Neural stem

cells improve cognition via BDNF in a transgenic model of Alzheimer disease. Proc Natl Acad Sci U S A 106:13594-13599.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-1362.

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell 123:1279-1291.

Bosch M, Hayashi Y (2012) Structural plasticity of dendritic spines. Curr Opin Neurobiol 22:383-388.

Bosman LW, Hartmann J, Barski JJ, Lepier A, Noll-Hussong M, Reichardt LF, Konnerth A (2006) Requirement of TrkB for synapse elimination in developing cerebellar Purkinje cells. Brain Cell Biol 35:87-101.

Boulay G, Zhu X, Peyton M, Jiang M, Hurst R, Stefani E, Birnbaumer L (1997) Cloning and expression of a novel mammalian homolog of Drosophila transient receptor potential (Trp) involved in calcium entry secondary to activation of receptors coupled by the Gq class of G protein. J Biol Chem 272:29672-29680.

Boutilier J, Ceni C, Pagdala PC, Forgie A, Neet KE, Barker PA (2008) Proneurotrophins require endocytosis and intracellular proteolysis to induce TrkA activation. J Biol Chem 283:12709-12716.

Bova R, Micheli MR, Qualadrucci P, Zucconi GG (1998) BDNF and trkB mRNAs oscillate in rat brain during the light-dark cycle. Brain Res Mol Brain Res 57:321-324.

Bozzi Y, Pizzorusso T, Cremisi F, Rossi FM, Barsacchi G, Maffei L (1995) Monocular deprivation decreases the expression of messenger RNA for brain-derived neurotrophic factor in the rat visual cortex. Neuroscience 69:1133-1144.

Bradshaw RA, Brickey WW, Walker KW (1998) N-terminal processing: the methionine aminopeptidase and N alpha-acetyl transferase families. Trends Biochem Sci 23:263-267.

Bramham CR, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol 76:99-125.

Brandon MP, Bogaard AR, Libby CP, Connerney MA, Gupta K, Hasselmo ME (2011) Reduction of theta rhythm dissociates grid cell spatial periodicity from directional tuning. Science 332:595-599.

Brigadski T, Hartmann M, Lessmann V (2005) Differential vesicular targeting and time course of synaptic secretion of the mammalian neurotrophins. J Neurosci 25:7601-7614.

Brittain JM, Chen L, Wilson SM, Brustovetsky T, Gao X, Ashpole NM, Molosh AI, You H, Hudmon A, Shekhar A, White FA, Zamponi GW, Brustovetsky N, Chen J, Khanna R (2011) Neuroprotection against traumatic brain injury by a peptide derived from the collapsin response mediator protein 2 (CRMP2). J Biol Chem 286:37778-37792.

Briz V, Hsu YT, Li Y, Lee E, Bi X, Baudry M (2013) Calpain-2-mediated PTEN degradation contributes to BDNF-induced stimulation of dendritic protein synthesis. J Neurosci 33:4317-4328.

Bronfman FC, Fainzilber M (2004) Multi-tasking by the p75 neurotrophin receptor: sortilin things out? EMBO Rep 5:867-871.

Brugg B, Matus A (1991) Phosphorylation determines the binding of microtubule-associated protein 2 (MAP2) to microtubules in living cells. J Cell Biol 114:735-743.

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297-305.

Brustovetsky T, Bolshakov A, Brustovetsky N (2010) Calpain activation and Na+/Ca2+ exchanger degradation occur downstream of calcium deregulation in hippocampal neurons exposed to excitotoxic glutamate. J Neurosci Res 88:1317-1328.

Burek MJ, Oppenheim RW (1996) Programmed cell death in the developing nervous system. Brain Pathol 6:427-446.

Butowt R, von Bartheld CS (2009) Fates of neurotrophins after retrograde axonal transport: phosphorylation of p75NTR is a sorting signal for delayed degradation. J Neurosci 29:10715-10729.

Cabrera N, Diaz-Rodriguez E, Becker E, Martin-Zanca D, Pandiella A (1996) TrkA receptor ectodomain cleavage generates a tyrosine-phosphorylated cell-associated fragment. J Cell Biol 132:427-436.

Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB (2007a) BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol Cell Neurosci 35:208-219.

Caldeira MV, Melo CV, Pereira DB, Carvalho R, Correia SS, Backos DS, Carvalho AL, Esteban JA, Duarte CB (2007b) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. J Biol Chem 282:12619-12628.

Caldeira MV, Curcio M, Leal G, Salazar IL, Mele M, Santos AR, Melo CV, Pereira P, Canzoniero LM, Duarte CB (2013) Excitotoxic stimulation downregulates the ubiquitin-proteasome system through activation of NMDA receptors in cultured hippocampal neurons. Biochim Biophys Acta 1832:263-274.

Camandola S, Mattson MP (2011) Aberrant subcellular neuronal calcium regulation in aging and Alzheimer's disease. Biochim Biophys Acta 1813:965-973.

Canals JM, Pineda JR, Torres-Peraza JF, Bosch M, Martin-Ibanez R, Munoz MT, Mengod G, Ernfors P, Alberch J (2004) Brain-derived neurotrophic factor regulates the onset and severity of motor dysfunction associated with enkephalinergic neuronal degeneration in Huntington's disease. J Neurosci 24:7727-7739.

Canossa M, Griesbeck O, Berninger B, Campana G, Kolbeck R, Thoenen H (1997) Neurotrophin release by neurotrophins: implications for activity-dependent neuronal plasticity. Proc Natl Acad Sci U S A 94:13279-13286.

Caputo V, Sinibaldi L, Fiorentino A, Parisi C, Catalanotto C, Pasini A, Cogoni C, Pizzuti A (2011) Brain derived neurotrophic factor (BDNF) expression is regulated by microRNAs miR-26a and miR-26b allele-specific binding. PLoS One 6:e28656.

Cargnello M, Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75:50-83.

Carim-Todd L, Bath KG, Fulgenzi G, Yanpallewar S, Jing D, Barrick CA, Becker J, Buckley H, Dorsey SG, Lee FS, Tessarollo L (2009) Endogenous truncated TrkB.T1 receptor regulates neuronal complexity and TrkB kinase receptor function in vivo. J Neurosci 29:678-685.

Carlsson Y, Schwendimann L, Vontell R, Rousset CI, Wang X, Lebon S, Charriaut-Marlangue C, Supramaniam V, Hagberg H, Gressens P, Jacotot E (2011) Genetic inhibition of caspase-2 reduces hypoxic-ischemic and excitotoxic neonatal brain injury. Ann Neurol 70:781-789.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Matthaei R, Baeuerle PA, Barde YA (1996) Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. Science 272:542-545.

Carvalho AL, Caldeira MV, Santos SD, Duarte CB (2008) Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br J Pharmacol 153 Suppl 1:S310-324.

Castren E, Zafra F, Thoenen H, Lindholm D (1992) Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proc Natl Acad Sci U S A 89:9444-9448.

Castren E, Pitkanen M, Sirvio J, Parsadanian A, Lindholm D, Thoenen H, Riekkinen PJ (1993) The induction of LTP increases BDNF and NGF mRNA but decreases NT-3 mRNA in the dentate gyrus. Neuroreport 4:895-898.

Ceni C, Kommaddi RP, Thomas R, Vereker E, Liu X, McPherson PS, Ritter B, Barker PA (2010) The p75NTR intracellular domain generated by neurotrophin-induced receptor cleavage potentiates Trk signaling. J Cell Sci 123:2299-2307.

Chandrasekar V, Dreyer JL (2009) microRNAs miR-124, let-7d and miR-181a regulate cocaine-induced plasticity. Mol Cell Neurosci 42:350-362.

Chang DT, Reynolds IJ (2006) Mitochondrial trafficking and morphology in healthy and injured neurons. Prog Neurobiol 80:241-268.

Chang L, Jones Y, Ellisman MH, Goldstein LS, Karin M (2003) JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. Dev Cell 4:521-533.

Chang R, Kwak Y, Gebremichael Y (2009) Structural properties of neurofilament sidearms: sequence-based modeling of neurofilament architecture. J Mol Biol 391:648-660.

Chao MV (2000) Trophic factors: An evolutionary cul-de-sac or door into higher neuronal function? J Neurosci Res 59:353-355.

Chapman BS (1995) A region of the 75 kDa neurotrophin receptor homologous to the death domains of TNFR-I and Fas. FEBS Lett 374:216-220.

Chen EY, Mufson EJ, Kordower JH (1996) TRK and p75 neurotrophin receptor systems in the developing human brain. J Comp Neurol 369:591-618.

Chen HH, Yu HI, Chiang WC, Lin YD, Shia BC, Tarn WY (2012) hnRNP Q regulates Cdc42-mediated neuronal morphogenesis. Mol Cell Biol 32:2224-2238.

Chen J, Chua KW, Chua CC, Yu H, Pei A, Chua BH, Hamdy RC, Xu X, Liu CF (2011) Antioxidant activity of 7,8-dihydroxyflavone provides neuroprotection against glutamate-induced toxicity. Neurosci Lett 499:181-185.

Chen MJ, Russo-Neustadt AA (2009) Running exercise-induced up-regulation of hippocampal brain-derived neurotrophic factor is CREB-dependent. Hippocampus 19:962-972.

Chen TJ, Wang DC, Chen SS (2009a) Amyloid-beta interrupts the PI3K-Akt-mTOR signaling pathway that could be involved in brain-derived neurotrophic factor-induced Arc expression in rat cortical neurons. J Neurosci Res 87:2297-2307.

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 302:885-889.

Chen Y, Zeng J, Cen L, Wang X, Yao G, Wang W, Qi W, Kong K (2009b) Multiple roles of the p75 neurotrophin receptor in the nervous system. J Int Med Res 37:281-288.

Chen Y, Wu H, Wang S, Koito H, Li J, Ye F, Hoang J, Escobar SS, Gow A, Arnett HA, Trapp BD, Karandikar NJ, Hsieh J, Lu QR (2009c) The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. Nat Neurosci 12:1398-1406.

Chen YS, Akula N, Detera-Wadleigh SD, Schulze TG, Thomas J, Potash JB, DePaulo JR, McInnis MG, Cox NJ, McMahon FJ (2004) Findings in an independent sample support an association between bipolar affective disorder and the G72/G30 locus on chromosome 13q33. Mol Psychiatry 9:87-92; image 85.

Chen Z, Simmons MS, Perry RT, Wiener HW, Harrell LE, Go RC (2008) Genetic association of neurotrophic tyrosine kinase receptor type 2 (NTRK2) With Alzheimer's disease. Am J Med Genet B Neuropsychiatr Genet 147:363-369.

Chen ZY, Ieraci A, Teng H, Dall H, Meng CX, Herrera DG, Nykjaer A, Hempstead BL, Lee FS (2005) Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. J Neurosci 25:6156-6166.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006) Genetic variant BDNF

(Val66Met) polymorphism alters anxiety-related behavior. Science 314:140-143.

Cheng PL, Song AH, Wong YH, Wang S, Zhang X, Poo MM (2011) Self-amplifying autocrine actions of BDNF in axon development. Proc Natl Acad Sci U S A 108:18430-18435.

Cheung EC, Melanson-Drapeau L, Cregan SP, Vanderluit JL, Ferguson KL, McIntosh WC, Park DS, Bennett SA, Slack RS (2005) Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. J Neurosci 25:1324-1334.

Chevalier-Larsen E, Holzbaur EL (2006) Axonal transport and neurodegenerative disease. Biochim Biophys Acta 1762:1094-1108.

Chiaruttini C, Sonego M, Baj G, Simonato M, Tongiorgi E (2008) BDNF mRNA splice variants display activity-dependent targeting to distinct hippocampal laminae. Mol Cell Neurosci 37:11-19.

Chiaruttini C, Vicario A, Li Z, Baj G, Braiuca P, Wu Y, Lee FS, Gardossi L, Baraban JM, Tongiorgi E (2009) Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. Proc Natl Acad Sci U S A 106:16481-16486.

Chung RS, McCormack GH, King AE, West AK, Vickers JC (2005) Glutamate induces rapid loss of axonal neurofilament proteins from cortical neurons in vitro. Exp Neurol 193:481-488.

Cirelli C, Tononi G (2000) Differential expression of plasticity-related genes in waking and sleep and their regulation by the noradrenergic system. J Neurosci 20:9187-9194.

Cirulli F, Berry A, Chiarotti F, Alleva E (2004) Intrahippocampal administration of BDNF in adult rats affects short-term behavioral plasticity in the Morris water maze and performance in the elevated plus-maze. Hippocampus 14:802-807.

Clapham DE (1996) TRP is cracked but is CRAC TRP? Neuron 16:1069-1072.

Clapham DE (2003) TRP channels as cellular sensors. Nature 426:517-524.

Clapham DE, Runnels LW, Strubing C (2001) The TRP ion channel family. Nat Rev Neurosci 2:387-396.

Clary DO, Reichardt LF (1994) An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc Natl Acad Sci U S A 91:11133-11137.

Cohen-Cory S, Fraser SE (1995) Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. Nature 378:192-196.

Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S (2010) Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol 70:271-288.

Collier TJ, Dung Ling Z, Carvey PM, Fletcher-Turner A, Yurek DM, Sladek JR, Jr., Kordower JH (2005) Striatal trophic factor activity in aging monkeys with unilateral MPTP-induced parkinsonism. Exp Neurol 191 Suppl 1:S60-67.

Conner JM, Lauterborn JC, Gall CM (1998) Anterograde transport of neurotrophin proteins in the CNS--a reassessment of the neurotrophic hypothesis. Rev Neurosci 9:91-103.

Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci 17:2295-2313.

Connor B, Young D, Yan Q, Faull RL, Synek B, Dragunow M (1997) Brain-derived neurotrophic factor is reduced in Alzheimer's disease. Brain Res Mol Brain Res 49:71-81.

Corbit KC, Foster DA, Rosner MR (1999) Protein kinase Cdelta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. Mol Cell Biol 19:4209-4218.

Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF, et al. (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173-183.

Costantini C, Della-Bianca V, Formaggio E, Chiamulera C, Montresor A, Rossi F (2005) The expression of p75 neurotrophin receptor protects against the neurotoxicity of soluble oligomers of beta-amyloid. Exp Cell Res 311:126-134.

Coughlan T, Gibson C, Murphy S (2009) Progesterone, BDNF and neuroprotection in the injured CNS. Int J Neurosci 119:1718-1740.

Coulson EJ, Reid K, Baca M, Shipham KA, Hulett SM, Kilpatrick TJ, Bartlett PF (2000) Chopper, a new death domain of the p75 neurotrophin receptor that mediates rapid neuronal cell death. J Biol Chem 275:30537-30545.

Crozier RA, Bi C, Han YR, Plummer MR (2008) BDNF modulation of NMDA receptors is activity dependent. J Neurophysiol 100:3264-3274.

Cui Q, Yu Z, Pan Y, Purisima EO, Wang E (2007) MicroRNAs preferentially target the genes with high transcriptional regulation complexity. Biochem Biophys Res Commun 352:733-738.

Cunha C, Brambilla R, Thomas KL (2010) A simple role for BDNF in learning and memory? Front Mol Neurosci 3:1.

Curtis R, Adryan KM, Stark JL, Park JS, Compton DL, Weskamp G, Huber LJ, Chao MV, Jaenisch R, Lee KF, et al. (1995) Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14:1201-1211.

Dajas-Bailador F, Bonev B, Garcez P, Stanley P, Guillemot F, Papalopulu N (2012) microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons. Nat Neurosci.

Danzer SC, Crooks KR, Lo DC, McNamara JO (2002) Increased expression of brain-derived neurotrophic factor induces formation of basal dendrites and axonal branching in dentate granule cells in hippocampal explant cultures. J Neurosci 22:9754-9763.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241.

David MD, Yeramian A, Dunach M, Llovera M, Canti C, de Herreros AG, Comella JX, Herreros J (2008) Signalling by neurotrophins and hepatocyte growth factor regulates axon morphogenesis by differential beta-catenin phosphorylation. J Cell Sci 121:2718-2730.

Davies AM, Thoenen H, Barde YA (1986) The response of chick sensory neurons to brain-derived neurotrophic factor. J Neurosci 6:1897-1904.

De Felice FG, Wu D, Lambert MP, Fernandez SJ, Velasco PT, Lacor PN, Bigio EH, Jerecic J, Acton PJ, Shughrue PJ, Chen-Dodson E, Kinney GG, Klein WL (2008) Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A beta oligomers. Neurobiol Aging 29:1334-1347.

De Girolamo LA, Billett EE (2006) Role of extracellular-regulated kinase and c-Jun NH2-terminal kinase in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurofilament phosphorylation. J Neurosci Res 83:680-693.

De Vos KJ, Grierson AJ, Ackerley S, Miller CC (2008) Role of axonal transport in neurodegenerative diseases. Annu Rev Neurosci 31:151-173.

Dean C, Liu H, Dunning FM, Chang PY, Jackson MB, Chapman ER (2009) Synaptotagmin-IV modulates synaptic function and long-term potentiation by regulating BDNF release. Nat Neurosci 12:767-776.

Dean C, Liu H, Staudt T, Stahlberg MA, Vingill S, Buckers J, Kamin D, Engelhardt J, Jackson MB, Hell SW, Chapman ER (2012) Distinct subsets of Syt-IV/BDNF vesicles are sorted to axons versus dendrites and recruited to synapses by activity. J Neurosci 32:5398-5413.

Dechant G, Barde YA (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. Nat Neurosci 5:1131-1136.

DeFreitas MF, McQuillen PS, Shatz CJ (2001) A novel p75NTR signaling pathway promotes survival, not death, of immunopurified neocortical subplate neurons. J Neurosci 21:5121-5129.

DeGracia DJ, Hu BR (2007) Irreversible translation arrest in the reperfused brain. J Cereb Blood Flow Metab 27:875-893.

Dehmelt L, Halpain S (2005) The MAP2/Tau family of microtubule-associated proteins. Genome Biol 6:204.

Deinhardt K, Kim T, Spellman DS, Mains RE, Eipper BA, Neubert TA, Chao MV, Hempstead BL (2011) Neuronal growth cone retraction relies on proneurotrophin receptor signaling through Rac. Sci Signal 4:ra82.

Dempster E, Toulopoulou T, McDonald C, Bramon E, Walshe M, Filbey F, Wickham H, Sham PC, Murray RM, Collier DA (2005) Association between BDNF val66 met genotype and episodic memory. Am J Med Genet B Neuropsychiatr Genet 134B:73-75.

Dennis SH, Jaafari N, Cimarosti H, Hanley JG, Henley JM, Mellor JR (2011) Oxygen/glucose deprivation induces a reduction in synaptic AMPA

receptors on hippocampal CA3 neurons mediated by mGluR1 and adenosine A3 receptors. J Neurosci 31:11941-11952.

Desai NS, Rutherford LC, Turrigiano GG (1999) BDNF regulates the intrinsic excitability of cortical neurons. Learn Mem 6:284-291.

Di Napoli M, McLaughlin B (2005) The ubiquitin-proteasome system as a drug target in cerebrovascular disease: therapeutic potential of proteasome inhibitors. Curr Opin Investig Drugs 6:686-699.

Diarra A, Geetha T, Potter P, Babu JR (2009) Signaling of the neurotrophin receptor p75 in relation to Alzheimer's disease. Biochem Biophys Res Commun 390:352-356.

Diaz-Rodriguez E, Cabrera N, Esparis-Ogando A, Montero JC, Pandiella A (1999) Cleavage of the TrkA neurotrophin receptor by multiple metalloproteases generates signalling-competent truncated forms. Eur J Neurosci 11:1421-1430.

Dieni S, Matsumoto T, Dekkers M, Rauskolb S, Ionescu MS, Deogracias R, Gundelfinger ED, Kojima M, Nestel S, Frotscher M, Barde YA (2012) BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. J Cell Biol 196:775-788.

Dimitropoulou A, Bixby JL (2000) Regulation of retinal neurite growth by alterations in MAPK/ERK kinase (MEK) activity. Brain Res 858:205-214.

Dincheva I, Glatt CE, Lee FS (2012) Impact of the BDNF Val66Met polymorphism on cognition: implications for behavioral genetics. Neuroscientist 18:439-451.

Diolaiti D, Bernardoni R, Trazzi S, Papa A, Porro A, Bono F, Herbert JM, Perini G, Della Valle G (2007) Functional cooperation between TrkA and p75(NTR) accelerates neuronal differentiation by increased transcription of GAP-43 and p21(CIP/WAF) genes via ERK1/2 and AP-1 activities. Exp Cell Res 313:2980-2992.

Dittrich F, Ochs G, Grosse-Wilde A, Berweiler U, Yan Q, Miller JA, Toyka KV, Sendtner M (1996) Pharmacokinetics of intrathecally applied BDNF and effects on spinal motoneurons. Exp Neurol 141:225-239.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596-1599.

Domeniconi M, Zampieri N, Spencer T, Hilaire M, Mellado W, Chao MV, Filbin MT (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. Neuron 46:849-855.

Dong XX, Wang Y, Qin ZH (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin 30:379-387.

Dono R (2003) Fibroblast growth factors as regulators of central nervous system development and function. Am J Physiol Regul Integr Comp Physiol 284:R867-881.

Dore S, Kar S, Quirion R (1997) Presence and differential internalization of two distinct insulin-like growth factor receptors in rat hippocampal neurons. Neuroscience 78:373-383.

Dorsey SG, Renn CL, Carim-Todd L, Barrick CA, Bambrick L, Krueger BK, Ward CW, Tessarollo L (2006) In vivo restoration of physiological levels of truncated TrkB.T1 receptor rescues neuronal cell death in a trisomic mouse model. Neuron 51:21-28.

Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. J Neurosci 8:1454-1468.

Dragunow M, Beilharz E, Mason B, Lawlor P, Abraham W, Gluckman P (1993) Brain-derived neurotrophic factor expression after long-term potentiation. Neurosci Lett 160:232-236.

Drake CT, Milner TA, Patterson SL (1999) Ultrastructural localization of full-length trkB immunoreactivity in rat hippocampus suggests multiple roles in modulating activity-dependent synaptic plasticity. J Neurosci 19:8009-8026.

Drewes G, Lichtenberg-Kraag B, Doring F, Mandelkow EM, Biernat J, Goris J, Doree M, Mandelkow E (1992) Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. EMBO J 11:2131-2138.

Du JL, Poo MM (2004) Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. Nature 429:878-883.

Du W, Huang J, Yao H, Zhou K, Duan B, Wang Y (2010) Inhibition of TRPC6 degradation suppresses ischemic brain damage in rats. J Clin Invest 120:3480-3492.

Egan MF, Weinberger DR, Lu B (2003a) Schizophrenia, III: brain-derived neurotropic factor and genetic risk. Am J Psychiatry 160:1242.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR (2003b) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 112:257-269.

Ehrnhoefer DE, Wong BK, Hayden MR (2011) Convergent pathogenic pathways in Alzheimer's and Huntington's diseases: shared targets for drug development. Nat Rev Drug Discov 10:853-867.

Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 16:3123-3129.

Eipper BA, Green CB, Campbell TA, Stoffers DA, Keutmann HT, Mains RE, Ouafik L (1992) Alternative splicing and endoproteolytic processing generate tissue-specific forms of pituitary peptidylglycine alpha-amidating monooxygenase (PAM). J Biol Chem 267:4008-4015.

Eisch AJ, Bolanos CA, de Wit J, Simonak RD, Pudiak CM, Barrot M, Verhaagen J, Nestler EJ (2003) Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. Biol Psychiatry 54:994-1005.

English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH (1999) New insights into the control of MAP kinase pathways. Exp Cell Res 253:255-270.

Ernfors P, Lee KF, Jaenisch R (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368:147-150.

Ernfors P, Wetmore C, Olson L, Persson H (1990) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron 5:511-526.

Escandon E, Soppet D, Rosenthal A, Mendoza-Ramirez JL, Szonyi E, Burton LE, Henderson CE, Parada LF, Nikolics K (1994) Regulation of neurotrophin receptor expression during embryonic and postnatal development. J Neurosci 14:2054-2068.

Faddis BT, Hasbani MJ, Goldberg MP (1997) Calpain activation contributes to dendritic remodeling after brief excitotoxic injury in vitro. J Neurosci 17:951-959.

Fan QW, Yu W, Gong JS, Zou K, Sawamura N, Senda T, Yanagisawa K, Michikawa M (2002) Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J Neurochem 80:178-190.

Farhadi HF, Mowla SJ, Petrecca K, Morris SJ, Seidah NG, Murphy RA (2000) Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J Neurosci 20:4059-4068.

Farkas LM, Dunker N, Roussa E, Unsicker K, Krieglstein K (2003) Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. J Neurosci 23:5178-5186.

Fasanaro P, Greco S, Lorenzi M, Pescatori M, Brioschi M, Kulshreshtha R, Banfi C, Stubbs A, Calin GA, Ivan M, Capogrossi MC, Martelli F (2009) An integrated approach for experimental target identification of hypoxia-induced miR-210. J Biol Chem 284:35134-35143.

Fawcett JP, Bamji SX, Causing CG, Aloyz R, Ase AR, Reader TA, McLean JH, Miller FD (1998) Functional evidence that BDNF is an anterograde neuronal trophic factor in the CNS. J Neurosci 18:2808-2821.

Fayard B, Loeffler S, Weis J, Vogelin E, Kruttgen A (2005) The secreted brain-derived neurotrophic factor precursor pro-BDNF binds to TrkB and p75NTR but not to TrkA or TrkC. J Neurosci Res 80:18-28.

Feng D, Kim T, Ozkan E, Light M, Torkin R, Teng KK, Hempstead BL, Garcia KC (2010) Molecular and structural insight into proNGF engagement of p75NTR and sortilin. J Mol Biol 396:967-984.

Ferenz KB, Gast RE, Rose K, Finger IE, Hasche A, Krieglstein J (2012) Nerve growth factor and brain-derived neurotrophic factor but not granulocyte colony-stimulating factor, nimodipine and dizocilpine, require ATP for neuroprotective activity after oxygen-glucose deprivation of primary neurons. Brain Res 1448:20-26.

Ferguson AR, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS (2008) Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J Neurosci 28:11391-11400.

Ferrer I, Goutan E, Marin C, Rey MJ, Ribalta T (2000) Brain-derived neurotrophic factor in Huntington disease. Brain Res 866:257-261.

Ferrer I, Krupinski J, Goutan E, Marti E, Ambrosio S, Arenas E (2001) Brain-derived neurotrophic factor reduces cortical cell death by ischemia after middle cerebral artery occlusion in the rat. Acta Neuropathol 101:229-238.

Ferrer I, Marin C, Rey MJ, Ribalta T, Goutan E, Blanco R, Tolosa E, Marti E (1999) BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies. J Neuropathol Exp Neurol 58:729-739.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706-709.

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9:102-114.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031-1047.

Fischer I, Romano-Clarke G, Grynspan F (1991) Calpain-mediated proteolysis of microtubule associated proteins MAP1B and MAP2 in developing brain. Neurochem Res 16:891-898.

Flavell SW, Greenberg ME (2008) Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Annu Rev Neurosci 31:563-590.

Flavell SW, Kim TK, Gray JM, Harmin DA, Hemberg M, Hong EJ, Markenscoff-Papadimitriou E, Bear DM, Greenberg ME (2008) Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. Neuron 60:1022-1038.

Fletcher JM, Morton CJ, Zwar RA, Murray SS, O'Leary PD, Hughes RA (2008) Design of a conformationally defined and proteolytically stable circular mimetic of brain-derived neurotrophic factor. J Biol Chem 283:33375-33383.

Foltynie T, Cheeran B, Williams-Gray CH, Edwards MJ, Schneider SA, Weinberger D, Rothwell JC, Barker RA, Bhatia KP (2009) BDNF val66met influences time to onset of levodopa induced dyskinesia in Parkinson's disease. J Neurol Neurosurg Psychiatry 80:141-144.

Foran E, Trotti D (2009) Glutamate transporters and the excitotoxic path to motor neuron degeneration in amyotrophic lateral sclerosis. Antioxid Redox Signal 11:1587-1602.

Fortin DA, Srivastava T, Dwarakanath D, Pierre P, Nygaard S, Derkach VA, Soderling TR (2012) Brain-derived neurotrophic factor activation of CaM-

kinase kinase via transient receptor potential canonical channels induces the translation and synaptic incorporation of GluA1-containing calcium-permeable AMPA receptors. J Neurosci 32:8127-8137.

Frade JM, Rodriguez-Tebar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383:166-168.

Frey U, Morris RG (1997) Synaptic tagging and long-term potentiation. Nature 385:533-536.

Friedman LM, Dror AA, Mor E, Tenne T, Toren G, Satoh T, Biesemeier DJ, Shomron N, Fekete DM, Hornstein E, Avraham KB (2009) MicroRNAs are essential for development and function of inner ear hair cells in vertebrates. Proc Natl Acad Sci U S A 106:7915-7920.

Friedrich P, Aszodi A (1991) MAP2: a sensitive cross-linker and adjustable spacer in dendritic architecture. FEBS Lett 295:5-9.

Frustaci A, Pozzi G, Gianfagna F, Manzoli L, Boccia S (2008) Meta-analysis of the brain-derived neurotrophic factor gene (BDNF) Val66Met polymorphism in anxiety disorders and anxiety-related personality traits. Neuropsychobiology 58:163-170.

Fryer RH, Kaplan DR, Feinstein SC, Radeke MJ, Grayson DR, Kromer LF (1996) Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. J Comp Neurol 374:21-40.

Fujihara H, Sei H, Morita Y, Ueta Y, Morita K (2003) Short-term sleep disturbance enhances brain-derived neurotrophic factor gene expression in rat hippocampus by acting as internal stressor. J Mol Neurosci 21:223-232.

Fyffe-Maricich SL, Karlo JC, Landreth GE, Miller RH (2011) The ERK2 mitogen-activated protein kinase regulates the timing of oligodendrocyte differentiation. J Neurosci 31:843-850.

Galvin KA, Oorschot DE (2003) Continuous low-dose treatment with brain-derived neurotrophic factor or neurotrophin-3 protects striatal medium spiny neurons from mild neonatal hypoxia/ischemia: a stereological study. Neuroscience 118:1023-1032.

Garner AS, Large TH (1994) Isoforms of the avian TrkC receptor: a novel kinase insertion dissociates transformation and process outgrowth from survival. Neuron 13:457-472.

Gartner A, Polnau DG, Staiger V, Sciarretta C, Minichiello L, Thoenen H, Bonhoeffer T, Korte M (2006) Hippocampal long-term potentiation is supported by presynaptic and postsynaptic tyrosine receptor kinase B-mediated phospholipase Cgamma signaling. J Neurosci 26:3496-3504.

Gascon S, Sobrado M, Roda JM, Rodriguez-Pena A, Diaz-Guerra M (2008) Excitotoxicity and focal cerebral ischemia induce truncation of the NR2A and NR2B subunits of the NMDA receptor and cleavage of the scaffolding protein PSD-95. Mol Psychiatry 13:99-114.

Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, De Mey J, MacDonald ME, Lessmann V, Humbert S, Saudou F (2004)

Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell 118:127-138.

Ge P, Luo Y, Liu CL, Hu B (2007) Protein aggregation and proteasome dysfunction after brain ischemia. Stroke 38:3230-3236.

Geetha T, Kenchappa RS, Wooten MW, Carter BD (2005) TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. EMBO J 24:3859-3868.

Gehler S, Gallo G, Veien E, Letourneau PC (2004a) p75 neurotrophin receptor signaling regulates growth cone filopodial dynamics through modulating RhoA activity. J Neurosci 24:4363-4372.

Gehler S, Shaw AE, Sarmiere PD, Bamburg JR, Letourneau PC (2004b) Brain-derived neurotrophic factor regulation of retinal growth cone filopodial dynamics is mediated through actin depolymerizing factor/cofilin. J Neurosci 24:10741-10749.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618-1623.

Gibney J, Zheng JQ (2003) Cytoskeletal dynamics underlying collateral membrane protrusions induced by neurotrophins in cultured Xenopus embryonic neurons. J Neurobiol 54:393-405.

Giralt A, Carreton O, Lao-Peregrin C, Martin ED, Alberch J (2011) Conditional BDNF release under pathological conditions improves Huntington's disease pathology by delaying neuronal dysfunction. Mol Neurodegener 6:71.

Glass DJ, Nye SH, Hantzopoulos P, Macchi MJ, Squinto SP, Goldfarb M, Yancopoulos GD (1991) TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell 66:405-413.

Glazner GW, Chan SL, Lu C, Mattson MP (2000) Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. J Neurosci 20:3641-3649.

Gomes JR, Lobo AC, Melo CV, Inacio AR, Takano J, Iwata N, Saido TC, de Almeida LP, Wieloch T, Duarte CB (2011) Cleavage of the vesicular GABA transporter under excitotoxic conditions is followed by accumulation of the truncated transporter in nonsynaptic sites. J Neurosci 31:4622-4635.

Gomes JR, Costa JT, Melo CV, Felizzi F, Monteiro P, Pinto MJ, Inacio AR, Wieloch T, Almeida RD, Graos M, Duarte CB (2012) Excitotoxicity downregulates TrkB.FL signaling and upregulates the neuroprotective truncated TrkB receptors in cultured hippocampal and striatal neurons. J Neurosci 32:4610-4622.

Gomes RA, Hampton C, El-Sabeawy F, Sabo SL, McAllister AK (2006) The dynamic distribution of TrkB receptors before, during, and after synapse formation between cortical neurons. J Neurosci 26:11487-11500.

Gomez-Pinilla F, Zhuang Y, Feng J, Ying Z, Fan G (2011) Exercise impacts brain-derived neurotrophic factor plasticity by engaging mechanisms of epigenetic regulation. Eur J Neurosci 33:383-390.

Gong Y, Cao P, Yu HJ, Jiang T (2008) Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. Nature 454:789-793.

Goodman LJ, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, Hefti F (1996) Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol Cell Neurosci 7:222-238.

Gotz R, Raulf F, Schartl M (1992a) Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. J Neurochem 59:432-442.

Gotz R, Kolbeck R, Lottspeich F, Barde YA (1992b) Production and characterization of recombinant mouse neurotrophin-3. Eur J Biochem 204:745-749.

Gotz R, Koster R, Winkler C, Raulf F, Lottspeich F, Schartl M, Thoenen H (1994) Neurotrophin-6 is a new member of the nerve growth factor family. Nature 372:266-269.

Goutan E, Marti E, Ferrer I (1998) BDNF, and full length and truncated TrkB expression in the hippocampus of the rat following kainic acid excitotoxic damage. Evidence of complex time-dependent and cell-specific responses. Brain Res Mol Brain Res 59:154-164.

Gratacos E, Checa N, Perez-Navarro E, Alberch J (2001a) Brain-derived neurotrophic factor (BDNF) mediates bone morphogenetic protein-2 (BMP-2) effects on cultured striatal neurones. J Neurochem 79:747-755.

Gratacos E, Perez-Navarro E, Tolosa E, Arenas E, Alberch J (2001b) Neuroprotection of striatal neurons against kainate excitotoxicity by neurotrophins and GDNF family members. J Neurochem 78:1287-1296.

Gratacos M, Soria V, Urretavizcaya M, Gonzalez JR, Crespo JM, Bayes M, de Cid R, Menchon JM, Vallejo J, Estivill X (2008) A brain-derived neurotrophic factor (BDNF) haplotype is associated with antidepressant treatment outcome in mood disorders. Pharmacogenomics J 8:101-112.

Gray J, Yeo GS, Cox JJ, Morton J, Adlam AL, Keogh JM, Yanovski JA, El Gharbawy A, Han JC, Tung YC, Hodges JR, Raymond FL, O'Rahilly S, Farooqi IS (2006) Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. Diabetes 55:3366-3371.

Greenberg ME, Xu B, Lu B, Hempstead BL (2009) New insights in the biology of BDNF synthesis and release: implications in CNS function. J Neurosci 29:12764-12767.

Greenwood SM, Mizielinska SM, Frenguelli BG, Harvey J, Connolly CN (2007) Mitochondrial dysfunction and dendritic beading during neuronal toxicity. J Biol Chem 282:26235-26244.

Grewal SS, York RD, Stork PJ (1999) Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol 9:544-553.

Grewer C, Gameiro A, Zhang Z, Tao Z, Braams S, Rauen T (2008) Glutamate forward and reverse transport: from molecular mechanism to transporter-mediated release after ischemia. IUBMB Life 60:609-619.

Griesbeck O, Canossa M, Campana G, Gartner A, Hoener MC, Nawa H, Kolbeck R, Thoenen H (1999) Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microsc Res Tech 45:262-275.

Gruart A, Sciarretta C, Valenzuela-Harrington M, Delgado-Garcia JM, Minichiello L (2007) Mutation at the TrkB PLC{gamma}-docking site affects hippocampal LTP and associative learning in conscious mice. Learn Mem 14:54-62.

Gubellini P, Ben-Ari Y, Gaiarsa JL (2005) Endogenous neurotrophins are required for the induction of GABAergic long-term potentiation in the neonatal rat hippocampus. J Neurosci 25:5796-5802.

Guirland C, Suzuki S, Kojima M, Lu B, Zheng JQ (2004) Lipid rafts mediate chemotropic guidance of nerve growth cones. Neuron 42:51-62.

Guo X, Ramirez A, Waddell DS, Li Z, Liu X, Wang XF (2008) Axin and GSK3-control Smad3 protein stability and modulate TGF- signaling. Genes Dev 22:106-120.

Gupta SK, Mishra R, Kusum S, Spedding M, Meiri KF, Gressens P, Mani S (2009) GAP-43 is essential for the neurotrophic effects of BDNF and positive AMPA receptor modulator S18986. Cell Death Differ 16:624-637.

Haapasalo A, Sipola I, Larsson K, Akerman KE, Stoilov P, Stamm S, Wong G, Castren E (2002) Regulation of TRKB surface expression by brain-derived neurotrophic factor and truncated TRKB isoforms. J Biol Chem 277:43160-43167.

Haj-Dahmane S, Andrade R (1996) Muscarinic activation of a voltage-dependent cation nonselective current in rat association cortex. J Neurosci 16:3848-3861.

Hall D, Dhilla A, Charalambous A, Gogos JA, Karayiorgou M (2003) Sequence variants of the brain-derived neurotrophic factor (BDNF) gene are strongly associated with obsessive-compulsive disorder. Am J Hum Genet 73:370-376.

Hallbook F (1999) Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr Opin Neurobiol 9:616-621.

Hallbook F, Ibanez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-858.

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Mol Cell Neurosci 14:28-40.

Hamburger V, Levi-Montalcini R (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J Exp Zool 111:457-501.

Han BH, Holtzman DM (2000) BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. J Neurosci 20:5775-5781.

Haniu M, Montestruque S, Bures EJ, Talvenheimo J, Toso R, Lewis-Sandy S, Welcher AA, Rohde MF (1997) Interactions between brain-derived neurotrophic factor and the TRKB receptor. Identification of two ligand binding domains in soluble TRKB by affinity separation and chemical cross-linking. J Biol Chem 272:25296-25303.

Hardingham GE, Chawla S, Cruzalegui FH, Bading H (1999) Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron 22:789-798.

Hariri AR, Goldberg TE, Mattay VS, Kolachana BS, Callicott JH, Egan MF, Weinberger DR (2003) Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. J Neurosci 23:6690-6694.

Harper MM, Adamson L, Blits B, Bunge MB, Grozdanic SD, Sakaguchi DS (2009) Brain-derived neurotrophic factor released from engineered mesenchymal stem cells attenuates glutamate- and hydrogen peroxide-mediated death of staurosporine-differentiated RGC-5 cells. Exp Eye Res 89:538-548.

Harrington AW, Kim JY, Yoon SO (2002) Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22:156-166.

Hartmann D, Drummond J, Handberg E, Ewell S, Pozzo-Miller L (2012) Multiple approaches to investigate the transport and activity-dependent release of BDNF and their application in neurogenetic disorders. Neural Plast 2012:203734.

Hartmann M, Heumann R, Lessmann V (2001) Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J 20:5887-5897.

Harvey BK, Airavaara M, Hinzman J, Wires EM, Chiocco MJ, Howard DB, Shen H, Gerhardt G, Hoffer BJ, Wang Y (2011) Targeted over-expression of glutamate transporter 1 (GLT-1) reduces ischemic brain injury in a rat model of stroke. PLoS One 6:e22135.

Hashimoto R, Takei N, Shimazu K, Christ L, Lu B, Chuang DM (2002) Lithium induces brain-derived neurotrophic factor and activates TrkB in rodent cortical neurons: an essential step for neuroprotection against glutamate excitotoxicity. Neuropharmacology 43:1173-1179.

Haubensak W, Narz F, Heumann R, Lessmann V (1998) BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. J Cell Sci 111 (Pt 11):1483-1493.

He XL, Garcia KC (2004) Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. Science 304:870-875.

He XP, Kotloski R, Nef S, Luikart BW, Parada LF, McNamara JO (2004) Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. Neuron 43:31-42.

Heinrich G, Pagtakhan CJ (2004) Both 5' and 3' flanks regulate Zebrafish brain-derived neurotrophic factor gene expression. BMC Neurosci 5:19.

Helton TD, Otsuka T, Lee MC, Mu Y, Ehlers MD (2008) Pruning and loss of excitatory synapses by the parkin ubiquitin ligase. Proc Natl Acad Sci U S A 105:19492-19497.

Hely TA, Graham B, Ooyen AV (2001) A computational model of dendrite elongation and branching based on MAP2 phosphorylation. J Theor Biol 210:375-384.

Hempstead BL (2002) The many faces of p75NTR. Curr Opin Neurobiol 12:260-267.

Hetman M, Kanning K, Cavanaugh JE, Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem 274:22569-22580.

Higuchi H, Yamashita T, Yoshikawa H, Tohyama M (2003) PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts. EMBO J 22:1790-1800.

Higuchi M, Tomioka M, Takano J, Shirotani K, Iwata N, Masumoto H, Maki M, Itohara S, Saido TC (2005) Distinct mechanistic roles of calpain and caspase activation in neurodegeneration as revealed in mice overexpressing their specific inhibitors. J Biol Chem 280:15229-15237.

Hilton GD, Nunez JL, Bambrick L, Thompson SM, McCarthy MM (2006) Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca++ from intracellular stores and is prevented by estradiol. Eur J Neurosci 24:3008-3016.

Ho BC, Andreasen NC, Dawson JD, Wassink TH (2007) Association between brain-derived neurotrophic factor Val66Met gene polymorphism and progressive brain volume changes in schizophrenia. Am J Psychiatry 164:1890-1899.

Ho BC, Milev P, O'Leary DS, Librant A, Andreasen NC, Wassink TH (2006) Cognitive and magnetic resonance imaging brain morphometric correlates of brain-derived neurotrophic factor Val66Met gene polymorphism in patients with schizophrenia and healthy volunteers. Arch Gen Psychiatry 63:731-740.

Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397:259-263.

Hogins J, Crawford DC, Jiang X, Mennerick S (2011) Presynaptic silencing is an endogenous neuroprotectant during excitotoxic insults. Neurobiol Dis 43:516-525.

Hohn A, Leibrock J, Bailey K, Barde YA (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 344:339-341.

Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ (1997) Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci U S A 94:12419-12424.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31-108.

Hong EJ, McCord AE, Greenberg ME (2008) A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron 60:610-624.

Horch HW, Katz LC (2002) BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat Neurosci 5:1177-1184.

Hossmann KA (1994) Viability thresholds and the penumbra of focal ischemia. Ann Neurol 36:557-565.

Huang CS, Zhou J, Feng AK, Lynch CC, Klumperman J, DeArmond SJ, Mobley WC (1999a) Nerve growth factor signaling in caveolae-like domains at the plasma membrane. J Biol Chem 274:36707-36714.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609-642.

Huang J, Manning BD (2009) A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans 37:217-222.

Huang J, Furuya A, Furuichi T (2007a) Very-KIND, a KIND domain containing RasGEF, controls dendrite growth by linking Ras small GTPases and MAP2. J Cell Biol 179:539-552.

Huang J, Furuya A, Hayashi K, Furuichi T (2011a) Interaction between very-KIND Ras guanine exchange factor and microtubule-associated protein 2, and its role in dendrite growth--structure and function of the second kinase noncatalytic C-lobe domain. FEBS J 278:1651-1661.

Huang JK, Dorey K, Ishibashi S, Amaya E (2007b) BDNF promotes target innervation of Xenopus mandibular trigeminal axons in vivo. BMC Dev Biol 7:59.

Huang SH, Zhao L, Sun ZP, Li XZ, Geng Z, Zhang KD, Chao MV, Chen ZY (2009) Essential role of Hrs in endocytic recycling of full-length TrkB receptor but not its isoform TrkB.T1. J Biol Chem 284:15126-15136.

Huang SH, Duan S, Sun T, Wang J, Zhao L, Geng Z, Yan J, Sun HJ, Chen ZY (2011b) JIP3 mediates TrkB axonal anterograde transport and enhances BDNF signaling by directly bridging TrkB with kinesin-1. J Neurosci 31:10602-10614.

Huang ZJ, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L, Tonegawa S (1999b) BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. Cell 98:739-755.

Huber KM, Sawtell NB, Bear MF (1998) Brain-derived neurotrophic factor alters the synaptic modification threshold in visual cortex. Neuropharmacology 37:571-579.

Hwang JJ, Park MH, Choi SY, Koh JY (2005) Activation of the Trk signaling pathway by extracellular zinc. Role of metalloproteinases. J Biol Chem 280:11995-12001.

Hyman C, Hofer M, Barde YA, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230-232.

Hynd MR, Scott HL, Dodd PR (2004) Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. Neurochem Int 45:583-595.

Ibanez CF (2004) Lipid rafts as organizing platforms for cell chemotaxis and axon guidance. Neuron 42:3-5.

Ibanez CF, Ilag LL, Murray-Rust J, Persson H (1993) An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. EMBO J 12:2281-2293.

Ikeda O, Murakami M, Ino H, Yamazaki M, Koda M, Nakayama C, Moriya H (2002) Effects of brain-derived neurotrophic factor (BDNF) on compression-induced spinal cord injury: BDNF attenuates down-regulation of superoxide dismutase expression and promotes up-regulation of myelin basic protein expression. J Neuropathol Exp Neurol 61:142-153.

Im HI, Hollander JA, Bali P, Kenny PJ (2010) MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. Nat Neurosci 13:1120-1127.

Ip NY, Yancopoulos GD (1996) The neurotrophins and CNTF: two families of collaborative neurotrophic factors. Annu Rev Neurosci 19:491-515.

Ip NY, Li Y, Yancopoulos GD, Lindsay RM (1993a) Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J Neurosci 13:3394-3405.

Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, Fandl J, Greene LA, Barbacid M, Yancopoulos GD (1993b) Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. Neuron 10:137-149.

Ip NY, Ibanez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa R, 3rd, Squinto SP, et al. (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci U S A 89:3060-3064.

Itami C, Kimura F, Kohno T, Matsuoka M, Ichikawa M, Tsumoto T, Nakamura S (2003) Brain-derived neurotrophic factor-dependent unmasking of "silent" synapses in the developing mouse barrel cortex. Proc Natl Acad Sci U S A 100:13069-13074.

Jaaro H, Beck G, Conticello SG, Fainzilber M (2001) Evolving better brains: a need for neurotrophins? Trends Neurosci 24:79-85.

Jakawich SK, Nasser HB, Strong MJ, McCartney AJ, Perez AS, Rakesh N, Carruthers CJ, Sutton MA (2010) Local presynaptic activity gates homeostatic changes in presynaptic function driven by dendritic BDNF synthesis. Neuron 68:1143-1158.

Jang SW, Liu X, Yepes M, Shepherd KR, Miller GW, Liu Y, Wilson WD, Xiao G, Blanchi B, Sun YE, Ye K (2010) A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. Proc Natl Acad Sci U S A 107:2687-2692.

Jantas D, Szymanska M, Budziszewska B, Lason W (2009) An involvement of BDNF and PI3-K/Akt in the anti-apoptotic effect of memantine on staurosporine-evoked cell death in primary cortical neurons. Apoptosis 14:900-912.

Jeon CY, Kim HJ, Morii H, Mori N, Settleman J, Lee JY, Kim J, Kim SC, Park JB (2010) Neurite outgrowth from PC12 cells by basic fibroblast growth factor (bFGF) is mediated by RhoA inactivation through p190RhoGAP and ARAP3. J Cell Physiol 224:786-794.

Ji Y, Pang PT, Feng L, Lu B (2005) Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat Neurosci 8:164-172.

Ji Y, Lu Y, Yang F, Shen W, Tang TT, Feng L, Duan S, Lu B (2010) Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons. Nat Neurosci 13:302-309.

Jia Y, Gall CM, Lynch G (2010) Presynaptic BDNF promotes postsynaptic long-term potentiation in the dorsal striatum. J Neurosci 30:14440-14445.

Jia Y, Zhou J, Tai Y, Wang Y (2007) TRPC channels promote cerebellar granule neuron survival. Nat Neurosci 10:559-567.

Jiang X, Xu K, Hoberman J, Tian F, Marko AJ, Waheed JF, Harris CR, Marini AM, Enoch MA, Lipsky RH (2005) BDNF variation and mood disorders: a novel functional promoter polymorphism and Val66Met are associated with anxiety but have opposing effects. Neuropsychopharmacology 30:1353-1361.

Jiang X, Tian F, Du Y, Copeland NG, Jenkins NA, Tessarollo L, Wu X, Pan H, Hu XZ, Xu K, Kenney H, Egan SE, Turley H, Harris AL, Marini AM, Lipsky RH (2008) BHLHB2 controls Bdnf promoter 4 activity and neuronal excitability. J Neurosci 28:1118-1130.

Johnson-Farley NN, Patel K, Kim D, Cowen DS (2007) Interaction of FGF-2 with IGF-1 and BDNF in stimulating Akt, ERK, and neuronal survival in hippocampal cultures. Brain Res 1154:40-49.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M (1986a) Expression and structure of the human NGF receptor. Cell 47:545-554.

Johnson GV, Foley VG (1993) Calpain-mediated proteolysis of microtubule-associated protein 2 (MAP-2) is inhibited by phosphorylation by cAMP-dependent protein kinase, but not by Ca2+/calmodulin-dependent protein kinase II. J Neurosci Res 34:642-647.

Johnson JE, Barde YA, Schwab M, Thoenen H (1986b) Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion cells. J Neurosci 6:3031-3038.

Johnson MD, Kinoshita Y, Xiang H, Ghatan S, Morrison RS (1999) Contribution of p53-dependent caspase activation to neuronal cell death declines with neuronal maturation. J Neurosci 19:2996-3006.

Jones KR, Reichardt LF (1990) Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc Natl Acad Sci U S A 87:8060-8064.

Jones SB, Lu HY, Lu Q (2004) Abl tyrosine kinase promotes dendrogenesis by inducing actin cytoskeletal rearrangements in cooperation with Rho family small GTPases in hippocampal neurons. J Neurosci 24:8510-8521.

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat Neurosci 3:323-329.

Jung H, Yoon BC, Holt CE (2012) Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. Nat Rev Neurosci 13:308-324.

Jung KM, Tan S, Landman N, Petrova K, Murray S, Lewis R, Kim PK, Kim DS, Ryu SH, Chao MV, Kim TW (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. J Biol Chem 278:42161-42169.

Jung S, Strotmann R, Schultz G, Plant TD (2002) TRPC6 is a candidate channel involved in receptor-stimulated cation currents in A7r5 smooth muscle cells. Am J Physiol Cell Physiol 282:C347-359.

Jungbluth S, Bailey K, Barde YA (1994) Purification and characterisation of a brain-derived neurotrophic factor/neurotrophin-3 (BDNF/NT-3) heterodimer. Eur J Biochem 221:677-685.

Kaech S, Banker G (2006) Culturing hippocampal neurons. Nat Protoc 1:2406-2415.

Kaech S, Huang CF, Banker G (2012a) General considerations for live imaging of developing hippocampal neurons in culture. Cold Spring Harb Protoc 2012:312-318.

Kaech S, Huang CF, Banker G (2012b) Long-term time-lapse imaging of developing hippocampal neurons in culture. Cold Spring Harb Protoc 2012:335-339.

Kaech S, Huang CF, Banker G (2012c) Short-term high-resolution imaging of developing hippocampal neurons in culture. Cold Spring Harb Protoc 2012:340-343.

Kafitz KW, Rose CR, Thoenen H, Konnerth A (1999) Neurotrophin-evoked rapid excitation through TrkB receptors. Nature 401:918-921.

Kanamoto T, Mota M, Takeda K, Rubin LL, Miyazono K, Ichijo H, Bazenet CE (2000) Role of apoptosis signal-regulating kinase in regulation of the c-Jun N-terminal kinase pathway and apoptosis in sympathetic neurons. Mol Cell Biol 20:196-204.

Kanato Y, Kitajima K, Sato C (2008) Direct binding of polysialic acid to a brain-derived neurotrophic factor depends on the degree of polymerization. Glycobiology 18:1044-1053.

Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. Science 294:1030-1038.

Kaneko M, Xie Y, An JJ, Stryker MP, Xu B (2012) Dendritic BDNF synthesis is required for late-phase spine maturation and recovery of cortical responses following sensory deprivation. J Neurosci 32:4790-4802.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267:1658-1662.

Kang H, Welcher AA, Shelton D, Schuman EM (1997) Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron 19:653-664.

Kanhema T, Dagestad G, Panja D, Tiron A, Messaoudi E, Havik B, Ying SW, Nairn AC, Sonenberg N, Bramham CR (2006) Dual regulation of translation initiation and peptide chain elongation during BDNF-induced LTP in vivo: evidence for compartment-specific translation control. J Neurochem 99:1328-1337.

Kanning KC, Hudson M, Amieux PS, Wiley JC, Bothwell M, Schecterson LC (2003) Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J Neurosci 23:5425-5436.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554-558.

Karmarkar SW, Bottum KM, Krager SL, Tischkau SA (2011) ERK/MAPK is essential for endogenous neuroprotection in SCN2.2 cells. PLoS One 6:e23493.

Karpova NN, Rantamaki T, Di Lieto A, Lindemann L, Hoener MC, Castren E (2010) Darkness reduces BDNF expression in the visual cortex and induces repressive chromatin remodeling at the BDNF gene in both hippocampus and visual cortex. Cell Mol Neurobiol 30:1117-1123.

Kasai A, Kinjo T, Ishihara R, Sakai I, Ishimaru Y, Yoshioka Y, Yamamuro A, Ishige K, Ito Y, Maeda S (2011) Apelin deficiency accelerates the progression of amyotrophic lateral sclerosis. PLoS One 6:e23968.

Kato AS, Gill MB, Ho MT, Yu H, Tu Y, Siuda ER, Wang H, Qian YW, Nisenbaum ES, Tomita S, Bredt DS (2010) Hippocampal AMPA receptor gating controlled by both TARP and cornichon proteins. Neuron 68:1082-1096.

Katoh-Semba R, Takeuchi IK, Semba R, Kato K (1997) Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. J Neurochem 69:34-42.

Kaufman J, Yang BZ, Douglas-Palumberi H, Grasso D, Lipschitz D, Houshyar S, Krystal JH, Gelernter J (2006) Brain-derived neurotrophic factor-5-HTTLPR gene interactions and environmental modifiers of depression in children. Biol Psychiatry 59:673-680.

Kawahara N, Croll SD, Wiegand SJ, Klatzo I (1997) Cortical spreading depression induces long-term alterations of BDNF levels in cortex and hippocampus distinct from lesion effects: implications for ischemic tolerance. Neurosci Res 29:37-47.

Kawamoto Y, Nakamura S, Nakano S, Oka N, Akiguchi I, Kimura J (1996) Immunohistochemical localization of brain-derived neurotrophic factor in adult rat brain. Neuroscience 74:1209-1226.

Kawashima H, Numakawa T, Kumamaru E, Adachi N, Mizuno H, Ninomiya M, Kunugi H, Hashido K (2010) Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression. Neuroscience 165:1301-1311.

Kelleher RJ, 3rd, Govindarajan A, Tonegawa S (2004a) Translational regulatory mechanisms in persistent forms of synaptic plasticity. Neuron 44:59-73.

Kelleher RJ, 3rd, Govindarajan A, Jung HY, Kang H, Tonegawa S (2004b) Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell 116:467-479.

Kells AP, Fong DM, Dragunow M, During MJ, Young D, Connor B (2004) AAV-mediated gene delivery of BDNF or GDNF is neuroprotective in a model of Huntington disease. Mol Ther 9:682-688.

Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, Hempstead BL, Carter BD (2006) Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50:219-232.

Kenchappa RS, Tep C, Korade Z, Urra S, Bronfman FC, Yoon SO, Carter BD (2010) p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-alpha-converting enzyme/ADAM17. J Biol Chem 285:20358-20368.

Khursigara G, Orlinick JR, Chao MV (1999) Association of the p75 neurotrophin receptor with TRAF6. J Biol Chem 274:2597-2600.

Khursigara G, Bertin J, Yano H, Moffett H, DiStefano PS, Chao MV (2001) A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. J Neurosci 21:5854-5863.

Khwaja F, Tabassum A, Allen J, Djakiew D (2006) The p75(NTR) tumor suppressor induces cell cycle arrest facilitating caspase mediated apoptosis in prostate tumor cells. Biochem Biophys Res Commun 341:1184-1192.

Kidane AH, Heinrich G, Dirks RP, de Ruyck BA, Lubsen NH, Roubos EW, Jenks BG (2009) Differential neuroendocrine expression of multiple brain-derived neurotrophic factor transcripts. Endocrinology 150:1361-1368.

Kim S, Chang R, Teunissen C, Gebremichael Y, Petzold A (2011) Neurofilament stoichiometry simulations during neurodegeneration suggest a remarkable self-sufficient and stable in vivo protein structure. J Neurol Sci 307:132-138.

Kingwell K (2010) Neurodegenerative disease: BDNF copycats. Nat Rev Drug Discov 9:433.

Kinoshita S, Yasuda H, Taniguchi N, Katoh-Semba R, Hatanaka H, Tsumoto T (1999) Brain-derived neurotrophic factor prevents low-frequency inputs from inducing long-term depression in the developing visual cortex. J Neurosci 19:2122-2130.

Kiselyov K, Xu X, Mozhayeva G, Kuo T, Pessah I, Mignery G, Zhu X, Birnbaumer L, Muallem S (1998) Functional interaction between InsP3 receptors and store-operated Htrp3 channels. Nature 396:478-482.

Klambt C, Glazer L, Shilo BZ (1992) breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. Genes Dev 6:1668-1678.

Klann E, Dever TE (2004) Biochemical mechanisms for translational regulation in synaptic plasticity. Nat Rev Neurosci 5:931-942.

Klein M, Hempstead BL, Teng KK (2005) Activation of STAT5-dependent transcription by the neurotrophin receptor Trk. J Neurobiol 63:159-171.

Klein ME, Lioy DT, Ma L, Impey S, Mandel G, Goodman RH (2007) Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci 10:1513-1514.

Klein R, Martin-Zanca D, Barbacid M, Parada LF (1990a) Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. Development 109:845-850.

Klein R, Conway D, Parada LF, Barbacid M (1990b) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647-656.

Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Klocker N, Kermer P, Weishaupt JH, Labes M, Ankerhold R, Bahr M (2000) Brain-derived neurotrophic factor-mediated neuroprotection of adult rat retinal ganglion cells in vivo does not exclusively depend on phosphatidyl-inositol-3'-kinase/protein kinase B signaling. J Neurosci 20:6962-6967.

Knusel B, Winslow JW, Rosenthal A, Burton LE, Seid DP, Nikolics K, Hefti F (1991) Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci U S A 88:961-965.

Kohara K, Kitamura A, Morishima M, Tsumoto T (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. Science 291:2419-2423.

Kohara K, Kitamura A, Adachi N, Nishida M, Itami C, Nakamura S, Tsumoto T (2003) Inhibitory but not excitatory cortical neurons require presynaptic brain-derived neurotrophic factor for dendritic development, as revealed by chimera cell culture. J Neurosci 23:6123-6131.

Kojima M, Takei N, Numakawa T, Ishikawa Y, Suzuki S, Matsumoto T, Katoh-Semba R, Nawa H, Hatanaka H (2001) Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J Neurosci Res 64:1-10.

Kokaia Z, Gido G, Ringstedt T, Bengzon J, Kokaia M, Siesjo BK, Persson H, Lindvall O (1993) Rapid increase of BDNF mRNA levels in cortical neurons

following spreading depression: regulation by glutamatergic mechanisms independent of seizure activity. Brain Res Mol Brain Res 19:277-286.

Kommaddi RP, Thomas R, Ceni C, Daigneault K, Barker PA (2011) Trk-dependent ADAM17 activation facilitates neurotrophin survival signaling. FASEB J 25:2061-2070.

Koppel I, Aid-Pavlidis T, Jaanson K, Sepp M, Palm K, Timmusk T (2010) BAC transgenic mice reveal distal cis-regulatory elements governing BDNF gene expression. Genesis 48:214-219.

Koppel I, Aid-Pavlidis T, Jaanson K, Sepp M, Pruunsild P, Palm K, Timmusk T (2009) Tissue-specific and neural activity-regulated expression of human BDNF gene in BAC transgenic mice. BMC Neurosci 10:68.

Korte M, Staiger V, Griesbeck O, Thoenen H, Bonhoeffer T (1996) The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. J Physiol Paris 90:157-164.

Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 92:8856-8860.

Kovalchuk Y, Hanse E, Kafitz KW, Konnerth A (2002) Postsynaptic Induction of BDNF-Mediated Long-Term Potentiation. Science 295:1729-1734.

Kramer ML, Schulz-Schaeffer WJ (2007) Presynaptic alpha-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with Lewy bodies. J Neurosci 27:1405-1410.

Krebs MO, Guillin O, Bourdell MC, Schwartz JC, Olie JP, Poirier MF, Sokoloff P (2000) Brain derived neurotrophic factor (BDNF) gene variants association with age at onset and therapeutic response in schizophrenia. Mol Psychiatry 5:558-562.

Krishna S, Maduzia LL, Padgett RW (1999) Specificity of TGFbeta signaling is conferred by distinct type I receptors and their associated SMAD proteins in Caenorhabditis elegans. Development 126:251-260.

Kryl D, Yacoubian T, Haapasalo A, Castren E, Lo D, Barker PA (1999) Subcellular localization of full-length and truncated Trk receptor isoforms in polarized neurons and epithelial cells. J Neurosci 19:5823-5833.

Kuczewski N, Porcher C, Lessmann V, Medina I, Gaiarsa JL (2008) Back-propagating action potential: A key contributor in activity-dependent dendritic release of BDNF. Commun Integr Biol 1:153-155.

Kuczewski N, Porcher C, Lessmann V, Medina I, Gaiarsa JL (2009) Activity-dependent dendritic release of BDNF and biological consequences. Mol Neurobiol 39:37-49.

Kullander K, Carlson B, Hallbook F (1997a) Molecular phylogeny and evolution of the neurotrophins from monotremes and marsupials. J Mol Evol 45:311-321.

Kullander K, Kaplan D, Ebendal T (1997b) Two restricted sites on the surface of the nerve growth factor molecule independently determine specific TrkA receptor binding and activation. J Biol Chem 272:9300-9307.

Kunugi H, Ueki A, Otsuka M, Isse K, Hirasawa H, Kato N, Nabika T, Kobayashi S, Nanko S (2001) A novel polymorphism of the brain-derived neurotrophic factor (BDNF) gene associated with late-onset Alzheimer's disease. Mol Psychiatry 6:83-86.

Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Kobune M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K, Honmou O, Houkin K, Date I, Hamada H (2004) BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. Mol Ther 9:189-197.

Kust BM, Copray JC, Brouwer N, Troost D, Boddeke HW (2002) Elevated levels of neurotrophins in human biceps brachii tissue of amyotrophic lateral sclerosis. Exp Neurol 177:419-427.

Kuzumaki N, Ikegami D, Tamura R, Hareyama N, Imai S, Narita M, Torigoe K, Niikura K, Takeshima H, Ando T, Igarashi K, Kanno J, Ushijima T, Suzuki T (2011) Hippocampal epigenetic modification at the brain-derived neurotrophic factor gene induced by an enriched environment. Hippocampus 21:127-132.

Kwon M, Fernandez JR, Zegarek GF, Lo SB, Firestein BL (2011) BDNF-promoted increases in proximal dendrites occur via CREB-dependent transcriptional regulation of cypin. J Neurosci 31:9735-9745.

Lai C, Xie C, McCormack SG, Chiang HC, Michalak MK, Lin X, Chandran J, Shim H, Shimoji M, Cookson MR, Huganir RL, Rothstein JD, Price DL, Wong PC, Martin LJ, Zhu JJ, Cai H (2006) Amyotrophic lateral sclerosis 2-deficiency leads to neuronal degeneration in amyotrophic lateral sclerosis through altered AMPA receptor trafficking. J Neurosci 26:11798-11806.

Lamballe F, Klein R, Barbacid M (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Lang SB, Bonhoeffer T, Lohmann C (2006) Simultaneous imaging of morphological plasticity and calcium dynamics in dendrites. Nat Protoc 1:1859-1864.

Lang SB, Stein V, Bonhoeffer T, Lohmann C (2007) Endogenous brain-derived neurotrophic factor triggers fast calcium transients at synapses in developing dendrites. J Neurosci 27:1097-1105.

Lang UE, Hellweg R, Kalus P, Bajbouj M, Lenzen KP, Sander T, Kunz D, Gallinat J (2005) Association of a functional BDNF polymorphism and anxiety-related personality traits. Psychopharmacology (Berl) 180:95-99.

Lau A, Tymianski M (2010) Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch 460:525-542.

Lau AG, Irier HA, Gu J, Tian D, Ku L, Liu G, Xia M, Fritsch B, Zheng JQ, Dingledine R, Xu B, Lu B, Feng Y (2010) Distinct 3'UTRs differentially regulate activity-dependent translation of brain-derived neurotrophic factor (BDNF). Proc Natl Acad Sci U S A 107:15945-15950.

Lauri SE, Palmer M, Segerstrale M, Vesikansa A, Taira T, Collingridge GL (2007) Presynaptic mechanisms involved in the expression of STP and LTP at CA1 synapses in the hippocampus. Neuropharmacology 52:1-11.

Lauterborn JC, Lynch G, Vanderklish P, Arai A, Gall CM (2000) Positive modulation of AMPA receptors increases neurotrophin expression by hippocampal and cortical neurons. J Neurosci 20:8-21.

Lee FS, Kim AH, Khursigara G, Chao MV (2001a) The uniqueness of being a neurotrophin receptor. Curr Opin Neurobiol 11:281-286.

Lee HJ, Lim IJ, Lee MC, Kim SU (2010) Human neural stem cells genetically modified to overexpress brain-derived neurotrophic factor promote functional recovery and neuroprotection in a mouse stroke model. J Neurosci Res 88:3282-3294.

Lee J, Duan W, Long JM, Ingram DK, Mattson MP (2000) Dietary restriction increases the number of newly generated neural cells, and induces BDNF expression, in the dentate gyrus of rats. J Mol Neurosci 15:99-108.

Lee JL, Everitt BJ, Thomas KL (2004) Independent cellular processes for hippocampal memory consolidation and reconsolidation. Science 304:839-843.

Lee R, Kermani P, Teng KK, Hempstead BL (2001b) Regulation of cell survival by secreted proneurotrophins. Science 294:1945-1948.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde YA (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149-152.

Lein ES, Shatz CJ (2000) Rapid regulation of brain-derived neurotrophic factor mRNA within eye-specific circuits during ocular dominance column formation. J Neurosci 20:1470-1483.

Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. Nature 440:352-357.

Lessmann V, Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res 65:11-22.

Levine ES, Dreyfus CF, Black IB, Plummer MR (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc Natl Acad Sci U S A 92:8074-8077.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115:787-798.

Lewis ML, Reynolds JL, Cubano LA, Hatton JP, Lawless BD, Piepmeier EH (1998) Spaceflight alters microtubules and increases apoptosis in human lymphocytes (Jurkat). FASEB J 12:1007-1018.

Li H, Waites CL, Staal RG, Dobryy Y, Park J, Sulzer DL, Edwards RH (2005a) Sorting of vesicular monoamine transporter 2 to the regulated secretory pathway confers the somatodendritic exocytosis of monoamines. Neuron 48:619-633.

Li HS, Xu XZ, Montell C (1999) Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. Neuron 24:261-273.

Li J, Horak KM, Su H, Sanbe A, Robbins J, Wang X (2011a) Enhancement of proteasomal function protects against cardiac proteinopathy and ischemia/reperfusion injury in mice. J Clin Invest 121:3689-3700.

Li M, Dai FR, Du XP, Yang QD, Zhang X, Chen Y (2012) Infusion of BDNF into the nucleus accumbens of aged rats improves cognition and structural synaptic plasticity through PI3K-ILK-Akt signaling. Behav Brain Res 231:146-153.

Li S, Stys PK (2001) Na(+)-K(+)-ATPase inhibition and depolarization induce glutamate release via reverse Na(+)-dependent transport in spinal cord white matter. Neuroscience 107:675-683.

Li W, Zheng Z, Keifer J (2011b) Transsynaptic EphB/Ephrin-B signaling regulates growth of presynaptic boutons required for classical conditioning. J Neurosci 31:8441-8449.

Li Y, Calfa G, Inoue T, Amaral MD, Pozzo-Miller L (2010) Activity-dependent release of endogenous BDNF from mossy fibers evokes a TRPC3 current and Ca2+ elevations in CA3 pyramidal neurons. J Neurophysiol 103:2846-2856.

Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ, Yuan XB (2005b) Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 434:894-898.

Li YX, Zhang Y, Lester HA, Schuman EM, Davidson N (1998) Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. J Neurosci 18:10231-10240.

Liang FQ, Walline R, Earnest DJ (1998) Circadian rhythm of brain-derived neurotrophic factor in the rat suprachiasmatic nucleus. Neurosci Lett 242:89-92.

Liao GY, An JJ, Gharami K, Waterhouse EG, Vanevski F, Jones KR, Xu B (2012) Dendritically targeted Bdnf mRNA is essential for energy balance and response to leptin. Nat Med 18:564-571.

Liepinsh E, Ilag LL, Otting G, Ibanez CF (1997) NMR structure of the death domain of the p75 neurotrophin receptor. EMBO J 16:4999-5005.

Lindefors N, Ballarin M, Ernfors P, Falkenberg T, Persson H (1992) Stimulation of glutamate receptors increases expression of brain-derived neurotrophic factor mRNA in rat hippocampus. Ann N Y Acad Sci 648:296-299.

Lindholm D, da Penha Berzaghi M, Cooper J, Thoenen H, Castren E (1994) Brain-derived neurotrophic factor and neurotrophin-4 increase neurotrophin-3 expression in the rat hippocampus. Int J Dev Neurosci 12:745-751.

Lindsay RM (1996) Therapeutic potential of the neurotrophins and neurotrophin-CNTF combinations in peripheral neuropathies and motor neuron diseases. Ciba Found Symp 196:39-48; discussion 48-53.

Lindsay RM, Yancopoulos GD (1996) GDNF in a bind with known orphan: accessory implicated in new twist. Neuron 17:571-574.

Lindsay RM, Thoenen H, Barde YA (1985) Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. Dev Biol 112:319-328.

Lindskog M, Li L, Groth RD, Poburko D, Thiagarajan TC, Han X, Tsien RW (2010) Postsynaptic GluA1 enables acute retrograde enhancement of presynaptic function to coordinate adaptation to synaptic inactivity. Proc Natl Acad Sci U S A 107:21806-21811.

Lindvall O, Ernfors P, Bengzon J, Kokaia Z, Smith ML, Siesjo BK, Persson H (1992) Differential regulation of mRNAs for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma. Proc Natl Acad Sci U S A 89:648-652.

Linggi MS, Burke TL, Williams BB, Harrington A, Kraemer R, Hempstead BL, Yoon SO, Carter BD (2005) Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. J Biol Chem 280:13801-13808.

Linnarsson S, Bjorklund A, Ernfors P (1997) Learning deficit in BDNF mutant mice. Eur J Neurosci 9:2581-2587.

Lipsky RH, Marini AM (2007) Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann N Y Acad Sci 1122:130-143.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613-622.

Liu HL, Zhao G, Cai K, Zhao HH, Shi LD (2011) Treadmill exercise prevents decline in spatial learning and memory in APP/PS1 transgenic mice through improvement of hippocampal long-term potentiation. Behav Brain Res 218:308-314.

Liu IY, Lyons WE, Mamounas LA, Thompson RF (2004) Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. J Neurosci 24:7958-7963.

Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR (2006) Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. Brain Res 1067:1-12.

Liu QR, Walther D, Drgon T, Polesskaya O, Lesnick TG, Strain KJ, de Andrade M, Bower JH, Maraganore DM, Uhl GR (2005) Human brain derived neurotrophic factor (BDNF) genes, splicing patterns, and assessments of associations with substance abuse and Parkinson's Disease. Am J Med Genet B Neuropsychiatr Genet 134B:93-103.

Liu X, Hashimoto M, Horii H, Yamaguchi A, Naito K, Yamashita T (2009a) Repulsive guidance molecule b inhibits neurite growth and is increased after spinal cord injury. Biochem Biophys Res Commun 382:795-800.

Liu X, Grishanin RN, Tolwani RJ, Renteria RC, Xu B, Reichardt LF, Copenhagen DR (2007) Brain-derived neurotrophic factor and TrkB modulate visual experience-dependent refinement of neuronal pathways in retina. J Neurosci 27:7256-7267.

Liu X, Robinson ML, Schreiber AM, Wu V, Lavail MM, Cang J, Copenhagen DR (2009b) Regulation of neonatal development of retinal ganglion cell dendrites by neurotrophin-3 overexpression. J Comp Neurol 514:449-458.

Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104:487-501.

Lodovichi C, Berardi N, Pizzorusso T, Maffei L (2000) Effects of neurotrophins on cortical plasticity: same or different? J Neurosci 20:2155-2165.

Lohof AM, Ip NY, Poo MM (1993) Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363:350-353.

Lom B, Cogen J, Sanchez AL, Vu T, Cohen-Cory S (2002) Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci 22:7639-7649.

Lou H, Kim SK, Zaitsev E, Snell CR, Lu B, Loh YP (2005) Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase e. Neuron 45:245-255.

Lu B (2003) BDNF and activity-dependent synaptic modulation. Learn Mem 10:86-98.

Lu C, Fu W, Zhao D, Mattson MP (2002) The DNA damaging agent etoposide activates a cell survival pathway involving alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors and mitogen-activated protein kinases in hippocampal neurons. J Neurosci Res 70:671-679.

Lu H, Cheng PL, Lim BK, Khoshnevisrad N, Poo MM (2010) Elevated BDNF after cocaine withdrawal facilitates LTP in medial prefrontal cortex by suppressing GABA inhibition. Neuron 67:821-833.

Lu Y, Christian K, Lu B (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol Learn Mem 89:312-323.

Lubin FD, Ren Y, Xu X, Anderson AE (2007) Nuclear factor-kappa B regulates seizure threshold and gene transcription following convulsant stimulation. J Neurochem 103:1381-1395.

Lucidi-Phillipi CA, Gage FH (1993) The neurotrophic hypothesis and the cholinergic basal forebrain projection. Prog Brain Res 98:241-249.

Luetjens CM, Bui NT, Sengpiel B, Munstermann G, Poppe M, Krohn AJ, Bauerbach E, Krieglstein J, Prehn JH (2000) Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. J Neurosci 20:5715-5723.

Luikart BW, Zhang W, Wayman GA, Kwon CH, Westbrook GL, Parada LF (2008) Neurotrophin-dependent dendritic filopodial motility: a convergence on PI3K signaling. J Neurosci 28:7006-7012.

Luikart BW, Nef S, Virmani T, Lush ME, Liu Y, Kavalali ET, Parada LF (2005) TrkB has a cell-autonomous role in the establishment of hippocampal Schaffer collateral synapses. J Neurosci 25:3774-3786.

Luo L (2000) Rho GTPases in neuronal morphogenesis. Nat Rev Neurosci 1:173-180.

Lynch G (2004) AMPA receptor modulators as cognitive enhancers. Curr Opin Pharmacol 4:4-11.

Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, Wihler C, Koliatsos VE, Tessarollo L (1999) Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. Proc Natl Acad Sci U S A 96:15239-15244.

Ma GQ, Wang B, Wang HB, Wang Q, Bao L (2008) Short elements with charged amino acids form clusters to sort protachykinin into large dense-core vesicles. Traffic 9:2165-2179.

Ma HT, Patterson RL, van Rossum DB, Birnbaumer L, Mikoshiba K, Gill DL (2000) Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels. Science 287:1647-1651.

MacPhee IJ, Barker PA (1997) Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduces TrkA signaling while increasing serine phosphorylation in the TrkA intracellular domain. J Biol Chem 272:23547-23551.

Magby JP, Bi C, Chen ZY, Lee FS, Plummer MR (2006) Single-cell characterization of retrograde signaling by brain-derived neurotrophic factor. J Neurosci 26:13531-13536.

Mai J, Fok L, Gao H, Zhang X, Poo MM (2009) Axon initiation and growth cone turning on bound protein gradients. J Neurosci 29:7450-7458.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Maisonpierre PC, Le Beau MM, Espinosa R, 3rd, Ip NY, Belluscio L, de la Monte SM, Squinto S, Furth ME, Yancopoulos GD (1991) Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics 10:558-568.

Makar TK, Bever CT, Singh IS, Royal W, Sahu SN, Sura TP, Sultana S, Sura KT, Patel N, Dhib-Jalbut S, Trisler D (2009) Brain-derived neurotrophic factor gene delivery in an animal model of multiple sclerosis using bone marrow stem cells as a vehicle. J Neuroimmunol 210:40-51.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Malinow R (2003) AMPA receptor trafficking and long-term potentiation. Philos Trans R Soc Lond B Biol Sci 358:707-714.

Mallucci GR, White MD, Farmer M, Dickinson A, Khatun H, Powell AD, Brandner S, Jefferys JG, Collinge J (2007) Targeting cellular prion protein reverses early cognitive deficits and neurophysiological dysfunction in prion-infected mice. Neuron 53:325-335.

Mantych KB, Ferreira A (2001) Agrin differentially regulates the rates of axonal and dendritic elongation in cultured hippocampal neurons. J Neurosci 21:6802-6809.

Mao QQ, Ip SP, Ko KM, Tsai SH, Che CT (2009) Peony glycosides produce antidepressant-like action in mice exposed to chronic unpredictable mild stress: effects on hypothalamic-pituitary-adrenal function and brain-derived neurotrophic factor. Prog Neuropsychopharmacol Biol Psychiatry 33:1211-1216.

Marler KJ, Becker-Barroso E, Martinez A, Llovera M, Wentzel C, Poopalasundaram S, Hindges R, Soriano E, Comella J, Drescher U (2008) A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis. J Neurosci 28:12700-12712.

Marsh HN, Palfrey HC (1996) Neurotrophin-3 and brain-derived neurotrophic factor activate multiple signal transduction events but are not survival factors for hippocampal pyramidal neurons. J Neurochem 67:952-963.

Martin KC, Kosik KS (2002) Synaptic tagging -- who's it? Nat Rev Neurosci 3:813-820.

Martin KR, Quigley HA, Zack DJ, Levkovitch-Verbin H, Kielczewski J, Valenta D, Baumrind L, Pease ME, Klein RL, Hauswirth WW (2003) Gene therapy with brain-derived neurotrophic factor as a protection: retinal ganglion cells in a rat glaucoma model. Invest Ophthalmol Vis Sci 44:4357-4365.

Martinez A, Alcantara S, Borrell V, Del Rio JA, Blasi J, Otal R, Campos N, Boronat A, Barbacid M, Silos-Santiago I, Soriano E (1998) TrkB and TrkC signaling are required for maturation and synaptogenesis of hippocampal connections. J Neurosci 18:7336-7350.

Martinowich K, Manji H, Lu B (2007) New insights into BDNF function in depression and anxiety. Nat Neurosci 10:1089-1093.

Massa SM, Yang T, Xie Y, Shi J, Bilgen M, Joyce JN, Nehama D, Rajadas J, Longo FM (2010) Small molecule BDNF mimetics activate TrkB signaling and prevent neuronal degeneration in rodents. J Clin Invest 120:1774-1785.

Matsuda N, Lu H, Fukata Y, Noritake J, Gao H, Mukherjee S, Nemoto T, Fukata M, Poo MM (2009) Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J Neurosci 29:14185-14198.

Matsumoto M, Kato K (2001) Altered calcium dynamics in cultured cerebellar cells from IP3R1-deficient mice. Neuroreport 12:3471-3474.

Mazzucchelli C, Vantaggiato C, Ciamei A, Fasano S, Pakhotin P, Krezel W, Welzl H, Wolfer DP, Pages G, Valverde O, Marowsky A, Porrazzo A, Orban PC, Maldonado R, Ehrengruber MU, Cestari V, Lipp HP, Chapman PF, Pouyssegur J, Brambilla R (2002) Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. Neuron 34:807-820.

McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15:791-803.

McAllister AK, Katz LC, Lo DC (1996) Neurotrophin regulation of cortical dendritic growth requires activity. Neuron 17:1057-1064.

McAllister AK, Katz LC, Lo DC (1997) Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. Neuron 18:767-778.

McDonald NQ, Hendrickson WA (1993) A structural superfamily of growth factors containing a cystine knot motif. Cell 73:421-424.

McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL (1991) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature 354:411-414.

McGeer EG, McGeer PL (1976) Duplication of biochemical changes of Huntington's chorea by intrastriatal injections of glutamic and kainic acids. Nature 263:517-519.

McQuillen PS, DeFreitas MF, Zada G, Shatz CJ (2002) A novel role for p75NTR in subplate growth cone complexity and visual thalamocortical innervation. J Neurosci 22:3580-3593.

Meakin SO, Shooter EM (1992) The nerve growth factor family of receptors. Trends Neurosci 15:323-331.

Mei F, Nagappan G, Ke Y, Sacktor TC, Lu B (2011) BDNF facilitates L-LTP maintenance in the absence of protein synthesis through PKMzeta. PLoS One 6:e21568.

Meller R, Cameron JA, Torrey DJ, Clayton CE, Ordonez AN, Henshall DC, Minami M, Schindler CK, Saugstad JA, Simon RP (2006) Rapid degradation of Bim by the ubiquitin-proteasome pathway mediates short-term ischemic tolerance in cultured neurons. J Biol Chem 281:7429-7436.

Mellios N, Huang HS, Baker SP, Galdzicka M, Ginns E, Akbarian S (2009) Molecular determinants of dysregulated GABAergic gene expression in the prefrontal cortex of subjects with schizophrenia. Biol Psychiatry 65:1006-1014.

Menard C, Hein P, Paquin A, Savelson A, Yang XM, Lederfein D, Barnabe-Heider F, Mir AA, Sterneck E, Peterson AC, Johnson PF, Vinson C, Miller FD (2002) An essential role for a MEK-C/EBP pathway during growth factor-regulated cortical neurogenesis. Neuron 36:597-610.

Menna E, Disanza A, Cagnoli C, Schenk U, Gelsomino G, Frittoli E, Hertzog M, Offenhauser N, Sawallisch C, Kreienkamp HJ, Gertler FB, Di Fiore PP, Scita G, Matteoli M (2009) Eps8 regulates axonal filopodia in hippocampal neurons in response to brain-derived neurotrophic factor (BDNF). PLoS Biol 7:e1000138.

Messaoudi E, Bardsen K, Srebro B, Bramham CR (1998) Acute intrahippocampal infusion of BDNF induces lasting potentiation of synaptic transmission in the rat dentate gyrus. J Neurophysiol 79:496-499.

Messaoudi E, Kanhema T, Soule J, Tiron A, Dagyte G, da Silva B, Bramham CR (2007) Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. J Neurosci 27:10445-10455.

Metsis M, Timmusk T, Arenas E, Persson H (1993) Differential usage of multiple brain-derived neurotrophic factor promoters in the rat brain following neuronal activation. Proc Natl Acad Sci U S A 90:8802-8806.

Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB (2004) LINGO-1 is

a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 7:221-228.

Miao N, Wang M, Ott JA, D'Alessandro JS, Woolf TM, Bumcrot DA, Mahanthappa NK, Pang K (1997) Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult In vitro. J Neurosci 17:5891-5899.

Middlemas DS, Lindberg RA, Hunter T (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 11:143-153.

Middleton G, Hamanoue M, Enokido Y, Wyatt S, Pennica D, Jaffray E, Hay RT, Davies AM (2000) Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons. J Cell Biol 148:325-332.

Miguel-Hidalgo JJ, Alvarez XA, Cacabelos R, Quack G (2002) Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1-40). Brain Res 958:210-221.

Miller FD, Gauthier AS (2007) Timing is everything: making neurons versus glia in the developing cortex. Neuron 54:357-369.

Miller S, Yasuda M, Coats JK, Jones Y, Martone ME, Mayford M (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron 36:507-519.

Mills J, Digicaylioglu M, Legg AT, Young CE, Young SS, Barr AM, Fletcher L, O'Connor TP, Dedhar S (2003) Role of integrin-linked kinase in nerve growth factor-stimulated neurite outgrowth. J Neurosci 23:1638-1648.

Minichiello L (2009) TrkB signalling pathways in LTP and learning. Nat Rev Neurosci 10:850-860.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36:121-137.

Minichiello L, Casagranda F, Tatche RS, Stucky CL, Postigo A, Lewin GR, Davies AM, Klein R (1998) Point mutation in trkB causes loss of NT4-dependent neurons without major effects on diverse BDNF responses. Neuron 21:335-345.

Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T, Klein R (1999) Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24:401-414.

Miura P, Amirouche A, Clow C, Belanger G, Jasmin BJ (2012) Brain-derived neurotrophic factor expression is repressed during myogenic differentiation by miR-206. J Neurochem 120:230-238.

Miyake K, Yamamoto W, Tadokoro M, Takagi N, Sasakawa K, Nitta A, Furukawa S, Takeo S (2002) Alterations in hippocampal GAP-43, BDNF, and L1 following sustained cerebral ischemia. Brain Res 935:24-31.

Mizoguchi H, Takuma K, Fukuzaki E, Ibi D, Someya E, Akazawa KH, Alkam T, Tsunekawa H, Mouri A, Noda Y, Nabeshima T, Yamada K (2009) Matrix

metalloprotease-9 inhibition improves amyloid beta-mediated cognitive impairment and neurotoxicity in mice. J Pharmacol Exp Ther 331:14-22.

Mizuno N, Kitayama S, Saishin Y, Shimada S, Morita K, Mitsuhata C, Kurihara H, Dohi T (1999) Molecular cloning and characterization of rat trp homologues from brain. Brain Res Mol Brain Res 64:41-51.

Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, Ichinose H, Nagatsu T (1999) Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. Neurosci Lett 270:45-48.

Mohajerani MH, Sivakumaran S, Zacchi P, Aguilera P, Cherubini E (2007) Correlated network activity enhances synaptic efficacy via BDNF and the ERK pathway at immature CA3 CA1 connections in the hippocampus. Proc Natl Acad Sci U S A 104:13176-13181.

Molz S, Decker H, Dal-Cim T, Cremonez C, Cordova FM, Leal RB, Tasca CI (2008) Glutamate-induced toxicity in hippocampal slices involves apoptotic features and p38 MAPK signaling. Neurochem Res 33:27-36.

Monnerie H, Le Roux PD (2008) Glutamate alteration of glutamic acid decarboxylase (GAD) in GABAergic neurons: the role of cysteine proteases. Exp Neurol 213:145-153.

Montag C, Weber B, Fliessbach K, Elger C, Reuter M (2009) The BDNF Val66Met polymorphism impacts parahippocampal and amygdala volume in healthy humans: incremental support for a genetic risk factor for depression. Psychol Med 39:1831-1839.

Monteleone P, Maj M (2013) Dysfunctions of leptin, ghrelin, BDNF and endocannabinoids in eating disorders: beyond the homeostatic control of food intake. Psychoneuroendocrinology 38:312-330.

Montell C, Birnbaumer L, Flockerzi V (2002) The TRP channels, a remarkably functional family. Cell 108:595-598.

Montell C, Jones K, Hafen E, Rubin G (1985) Rescue of the Drosophila phototransduction mutation trp by germline transformation. Science 230:1040-1043.

Morimoto K, Sato K, Sato S, Yamada N, Hayabara T (1998) Time-dependent changes in neurotrophic factor mRNA expression after kindling and long-term potentiation in rats. Brain Res Bull 45:599-605.

Morse JK, Wiegand SJ, Anderson K, You Y, Cai N, Carnahan J, Miller J, DiStefano PS, Altar CA, Lindsay RM, et al. (1993) Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. J Neurosci 13:4146-4156.

Motil J, Chan WK, Dubey M, Chaudhury P, Pimenta A, Chylinski TM, Ortiz DT, Shea TB (2006) Dynein mediates retrograde neurofilament transport within axons and anterograde delivery of NFs from perikarya into axons: regulation by multiple phosphorylation events. Cell Motil Cytoskeleton 63:266-286.

Mowla SJ, Farhadi HF, Pareek S, Atwal JK, Morris SJ, Seidah NG, Murphy RA (2001) Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J Biol Chem 276:12660-12666.

Mowla SJ, Pareek S, Farhadi HF, Petrecca K, Fawcett JP, Seidah NG, Morris SJ, Sossin WS, Murphy RA (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 19:2069-2080.

Mulcahy LR, Nillni EA (2007) Discovery of new peptides from old prohormones: insights for energy balance and beyond. Front Biosci 12:3545-3553.

Muller F, Bonigk W, Sesti F, Frings S (1998a) Phosphorylation of mammalian olfactory cyclic nucleotide-gated channels increases ligand sensitivity. J Neurosci 18:164-173.

Muller G, Storz P, Bourteele S, Doppler H, Pfizenmaier K, Mischak H, Philipp A, Kaiser C, Kolch W (1998b) Regulation of Raf-1 kinase by TNF via its second messenger ceramide and cross-talk with mitogenic signalling. EMBO J 17:732-742.

Murray PS, Holmes PV (2011) An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus. Int J Pept 2011:654085.

Mustafa AK, Ahmad AS, Zeynalov E, Gazi SK, Sikka G, Ehmsen JT, Barrow RK, Coyle JT, Snyder SH, Dore S (2010) Serine racemase deletion protects against cerebral ischemia and excitotoxicity. J Neurosci 30:1413-1416.

Mutoh T, Sobue G, Hamano T, Kuriyama M, Hirayama M, Yamamoto M, Mitsuma T (2000) Decreased phosphorylation levels of TrkB neurotrophin receptor in the spinal cords from patients with amyotrophic lateral sclerosis. Neurochem Res 25:239-245.

Myers EW et al. (2000) A whole-genome assembly of Drosophila. Science 287:2196-2204.

Nagahara AH, Tuszynski MH (2011) Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat Rev Drug Discov 10:209-219.

Nagahara AH, Merrill DA, Coppola G, Tsukada S, Schroeder BE, Shaked GM, Wang L, Blesch A, Kim A, Conner JM, Rockenstein E, Chao MV, Koo EH, Geschwind D, Masliah E, Chiba AA, Tuszynski MH (2009) Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. Nat Med 15:331-337.

Nagappan G, Lu B (2005) Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. Trends Neurosci 28:464-471.

Nagappan G, Zaitsev E, Senatorov VV, Jr., Yang J, Hempstead BL, Lu B (2009) Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. Proc Natl Acad Sci U S A 106:1267-1272.

Nakata H, Nakamura S (2007) Brain-derived neurotrophic factor regulates AMPA receptor trafficking to post-synaptic densities via IP3R and TRPC calcium signaling. FEBS Lett 581:2047-2054.

Namekata K, Harada C, Taya C, Guo X, Kimura H, Parada LF, Harada T (2010) Dock3 induces axonal outgrowth by stimulating membrane recruitment of the WAVE complex. Proc Natl Acad Sci U S A 107:7586-7591.

Namekata K, Harada C, Guo X, Kimura A, Kittaka D, Watanabe H, Harada T (2012) Dock3 stimulates axonal outgrowth via GSK-3beta-mediated microtubule assembly. J Neurosci 32:264-274.

Nanda SA, Mack KJ (2000) Seizures and sensory stimulation result in different patterns of brain derived neurotrophic factor protein expression in the barrel cortex and hippocampus. Brain Res Mol Brain Res 78:1-14.

Naska S, Lin DC, Miller FD, Kaplan DR (2010) p75NTR is an obligate signaling receptor required for cues that cause sympathetic neuron growth cone collapse. Mol Cell Neurosci 45:108-120.

Naylor RL, Robertson AG, Allen SJ, Sessions RB, Clarke AR, Mason GG, Burston JJ, Tyler SJ, Wilcock GK, Dawbarn D (2002) A discrete domain of the human TrkB receptor defines the binding sites for BDNF and NT-4. Biochem Biophys Res Commun 291:501-507.

Neasta J, Kiely PA, He DY, Adams DR, O'Connor R, Ron D (2012) Direct interaction between scaffolding proteins RACK1 and 14-3-3zeta regulates brain-derived neurotrophic factor (BDNF) transcription. J Biol Chem 287:322-336.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman C (1995) Exercise and brain neurotrophins. Nature 373:109.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman CW (1996) Physical activity increases mRNA for brain-derived neurotrophic factor and nerve growth factor in rat brain. Brain Res 726:49-56.

Neves-Pereira M, Mundo E, Muglia P, King N, Macciardi F, Kennedy JL (2002) The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: evidence from a family-based association study. Am J Hum Genet 71:651-655.

Ng BK, Chen L, Mandemakers W, Cosgaya JM, Chan JR (2007) Anterograde transport and secretion of brain-derived neurotrophic factor along sensory axons promote Schwann cell myelination. J Neurosci 27:7597-7603.

Nguyen PV, Abel T, Kandel ER (1994) Requirement of a critical period of transcription for induction of a late phase of LTP. Science 265:1104-1107.

Niewiadomska G, Mietelska-Porowska A, Mazurkiewicz M (2011) The cholinergic system, nerve growth factor and the cytoskeleton. Behav Brain Res 221:515-526.

Nilsson AS, Fainzilber M, Falck P, Ibanez CF (1998) Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. FEBS Lett 424:285-290.

Ninan I, Bath KG, Dagar K, Perez-Castro R, Plummer MR, Lee FS, Chao MV (2010) The BDNF Val66Met polymorphism impairs NMDA receptor-dependent synaptic plasticity in the hippocampus. J Neurosci 30:8866-8870.

Ninkina N, Adu J, Fischer A, Pinon LG, Buchman VL, Davies AM (1996) Expression and function of TrkB variants in developing sensory neurons. EMBO J 15:6385-6393.

Noble EE, Billington CJ, Kotz CM, Wang C (2011) The lighter side of BDNF. Am J Physiol Regul Integr Comp Physiol 300:R1053-1069.

Noh KM, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MV (2005) Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. Proc Natl Acad Sci U S A 102:12230-12235.

Nomoto H, Takaiwa M, Mouri A, Furukawa S (2007) Pro-region of neurotrophins determines the processing efficiency. Biochem Biophys Res Commun 356:919-924.

Nonaka S, Chuang DM (1998) Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. Neuroreport 9:2081-2084.

O'Leary PD, Hughes RA (2003) Design of potent peptide mimetics of brain-derived neurotrophic factor. J Biol Chem 278:25738-25744.

Ohara O, Gahara Y, Miyake T, Teraoka H, Kitamura T (1993) Neurofilament deficiency in quail caused by nonsense mutation in neurofilament-L gene. J Cell Biol 121:387-395.

Ohara PT, Lieberman AR (1993) Some aspects of the synaptic circuitry underlying inhibition in the ventrobasal thalamus. J Neurocytol 22:815-825.

Ohira K, Homma KJ, Hirai H, Nakamura S, Hayashi M (2006) TrkB-T1 regulates the RhoA signaling and actin cytoskeleton in glioma cells. Biochem Biophys Res Commun 342:867-874.

Ohkubo Y, Uchida AO, Shin D, Partanen J, Vaccarino FM (2004) Fibroblast growth factor receptor 1 is required for the proliferation of hippocampal progenitor cells and for hippocampal growth in mouse. J Neurosci 24:6057-6069.

Okada M, Hozumi Y, Tanaka T, Suzuki Y, Yanagida M, Araki Y, Evangelisti C, Yagisawa H, Topham MK, Martelli AM, Goto K (2012) DGKzeta is degraded through the cytoplasmic ubiquitin-proteasome system under excitotoxic conditions, which causes neuronal apoptosis because of aberrant cell cycle reentry. Cell Signal 24:1573-1582.

Oliff HS, Berchtold NC, Isackson P, Cotman CW (1998) Exercise-induced regulation of brain-derived neurotrophic factor (BDNF) transcripts in the rat hippocampus. Brain Res Mol Brain Res 61:147-153.

Oliva AA, Jr., Atkins CM, Copenagle L, Banker GA (2006) Activated c-Jun N-terminal kinase is required for axon formation. J Neurosci 26:9462-9470.

Oppenheim RW, Yin QW, Prevette D, Yan Q (1992) Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360:755-757.

Oppenheim RW, Prevette D, Yin QW, Collins F, MacDonald J (1991) Control of embryonic motoneuron survival in vivo by ciliary neurotrophic factor. Science 251:1616-1618.

Orban PC, Chapman PF, Brambilla R (1999) Is the Ras-MAPK signalling pathway necessary for long-term memory formation? Trends Neurosci 22:38-44.

Ordonez AN, Jessick VJ, Clayton CE, Ashley MD, Thompson SJ, Simon RP, Meller R (2010) Rapid ischemic tolerance induced by adenosine preconditioning results in Bcl-2 interacting mediator of cell death (Bim) degradation by the proteasome. Int J Physiol Pathophysiol Pharmacol 2:36-44.

Ortega F, Perez-Sen R, Delicado EG, Miras-Portugal MT (2009) P2X7 nucleotide receptor is coupled to GSK-3 inhibition and neuroprotection in cerebellar granule neurons. Neurotox Res 15:193-204.

Ouyang Y, Rosenstein A, Kreiman G, Schuman EM, Kennedy MB (1999) Tetanic stimulation leads to increased accumulation of Ca(2+)/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons. J Neurosci 19:7823-7833.

Ozer RS, Halpain S (2000) Phosphorylation-dependent localization of microtubule-associated protein MAP2c to the actin cytoskeleton. Mol Biol Cell 11:3573-3587.

Pae CU (2007) Comments on "Low plasma BDNF is associated with suicidal behavior in major depression" by Y.K. Kim et al. Progress in Neuropsychopharmacology and Biological Psychiatry 2006 Aug 9; [Epub ahead of print]. Prog Neuropsychopharmacol Biol Psychiatry 31:578-579; author reply 580-571.

Palko ME, Coppola V, Tessarollo L (1999) Evidence for a role of truncated trkC receptor isoforms in mouse development. J Neurosci 19:775-782.

Panagiotaki N, Dajas-Bailador F, Amaya E, Papalopulu N, Dorey K (2010) Characterisation of a new regulator of BDNF signalling, Sprouty3, involved in axonal morphogenesis in vivo. Development 137:4005-4015.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306:487-491.

Paquin A, Barnabe-Heider F, Kageyama R, Miller FD (2005) CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather than astrocytes in vivo. J Neurosci 25:10747-10758.

Parain K, Murer MG, Yan Q, Faucheux B, Agid Y, Hirsch E, Raisman-Vozari R (1999) Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. Neuroreport 10:557-561.

Pardridge WM, Kang YS, Buciak JL (1994) Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood-brain barrier in vivo using vector-mediated peptide drug delivery. Pharm Res 11:738-746.

Pardridge WM, Wu D, Sakane T (1998) Combined use of carboxyl-directed protein pegylation and vector-mediated blood-brain barrier drug delivery system optimizes brain uptake of brain-derived neurotrophic factor following intravenous administration. Pharm Res 15:576-582.

Park H, Poo MM (2013) Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci 14:7-23.

Park HY, Kim JH, Sun Kim H, Park CK (2012) Stem cell-based delivery of brain-derived neurotrophic factor gene in the rat retina. Brain Res 1469:10-23.

Park JJ, Cawley NX, Loh YP (2008) A bi-directional carboxypeptidase E-driven transport mechanism controls BDNF vesicle homeostasis in hippocampal neurons. Mol Cell Neurosci 39:63-73.

Pattabiraman PP, Tropea D, Chiaruttini C, Tongiorgi E, Cattaneo A, Domenici L (2005) Neuronal activity regulates the developmental expression and subcellular localization of cortical BDNF mRNA isoforms in vivo. Mol Cell Neurosci 28:556-570.

Patterson SL, Grover LM, Schwartzkroin PA, Bothwell M (1992) Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. Neuron 9:1081-1088.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16:1137-1145.

Peethumnongsin E, Yang L, Kallhoff-Munoz V, Hu L, Takashima A, Pautler RG, Zheng H (2010) Convergence of presenilin- and tau-mediated pathways on axonal trafficking and neuronal function. J Neurosci 30:13409-13418.

Peng S, Wuu J, Mufson EJ, Fahnestock M (2004) Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer disease. J Neuropathol Exp Neurol 63:641-649.

Pereira DB, Chao MV (2007) The tyrosine kinase Fyn determines the localization of TrkB receptors in lipid rafts. J Neurosci 27:4859-4869.

Pereira LO, Nabinger PM, Strapasson AC, Nardin P, Goncalves CA, Siqueira IR, Netto CA (2009) Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. Brain Res 1247:188-195.

Perrot R, Julien JP (2009) Real-time imaging reveals defects of fast axonal transport induced by disorganization of intermediate filaments. FASEB J 23:3213-3225.

Perrot R, Berges R, Bocquet A, Eyer J (2008) Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration. Mol Neurobiol 38:27-65.

Pettmann B, Henderson CE (1998) Neuronal cell death. Neuron 20:633-647.

Petzold A (2005) Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J Neurol Sci 233:183-198.

Pezawas L, Verchinski BA, Mattay VS, Callicott JH, Kolachana BS, Straub RE, Egan MF, Meyer-Lindenberg A, Weinberger DR (2004) The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. J Neurosci 24:10099-10102.

Pezet S, McMahon SB (2006) Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 29:507-538.

Philipp S, Hambrecht J, Braslavski L, Schroth G, Freichel M, Murakami M, Cavalie A, Flockerzi V (1998) A novel capacitative calcium entry channel expressed in excitable cells. EMBO J 17:4274-4282.

Phillips HS, Hains JM, Laramee GR, Rosenthal A, Winslow JW (1990) Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science 250:290-294.

Phillips JB, Williams AJ, Adams J, Elliott PJ, Tortella FC (2000) Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia. Stroke 31:1686-1693.

Philo J, Talvenheimo J, Wen J, Rosenfeld R, Welcher A, Arakawa T (1994) Interactions of neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and the NT-3.BDNF heterodimer with the extracellular domains of the TrkB and TrkC receptors. J Biol Chem 269:27840-27846.

Ploughman M, Windle V, MacLellan CL, White N, Dore JJ, Corbett D (2009) Brain-derived neurotrophic factor contributes to recovery of skilled reaching after focal ischemia in rats. Stroke 40:1490-1495.

Poduslo JF, Curran GL (1996) Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. Brain Res Mol Brain Res 36:280-286.

Pollock GS, Vernon E, Forbes ME, Yan Q, Ma YT, Hsieh T, Robichon R, Frost DO, Johnson JE (2001) Effects of early visual experience and diurnal rhythms on BDNF mRNA and protein levels in the visual system, hippocampus, and cerebellum. J Neurosci 21:3923-3931.

Potter SM, DeMarse TB (2001) A new approach to neural cell culture for long-term studies. J Neurosci Methods 110:17-24.

Poulain FE, Sobel A (2007) The "SCG10-LIke Protein" SCLIP is a novel regulator of axonal branching in hippocampal neurons, unlike SCG10. Mol Cell Neurosci 34:137-146.

Powell JC, Twomey C, Jain R, McCarthy JV (2009) Association between Presenilin-1 and TRAF6 modulates regulated intramembrane proteolysis of the p75NTR neurotrophin receptor. J Neurochem 108:216-230.

Pozo K, Goda Y (2010) Unraveling mechanisms of homeostatic synaptic plasticity. Neuron 66:337-351.

Pozzo-Miller LD, Pivovarova NB, Connor JA, Reese TS, Andrews SB (1999) Correlated measurements of free and total intracellular calcium concentration in central nervous system neurons. Microsc Res Tech 46:370-379.

Pruunsild P, Kazantseva A, Aid T, Palm K, Timmusk T (2007) Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. Genomics 90:397-406.

Putney JW, Jr. (2004) The enigmatic TRPCs: multifunctional cation channels. Trends Cell Biol 14:282-286.

Putney JW, Jr. (2007) Inositol lipids and TRPC channel activation. Biochem Soc Symp:37-45.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Radziejewski C, Robinson RC (1993) Heterodimers of the neurotrophic factors: formation, isolation, and differential stability. Biochemistry 32:13350-13356.

Raftery LA, Sutherland DJ (1999) TGF-beta family signal transduction in Drosophila development: from Mad to Smads. Dev Biol 210:251-268.

Rattiner LM, Davis M, Ressler KJ (2004) Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. Learn Mem 11:727-731.

Redondo RL, Morris RG (2011) Making memories last: the synaptic tagging and capture hypothesis. Nat Rev Neurosci 12:17-30.

Rehni AK, Singh TG, Kakkar T, Arora S (2011) Involvement of src-kinase activation in ischemic preconditioning induced protection of mouse brain. Life Sci 88:825-829.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361:1545-1564.

Remenyi J, Hunter CJ, Cole C, Ando H, Impey S, Monk CE, Martin KJ, Barton GJ, Hutvagner G, Arthur JS (2010) Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins. Biochem J 428:281-291.

Reymann KG, Frey JU (2007) The late maintenance of hippocampal LTP: requirements, phases, 'synaptic tagging', 'late-associativity' and implications. Neuropharmacology 52:24-40.

Ribases M, Gratacos M, Armengol L, de Cid R, Badia A, Jimenez L, Solano R, Vallejo J, Fernandez F, Estivill X (2003) Met66 in the brain-derived neurotrophic factor (BDNF) precursor is associated with anorexia nervosa restrictive type. Mol Psychiatry 8:745-751.

Riemenschneider M, Schwarz S, Wagenpfeil S, Diehl J, Muller U, Forstl H, Kurz A (2002) A polymorphism of the brain-derived neurotrophic factor (BDNF) is associated with Alzheimer's disease in patients lacking the Apolipoprotein E epsilon4 allele. Mol Psychiatry 7:782-785.

Rigamonti D, Bolognini D, Mutti C, Zuccato C, Tartari M, Sola F, Valenza M, Kazantsev AG, Cattaneo E (2007) Loss of huntingtin function complemented by small molecules acting as repressor element 1/neuron restrictive silencer element silencer modulators. J Biol Chem 282:24554-24562.

Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R (2001) Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. Mol Endocrinol 15:1748-1757.

Robinson RC, Radziejewski C, Stuart DI, Jones EY (1995) Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. Biochemistry 34:4139-4146.

Robinson RC, Radziejewski C, Spraggon G, Greenwald J, Kostura MR, Burtnick LD, Stuart DI, Choe S, Jones EY (1999) The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site. Protein Sci 8:2589-2597.

Rocamora N, Welker E, Pascual M, Soriano E (1996) Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation. J Neurosci 16:4411-4419.

Rodriguez-Tebar A, Dechant G, Barde YA (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Romera C, Hurtado O, Botella SH, Lizasoain I, Cardenas A, Fernandez-Tome P, Leza JC, Lorenzo P, Moro MA (2004) In vitro ischemic tolerance involves upregulation of glutamate transport partly mediated by the TACE/ADAM17-tumor necrosis factor-alpha pathway. J Neurosci 24:1350-1357.

Rosario M, Schuster S, Juttner R, Parthasarathy S, Tarabykin V, Birchmeier W (2012) Neocortical dendritic complexity is controlled during development by NOMA-GAP-dependent inhibition of Cdc42 and activation of cofilin. Genes Dev 26:1743-1757.

Rosas-Vargas H, Martinez-Ezquerro JD, Bienvenu T (2011) Brain-derived neurotrophic factor, food intake regulation, and obesity. Arch Med Res 42:482-494.

Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR, Nikolics K, Winslow JW (1990) Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4:767-773.

Rossi FM, Bozzi Y, Pizzorusso T, Maffei L (1999) Monocular deprivation decreases brain-derived neurotrophic factor immunoreactivity in the rat visual cortex. Neuroscience 90:363-368.

Rothman SM (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. J Neurosci 5:1483-1489.

Roubin R, Naert K, Popovici C, Vatcher G, Coulier F, Thierry-Mieg J, Pontarotti P, Birnbaum D, Baillie D, Thierry-Mieg D (1999) let-756, a C. elegans fgf essential for worm development. Oncogene 18:6741-6747.

Roux PP, Bhakar AL, Kennedy TE, Barker PA (2001) The p75 neurotrophin receptor activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway. J Biol Chem 276:23097-23104.

Rudge JS, Mather PE, Pasnikowski EM, Cai N, Corcoran T, Acheson A, Anderson K, Lindsay RM, Wiegand SJ (1998) Endogenous BDNF protein is increased in adult rat hippocampus after a kainic acid induced excitotoxic insult but exogenous BDNF is not neuroprotective. Exp Neurol 149:398-410.

Ruiz A, Matute C, Alberdi E (2009) Endoplasmic reticulum Ca(2+) release through ryanodine and IP(3) receptors contributes to neuronal excitotoxicity. Cell Calcium 46:273-281.

Russo-Neustadt A, Beard RC, Cotman CW (1999) Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. Neuropsychopharmacology 21:679-682.

Rutherford LC, Nelson SB, Turrigiano GG (1998) BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. Neuron 21:521-530.

Ruvkun G, Hobert O (1998) The taxonomy of developmental control in Caenorhabditis elegans. Science 282:2033-2041.

Sadakata T, Mizoguchi A, Sato Y, Katoh-Semba R, Fukuda M, Mikoshiba K, Furuichi T (2004) The secretory granule-associated protein CAPS2 regulates neurotrophin release and cell survival. J Neurosci 24:43-52.

Sadakata T, Kakegawa W, Mizoguchi A, Washida M, Katoh-Semba R, Shutoh F, Okamoto T, Nakashima H, Kimura K, Tanaka M, Sekine Y, Itohara S, Yuzaki M, Nagao S, Furuichi T (2007) Impaired cerebellar development and function in mice lacking CAPS2, a protein involved in neurotrophin release. J Neurosci 27:2472-2482.

Sahai E, Olson MF, Marshall CJ (2001) Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility. EMBO J 20:755-766.

Sajikumar S, Korte M (2011) Different compartments of apical CA1 dendrites have different plasticity thresholds for expressing synaptic tagging and capture. Learn Mem 18:327-331.

Samuels IS, Karlo JC, Faruzzi AN, Pickering K, Herrup K, Sweatt JD, Saitta SC, Landreth GE (2008) Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci 28:6983-6995.

Sano K, Nanba H, Tabuchi A, Tsuchiya T, Tsuda M (1996) BDNF gene can Be activated by Ca2+ signals without involvement of de novo AP-1 synthesis. Biochem Biophys Res Commun 229:788-793.

Santi S, Cappello S, Riccio M, Bergami M, Aicardi G, Schenk U, Matteoli M, Canossa M (2006) Hippocampal neurons recycle BDNF for activity-dependent secretion and LTP maintenance. EMBO J 25:4372-4380.

Santos AR, Comprido D, Duarte CB (2010a) Regulation of local translation at the synapse by BDNF. Prog Neurobiol 92:505-516.

Santos SD, Manadas B, Duarte CB, Carvalho AL (2010b) Proteomic analysis of an interactome for long-form AMPA receptor subunits. J Proteome Res 9:1670-1682.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098-1101.

Sasaki M, Radtke C, Tan AM, Zhao P, Hamada H, Houkin K, Honmou O, Kocsis JD (2009) BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. J Neurosci 29:14932-14941.

Sattler R, Tymianski M (2001) Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol 24:107-129.

Schabitz WR, Schwab S, Spranger M, Hacke W (1997) Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. J Cereb Blood Flow Metab 17:500-506.

Schabitz WR, Sommer C, Zoder W, Kiessling M, Schwaninger M, Schwab S (2000) Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. Stroke 31:2212-2217.

Schecterson LC, Bothwell M (2010) Neurotrophin receptors: Old friends with new partners. Dev Neurobiol 70:332-338.

Schmid DA, Yang T, Ogier M, Adams I, Mirakhur Y, Wang Q, Massa SM, Longo FM, Katz DM (2012) A TrkB small molecule partial agonist rescues TrkB phosphorylation deficits and improves respiratory function in a mouse model of Rett syndrome. J Neurosci 32:1803-1810.

Schor NF (2005) The p75 neurotrophin receptor in human development and disease. Prog Neurobiol 77:201-214.

Schoups AA, Elliott RC, Friedman WJ, Black IB (1995) NGF and BDNF are differentially modulated by visual experience in the developing geniculocortical pathway. Brain Res Dev Brain Res 86:326-334.

Schratt GM, Nigh EA, Chen WG, Hu L, Greenberg ME (2004) BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. J Neurosci 24:7366-7377.

Schurr A, Payne RS, Rigor BM (1995) Synergism between diltiazem and MK-801 but not APV in protecting hippocampal slices against hypoxic damage. Brain Res 684:233-236.

Seidah NG, Benjannet S, Pareek S, Chretien M, Murphy RA (1996) Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett 379:247-250.

Seidah NG, Mowla SJ, Hamelin J, Mamarbachi AM, Benjannet S, Toure BB, Basak A, Munzer JS, Marcinkiewicz J, Zhong M, Barale JC, Lazure C, Murphy RA, Chretien M, Marcinkiewicz M (1999) Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. Proc Natl Acad Sci U S A 96:1321-1326.

Semenova MM, Maki-Hokkonen AM, Cao J, Komarovski V, Forsberg KM, Koistinaho M, Coffey ET, Courtney MJ (2007) Rho mediates calcium-dependent activation of p38alpha and subsequent excitotoxic cell death. Nat Neurosci 10:436-443.

Sen S, Nesse RM, Stoltenberg SF, Li S, Gleiberman L, Chakravarti A, Weder AB, Burmeister M (2003) A BDNF coding variant is associated with the NEO personality inventory domain neuroticism, a risk factor for depression. Neuropsychopharmacology 28:397-401.

Seo JY, Lee CH, Cho JH, Choi JH, Yoo KY, Kim DW, Park OK, Li H, Choi SY, Hwang IK, Won MH (2009) Neuroprotection of ebselen against

ischemia/reperfusion injury involves GABA shunt enzymes. J Neurol Sci 285:88-94.

Sha D, Jin Y, Wu H, Wei J, Lin CH, Lee YH, Buddhala C, Kuchay S, Chishti AH, Wu JY (2008) Role of mu-calpain in proteolytic cleavage of brain L-glutamic acid decarboxylase. Brain Res 1207:9-18.

Sha H, Xu J, Tang J, Ding J, Gong J, Ge X, Kong D, Gao X (2007) Disruption of a novel regulatory locus results in decreased Bdnf expression, obesity, and type 2 diabetes in mice. Physiol Genomics 31:252-263.

Shah JV, Flanagan LA, Janmey PA, Leterrier JF (2000) Bidirectional translocation of neurofilaments along microtubules mediated in part by dynein/dynactin. Mol Biol Cell 11:3495-3508.

Shamovsky IL, Ross GM, Riopelle RJ, Weaver DF (1999) The interaction of neurotrophins with the p75NTR common neurotrophin receptor: a comprehensive molecular modeling study. Protein Sci 8:2223-2233.

Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821-861.

Shea TB, Chan WK, Kushkuley J, Lee S (2009) Organizational dynamics, functions, and pathobiological dysfunctions of neurofilaments. Results Probl Cell Differ 48:29-45.

Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int 51:333-355.

Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, Carroll K, Spencer SD, Levinson AD (1995) Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci 15:477-491.

Shen B, Kwan HY, Ma X, Wong CO, Du J, Huang Y, Yao X (2011) cAMP activates TRPC6 channels via the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)-mitogen-activated protein kinase kinase (MEK)-ERK1/2 signaling pathway. J Biol Chem 286:19439-19445.

Shen J, Maruyama IN (2012) Brain-derived neurotrophic factor receptor TrkB exists as a preformed dimer in living cells. J Mol Signal 7:2.

Shen W, Wu B, Zhang Z, Dou Y, Rao ZR, Chen YR, Duan S (2006) Activity-induced rapid synaptic maturation mediated by presynaptic cdc42 signaling. Neuron 50:401-414.

Shi Q, Zhang P, Zhang J, Chen X, Lu H, Tian Y, Parker TL, Liu Y (2009a) Adenovirus-mediated brain-derived neurotrophic factor expression regulated by hypoxia response element protects brain from injury of transient middle cerebral artery occlusion in mice. Neurosci Lett 465:220-225.

Shi SH, Jan LY, Jan YN (2003) Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell 112:63-75.

Shi Y, Lu W, Milstein AD, Nicoll RA (2009b) The stoichiometry of AMPA receptors and TARPs varies by neuronal cell type. Neuron 62:633-640.

Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727-740.

Shimada A, Mason CA, Morrison ME (1998) TrkB signaling modulates spine density and morphology independent of dendrite structure in cultured neonatal Purkinje cells. J Neurosci 18:8559-8570.

Shinoda Y, Sadakata T, Nakao K, Katoh-Semba R, Kinameri E, Furuya A, Yanagawa Y, Hirase H, Furuichi T (2011) Calcium-dependent activator protein for secretion 2 (CAPS2) promotes BDNF secretion and is critical for the development of GABAergic interneuron network. Proc Natl Acad Sci U S A 108:373-378.

Sihag RK, Inagaki M, Yamaguchi T, Shea TB, Pant HC (2007) Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. Exp Cell Res 313:2098-2109.

Simmons DA, Rex CS, Palmer L, Pandyarajan V, Fedulov V, Gall CM, Lynch G (2009) Up-regulating BDNF with an ampakine rescues synaptic plasticity and memory in Huntington's disease knockin mice. Proc Natl Acad Sci U S A 106:4906-4911.

Simo S, Cooper JA (2012) Regulation of dendritic branching by Cdc42 GAPs. Genes Dev 26:1653-1658.

Simpkins KL, Guttmann RP, Dong Y, Chen Z, Sokol S, Neumar RW, Lynch DR (2003) Selective activation induced cleavage of the NR2B subunit by calpain. J Neurosci 23:11322-11331.

Sirianni RW, Olausson P, Chiu AS, Taylor JR, Saltzman WM (2010) The behavioral and biochemical effects of BDNF containing polymers implanted in the hippocampus of rats. Brain Res 1321:40-50.

Skaper SD (2012) The neurotrophin family of neurotrophic factors: an overview. Methods Mol Biol 846:1-12.

Skeldal S, Matusica D, Nykjaer A, Coulson EJ (2011) Proteolytic processing of the p75 neurotrophin receptor: A prerequisite for signalling?: Neuronal life, growth and death signalling are crucially regulated by intra-membrane proteolysis and trafficking of p75(NTR). Bioessays 33:614-625.

Skeldal S, Sykes AM, Glerup S, Matusica D, Palstra N, Autio H, Boskovic Z, Madsen P, Castren E, Nykjaer A, Coulson EJ (2012) Mapping of the interaction site between sortilin and the p75 neurotrophin receptor reveals a regulatory role for the sortilin intracellular domain in p75 neurotrophin receptor shedding and apoptosis. J Biol Chem 287:43798-43809.

Sklar P, Gabriel SB, McInnis MG, Bennett P, Lim Y, Tsan G, Schaffner S, Kirov G, Jones I, Owen M, Craddock N, DePaulo JR, Lander ES (2002) Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. Brain-derived neutrophic factor. Mol Psychiatry 7:579-593.

Skledar M, Nikolac M, Dodig-Curkovic K, Curkovic M, Borovecki F, Pivac N (2012) Association between brain-derived neurotrophic factor Val66Met and obesity in children and adolescents. Prog Neuropsychopharmacol Biol Psychiatry 36:136-140.

Smart FM, Edelman GM, Vanderklish PW (2003) BDNF induces translocation of initiation factor 4E to mRNA granules: evidence for a role of synaptic microfilaments and integrins. Proc Natl Acad Sci U S A 100:14403-14408.

Smith MA, Makino S, Kvetnansky R, Post RM (1995) Effects of stress on neurotrophic factor expression in the rat brain. Ann N Y Acad Sci 771:234-239.

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627-638.

Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24:1217-1281.

Sohrabji F, Lewis DK (2006) Estrogen-BDNF interactions: implications for neurodegenerative diseases. Front Neuroendocrinol 27:404-414.

Sokka AL, Putkonen N, Mudo G, Pryazhnikov E, Reijonen S, Khiroug L, Belluardo N, Lindholm D, Korhonen L (2007) Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain. J Neurosci 27:901-908.

Song MS, Posse de Chaves EI (2003) Inhibition of rat sympathetic neuron apoptosis by ceramide. Role of p75NTR in ceramide generation. Neuropharmacology 45:1130-1150.

Song XY, Li F, Zhang FH, Zhong JH, Zhou XF (2008) Peripherally-derived BDNF promotes regeneration of ascending sensory neurons after spinal cord injury. PLoS One 3:e1707.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, Nikolics K, Parada LF (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell 65:895-903.

Soule J, Messaoudi E, Bramham CR (2006) Brain-derived neurotrophic factor and control of synaptic consolidation in the adult brain. Biochem Soc Trans 34:600-604.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, et al. (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-893.

Srinivasan B, Wang Z, Brun-Zinkernagel AM, Collier RJ, Black RA, Frank SJ, Barker PA, Roque RS (2007) Photic injury promotes cleavage of p75NTR by TACE and nuclear trafficking of the p75 intracellular domain. Mol Cell Neurosci 36:449-461.

Stack EC, Dedeoglu A, Smith KM, Cormier K, Kubilus JK, Bogdanov M, Matson WR, Yang L, Jenkins BG, Luthi-Carter R, Kowall NW, Hersch SM, Beal MF, Ferrante RJ (2007) Neuroprotective effects of synaptic modulation in Huntington's disease R6/2 mice. J Neurosci 27:12908-12915.

Stadelmann C, Kerschensteiner M, Misgeld T, Bruck W, Hohlfeld R, Lassmann H (2002) BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? Brain 125:75-85.

Stanzione F, Esposito L, Paladino A, Pedone C, Morelli G, Vitagliano L (2010) Role of the conformational versatility of the neurotrophin N-terminal regions in their recognition by Trk receptors. Biophys J 99:2273-2278.

Steward O, Schuman EM (2001) Protein synthesis at synaptic sites on dendrites. Annu Rev Neurosci 24:299-325.

Strohmaier C, Carter BD, Urfer R, Barde YA, Dechant G (1996) A splice variant of the neurotrophin receptor trkB with increased specificity for brain-derived neurotrophic factor. EMBO J 15:3332-3337.

Suh J, Im DS, Moon GJ, Ryu KS, de Silva R, Choi IS, Lees AJ, Guenette SY, Tanzi RE, Gwag BJ (2010) Hypoxic ischemia and proteasome dysfunction alter tau isoform ratio by inhibiting exon 10 splicing. J Neurochem 114:160-170.

Suh LH, Oster SF, Soehrman SS, Grenningloh G, Sretavan DW (2004) L1/Laminin modulation of growth cone response to EphB triggers growth pauses and regulates the microtubule destabilizing protein SCG10. J Neurosci 24:1976-1986.

Sun X, Zhou H, Luo X, Li S, Yu D, Hua J, Mu D, Mao M (2008) Neuroprotection of brain-derived neurotrophic factor against hypoxic injury in vitro requires activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. Int J Dev Neurosci 26:363-370.

Sun Y, Lim Y, Li F, Liu S, Lu JJ, Haberberger R, Zhong JH, Zhou XF (2012) ProBDNF collapses neurite outgrowth of primary neurons by activating RhoA. PLoS One 7:e35883.

Suzuki S, Kiyosue K, Hazama S, Ogura A, Kashihara M, Hara T, Koshimizu H, Kojima M (2007) Brain-derived neurotrophic factor regulates cholesterol metabolism for synapse development. J Neurosci 27:6417-6427.

Suzuki S, Numakawa T, Shimazu K, Koshimizu H, Hara T, Hatanaka H, Mei L, Lu B, Kojima M (2004) BDNF-induced recruitment of TrkB receptor into neuronal lipid rafts: roles in synaptic modulation. J Cell Biol 167:1205-1215.

Swanwick CC, Murthy NR, Kapur J (2006) Activity-dependent scaling of GABAergic synapse strength is regulated by brain-derived neurotrophic factor. Mol Cell Neurosci 31:481-492.

Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 14:311-317.

Szaro BG, Strong MJ (2010) Post-transcriptional control of neurofilaments: New roles in development, regeneration and neurodegenerative disease. Trends Neurosci 33:27-37.

Tabuchi A, Sakaya H, Kisukeda T, Fushiki H, Tsuda M (2002) Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. J Biol Chem 277:35920-35931.

Tabuchi A, Nakaoka R, Amano K, Yukimine M, Andoh T, Kuraishi Y, Tsuda M (2000) Differential activation of brain-derived neurotrophic factor gene promoters I and III by Ca2+ signals evoked via L-type voltage-dependent and N-methyl-D-aspartate receptor Ca2+ channels. J Biol Chem 275:17269-17275.

Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. Physiol Rev 81:153-208.

Takano J, Tomioka M, Tsubuki S, Higuchi M, Iwata N, Itohara S, Maki M, Saido TC (2005) Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. J Biol Chem 280:16175-16184.

Takeda S, Okabe S, Funakoshi T, Hirokawa N (1994) Differential dynamics of neurofilament-H protein and neurofilament-L protein in neurons. J Cell Biol 127:173-185.

Takei N, Inamura N, Kawamura M, Namba H, Hara K, Yonezawa K, Nawa H (2004) Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. J Neurosci 24:9760-9769.

Takemoto-Kimura S, Ageta-Ishihara N, Nonaka M, Adachi-Morishima A, Mano T, Okamura M, Fujii H, Fuse T, Hoshino M, Suzuki S, Kojima M, Mishina M, Okuno H, Bito H (2007) Regulation of dendritogenesis via a lipid-raft-associated Ca2+/calmodulin-dependent protein kinase CLICK-III/CaMKIgamma. Neuron 54:755-770.

Takeshima Y, Nakamura M, Miyake H, Tamaki R, Inui T, Horiuchi K, Wajima D, Nakase H (2011) Neuroprotection with intraventricular brain-derived neurotrophic factor in rat venous occlusion model. Neurosurgery 68:1334-1341.

Takeuchi H, Mizuno T, Zhang G, Wang J, Kawanokuchi J, Kuno R, Suzumura A (2005) Neuritic beading induced by activated microglia is an early feature of neuronal dysfunction toward neuronal death by inhibition of mitochondrial respiration and axonal transport. J Biol Chem 280:10444-10454.

Taliaz D, Loya A, Gersner R, Haramati S, Chen A, Zangen A (2011) Resilience to chronic stress is mediated by hippocampal brain-derived neurotrophic factor. J Neurosci 31:4475-4483.

Tan YL, Zhou DF, Cao LY, Zou YZ, Wu GY, Zhang XY (2005) Effect of the BDNF Val66Met genotype on episodic memory in schizophrenia. Schizophr Res 77:355-356.

Tanaka J, Horiike Y, Matsuzaki M, Miyazaki T, Ellis-Davies GC, Kasai H (2008) Protein synthesis and neurotrophin-dependent structural plasticity of single dendritic spines. Science 319:1683-1687.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709-726.

Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33:383-395.

Tararuk T, Ostman N, Li W, Bjorkblom B, Padzik A, Zdrojewska J, Hongisto V, Herdegen T, Konopka W, Courtney MJ, Coffey ET (2006) JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length. J Cell Biol 173:265-277.

Tardito D, Perez J, Tiraboschi E, Musazzi L, Racagni G, Popoli M (2006) Signaling pathways regulating gene expression, neuroplasticity, and neurotrophic mechanisms in the action of antidepressants: a critical overview. Pharmacol Rev 58:115-134.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 25:5455-5463.

Teng KK, Felice S, Kim T, Hempstead BL (2010) Understanding proneurotrophin actions: Recent advances and challenges. Dev Neurobiol 70:350-359.

Tettamanti G, Cattaneo AG, Gornati R, de Eguileor M, Bernardini G, Binelli G (2010) Phylogenesis of brain-derived neurotrophic factor (BDNF) in vertebrates. Gene 450:85-93.

Thiriet N, Amar L, Toussay X, Lardeux V, Ladenheim B, Becker KG, Cadet JL, Solinas M, Jaber M (2008) Environmental enrichment during adolescence regulates gene expression in the striatum of mice. Brain Res 1222:31-41.

Thoenen H, Edgar D (1985) Neurotrophic factors. Science 229:238-242.

Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173-183.

Thomas K, Davies A (2005) Neurotrophins: a ticket to ride for BDNF. Curr Biol 15:R262-264.

Thompson RJ, Zhou N, MacVicar BA (2006) Ischemia opens neuronal gap junction hemichannels. Science 312:924-927.

Thomson RE, Pellicano F, Iwata T (2007) Fibroblast growth factor receptor 3 kinase domain mutation increases cortical progenitor proliferation via mitogen-activated protein kinase activation. J Neurochem 100:1565-1578.

Timmusk T, Persson H, Metsis M (1994) Analysis of transcriptional initiation and translatability of brain-derived neurotrophic factor mRNAs in the rat brain. Neurosci Lett 177:27-31.

Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10:475-489.

Toledo-Aral JJ, Brehm P, Halegoua S, Mandel G (1995) A single pulse of nerve growth factor triggers long-term neuronal excitability through sodium channel gene induction. Neuron 14:607-611.

Tomita S, Fukata M, Nicoll RA, Bredt DS (2004) Dynamic interaction of stargazin-like TARPs with cycling AMPA receptors at synapses. Science 303:1508-1511.

Tongiorgi E (2008) Activity-dependent expression of brain-derived neurotrophic factor in dendrites: facts and open questions. Neurosci Res 61:335-346.

Tongiorgi E, Righi M, Cattaneo A (1997) Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. J Neurosci 17:9492-9505.

Tongiorgi E, Domenici L, Simonato M (2006) What is the biological significance of BDNF mRNA targeting in the dendrites? Clues from epilepsy and cortical development. Mol Neurobiol 33:17-32.

Tornero D, Posadas I, Cena V (2011) Bcl-x(L) blocks a mitochondrial inner membrane channel and prevents Ca2+ overload-mediated cell death. PLoS One 6:e20423.

Treanor JJ, Schmelzer C, Knusel B, Winslow JW, Shelton DL, Hefti F, Nikolics K, Burton LE (1995) Heterodimeric neurotrophins induce phosphorylation of Trk receptors and promote neuronal differentiation in PC12 cells. J Biol Chem 270:23104-23110.

Tremblay R, Hewitt K, Lesiuk H, Mealing G, Morley P, Durkin JP (1999) Evidence that brain-derived neurotrophic factor neuroprotection is linked to its ability to reverse the NMDA-induced inactivation of protein kinase C in cortical neurons. J Neurochem 72:102-111.

Tsoulfas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez JL, Rosenthal A, Nikolics K, Parada LF (1993) The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron 10:975-990.

Tyler WJ, Pozzo-Miller LD (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. J Neurosci 21:4249-4258.

Ueyama T, Kawai Y, Nemoto K, Sekimoto M, Tone S, Senba E (1997) Immobilization stress reduced the expression of neurotrophins and their receptors in the rat brain. Neurosci Res 28:103-110.

Ultsch MH, Wiesmann C, Simmons LC, Henrich J, Yang M, Reilly D, Bass SH, de Vos AM (1999) Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC. J Mol Biol 290:149-159.

Underwood CK, Reid K, May LM, Bartlett PF, Coulson EJ (2008) Palmitoylation of the C-terminal fragment of p75(NTR) regulates death signaling and is required for subsequent cleavage by gamma-secretase. Mol Cell Neurosci 37:346-358.

Unger TJ, Calderon GA, Bradley LC, Sena-Esteves M, Rios M (2007) Selective deletion of Bdnf in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity. J Neurosci 27:14265-14274.

Urdiales JL, Becker E, Andrieu M, Thomas A, Jullien J, van Grunsven LA, Menut S, Evan GI, Martin-Zanca D, Rudkin BB (1998) Cell cycle phase-specific

surface expression of nerve growth factor receptors TrkA and p75(NTR). J Neurosci 18:6767-6775.

Urfer R, Tsoulfas P, O'Connell L, Shelton DL, Parada LF, Presta LG (1995) An immunoglobulin-like domain determines the specificity of neurotrophin receptors. EMBO J 14:2795-2805.

Uversky VN (2002) Natively unfolded proteins: a point where biology waits for physics. Protein Sci 11:739-756.

Vaccarino FM, Schwartz ML, Raballo R, Rhee J, Lyn-Cook R (1999) Fibroblast growth factor signaling regulates growth and morphogenesis at multiple steps during brain development. Curr Top Dev Biol 46:179-200.

Valenzuela DM, Maisonpierre PC, Glass DJ, Rojas E, Nunez L, Kong Y, Gies DR, Stitt TN, Ip NY, Yancopoulos GD (1993) Alternative forms of rat TrkC with different functional capabilities. Neuron 10:963-974.

Vandenplas ML, Mamidipudi V, Lamar Seibenhener M, Wooten MW (2002) Nerve growth factor activates kinases that phosphorylate atypical protein kinase C. Cell Signal 14:359-363.

Vanderklish PW, Bahr BA (2000) The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 81:323-339.

Vargas-Perez H, Ting AKR, Walton CH, Hansen DM, Razavi R, Clarke L, Bufalino MR, Allison DW, Steffensen SC, van der Kooy D (2009) Ventral tegmental area BDNF induces an opiate-dependent-like reward state in naive rats. Science 324:1732-1734.

Venkatachalam K, Zheng F, Gill DL (2003) Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. J Biol Chem 278:29031-29040.

Ventriglia M, Bocchio Chiavetto L, Benussi L, Binetti G, Zanetti O, Riva MA, Gennarelli M (2002) Association between the BDNF 196 A/G polymorphism and sporadic Alzheimer's disease. Mol Psychiatry 7:136-137.

Vicario-Abejon C, Collin C, McKay RD, Segal M (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. J Neurosci 18:7256-7271.

Vilar M, Charalampopoulos I, Kenchappa RS, Reversi A, Klos-Applequist JM, Karaca E, Simi A, Spuch C, Choi S, Friedman WJ, Ericson J, Schiavo G, Carter BD, Ibanez CF (2009a) Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor. J Cell Sci 122:3351-3357.

Vilar M, Charalampopoulos I, Kenchappa RS, Simi A, Karaca E, Reversi A, Choi S, Bothwell M, Mingarro I, Friedman WJ, Schiavo G, Bastiaens PI, Verveer PJ, Carter BD, Ibanez CF (2009b) Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. Neuron 62:72-83.

Villarreal DM, Do V, Haddad E, Derrick BE (2002) NMDA receptor antagonists sustain LTP and spatial memory: active processes mediate LTP decay. Nat Neurosci 5:48-52.

Vincent P, Mulle C (2009) Kainate receptors in epilepsy and excitotoxicity. Neuroscience 158:309-323.

Vollmayr B, Faust H, Lewicka S, Henn FA (2001) Brain-derived-neurotrophic-factor (BDNF) stress response in rats bred for learned helplessness. Mol Psychiatry 6:471-474, 358.

von Bartheld CS, Wang X, Butowt R (2001) Anterograde axonal transport, transcytosis, and recycling of neurotrophic factors: the concept of trophic currencies in neural networks. Mol Neurobiol 24:1-28.

von Bartheld CS, Williams R, Lefcort F, Clary DO, Reichardt LF, Bothwell M (1996) Retrograde transport of neurotrophins from the eye to the brain in chick embryos: roles of the p75NTR and trkB receptors. J Neurosci 16:2995-3008.

von Schack D, Casademunt E, Schweigreiter R, Meyer M, Bibel M, Dechant G (2001) Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. Nat Neurosci 4:977-978.

Wahrle S, Das P, Nyborg AC, McLendon C, Shoji M, Kawarabayashi T, Younkin LH, Younkin SG, Golde TE (2002) Cholesterol-dependent gamma-secretase activity in buoyant cholesterol-rich membrane microdomains. Neurobiol Dis 9:11-23.

Walicke P, Cowan WM, Ueno N, Baird A, Guillemin R (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci U S A 83:3012-3016.

Walz C, Jungling K, Lessmann V, Gottmann K (2006) Presynaptic plasticity in an immature neocortical network requires NMDA receptor activation and BDNF release. J Neurophysiol 96:3512-3516.

Wanet A, Tacheny A, Arnould T, Renard P (2012) miR-212/132 expression and functions: within and beyond the neuronal compartment. Nucleic Acids Res 40:4742-4753.

Wang GX, Poo MM (2005) Requirement of TRPC channels in netrin-1-induced chemotropic turning of nerve growth cones. Nature 434:898-904.

Wang H, Ward N, Boswell M, Katz DM (2006) Secretion of brain-derived neurotrophic factor from brain microvascular endothelial cells. Eur J Neurosci 23:1665-1670.

Wang H, Yu SW, Koh DW, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neurosci 24:10963-10973.

Wang JJ, Tasinato A, Ethell DW, Testa MP, Bredesen DE (2000) Phosphorylation of the common neurotrophin receptor p75 by p38beta2 kinase affects NF-kappaB and AP-1 activities. J Mol Neurosci 15:19-29.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z (2002) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420:74-78.

Wang L, Fu C, Cui Y, Xie Y, Yuan Y, Wang X, Chen H, Huang BR (2012) The Ran-binding protein RanBPM can depress the NF-kappaB pathway by interacting with TRAF6. Mol Cell Biochem 359:83-94.

Wang LH, Paden AJ, Johnson EM, Jr. (2005) Mixed-lineage kinase inhibitors require the activation of Trk receptors to maintain long-term neuronal trophism and survival. J Pharmacol Exp Ther 312:1007-1019.

Wang W, Dow KE, Riopelle RJ, Ross GM (2001) The common neurotrophin receptor p75NTR enhances the ability of PC12 cells to resist oxidative stress by a trkA-dependent mechanism. Neurotox Res 3:485-499.

Wang X, Teng L, Li A, Ge J, Laties AM, Zhang X (2010a) TRPC6 channel protects retinal ganglion cells in a rat model of retinal ischemia/reperfusion-induced cell death. Invest Ophthalmol Vis Sci 51:5751-5758.

Wang ZY, Miki T, Lee KY, Yokoyama T, Kusaka T, Sumitani K, Warita K, Matsumoto Y, Yakura T, Hosomi N, Ameno K, Bedi KS, Takeuchi Y (2010b) Short-term exposure to ethanol causes a differential response between nerve growth factor and brain-derived neurotrophic factor ligand/receptor systems in the mouse cerebellum. Neuroscience 165:485-491.

Wehrman T, He X, Raab B, Dukipatti A, Blau H, Garcia KC (2007) Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. Neuron 53:25-38.

Wei J, Lin CH, Wu H, Jin Y, Lee YH, Wu JY (2006) Activity-dependent cleavage of brain glutamic acid decarboxylase 65 by calpain. J Neurochem 98:1688-1695.

West AE, Griffith EC, Greenberg ME (2002) Regulation of transcription factors by neuronal activity. Nat Rev Neurosci 3:921-931.

West AE, Chen WG, Dalva MB, Dolmetsch RE, Kornhauser JM, Shaywitz AJ, Takasu MA, Tao X, Greenberg ME (2001) Calcium regulation of neuronal gene expression. Proc Natl Acad Sci U S A 98:11024-11031.

Wiesmann C, Muller YA, de Vos AM (2000) Ligand-binding sites in Ig-like domains of receptor tyrosine kinases. J Mol Med (Berl) 78:247-260.

Wiesmann C, Ultsch MH, Bass SH, de Vos AM (1999) Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature 401:184-188.

Williams AJ, Hale SL, Moffett JR, Dave JR, Elliott PJ, Adams J, Tortella FC (2003) Delayed treatment with MLN519 reduces infarction and associated neurologic deficit caused by focal ischemic brain injury in rats via antiinflammatory mechanisms involving nuclear factor-kappaB activation, gliosis, and leukocyte infiltration. J Cereb Blood Flow Metab 23:75-87.

Williams EJ, Furness J, Walsh FS, Doherty P (1994) Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron 13:583-594.

Windisch JM, Marksteiner R, Schneider R (1995) Nerve growth factor binding site on TrkA mapped to a single 24-amino acid leucine-rich motif. J Biol Chem 270:28133-28138.

Wirth MJ, Brun A, Grabert J, Patz S, Wahle P (2003) Accelerated dendritic development of rat cortical pyramidal cells and interneurons after biolistic transfection with BDNF and NT4/5. Development 130:5827-5838.

Wong J, Hyde TM, Cassano HL, Deep-Soboslay A, Kleinman JE, Weickert CS (2010) Promoter specific alterations of brain-derived neurotrophic factor mRNA in schizophrenia. Neuroscience 169:1071-1084.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5:1302-1308.

Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8:1069-1077.

Wooten MW, Vandenplas ML, Seibenhener ML, Geetha T, Diaz-Meco MT (2001a) Nerve growth factor stimulates multisite tyrosine phosphorylation and activation of the atypical protein kinase C's via a src kinase pathway. Mol Cell Biol 21:8414-8427.

Wooten MW, Seibenhener ML, Mamidipudi V, Diaz-Meco MT, Barker PA, Moscat J (2001b) The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. J Biol Chem 276:7709-7712.

Wright DE, Zhou L, Kucera J, Snider WD (1997) Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3. Neuron 19:503-517.

Wu D (2005) Neuroprotection in experimental stroke with targeted neurotrophins. NeuroRx 2:120-128.

Wu D, Pardridge WM (1999) Neuroprotection with noninvasive neurotrophin delivery to the brain. Proc Natl Acad Sci U S A 96:254-259.

Wu YC, Williamson R, Li Z, Vicario A, Xu J, Kasai M, Chern Y, Tongiorgi E, Baraban JM (2011) Dendritic trafficking of brain-derived neurotrophic factor mRNA: regulation by translin-dependent and -independent mechanisms. J Neurochem 116:1112-1121.

Wu YJ, Kruttgen A, Moller JC, Shine D, Chan JR, Shooter EM, Cosgaya JM (2004) Nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 are sorted to dense-core vesicles and released via the regulated pathway in primary rat cortical neurons. J Neurosci Res 75:825-834.

Wurm A, Lipp S, Pannicke T, Linnertz R, Krugel U, Schulz A, Farber K, Zahn D, Grosse J, Wiedemann P, Chen J, Schoneberg T, Illes P, Reichenbach A, Bringmann A (2010) Endogenous purinergic signaling is required for osmotic volume regulation of retinal glial cells. J Neurochem 112:1261-1272.

Xie Y, Hayden MR, Xu B (2010) BDNF overexpression in the forebrain rescues Huntington's disease phenotypes in YAC128 mice. J Neurosci 30:14708-14718.

Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH, Reichardt LF (2003) Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci 6:736-742.

Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF (2000) The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J Neurosci 20:6888-6897.

Xu J, Kurup P, Zhang Y, Goebel-Goody SM, Wu PH, Hawasli AH, Baum ML, Bibb JA, Lombroso PJ (2009) Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP. J Neurosci 29:9330-9343.

Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M (2007) Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. Neuron 53:399-412.

Xue L, Murray JH, Tolkovsky AM (2000) The Ras/phosphatidylinositol 3-kinase and Ras/ERK pathways function as independent survival modules each of which inhibits a distinct apoptotic signaling pathway in sympathetic neurons. J Biol Chem 275:8817-8824.

Yabe JT, Chylinski T, Wang FS, Pimenta A, Kattar SD, Linsley MD, Chan WK, Shea TB (2001) Neurofilaments consist of distinct populations that can be distinguished by C-terminal phosphorylation, bundling, and axonal transport rate in growing axonal neurites. J Neurosci 21:2195-2205.

Yamada M, Ohnishi H, Sano S, Nakatani A, Ikeuchi T, Hatanaka H (1997) Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. J Biol Chem 272:30334-30339.

Yamasaki T, Kawasaki H, Arakawa S, Shimizu K, Shimizu S, Reiner O, Okano H, Nishina S, Azuma N, Penninger JM, Katada T, Nishina H (2011) Stress-activated protein kinase MKK7 regulates axon elongation in the developing cerebral cortex. J Neurosci 31:16872-16883.

Yamashita K, Wiessner C, Lindholm D, Thoenen H, Hossmann KA (1997) Post-occlusion treatment with BDNF reduces infarct size in a model of permanent occlusion of the middle cerebral artery in rat. Metab Brain Dis 12:271-280.

Yamashita T, Tohyama M (2003) The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nat Neurosci 6:461-467.

Yamashita T, Tucker KL, Barde YA (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. Neuron 24:585-593.

Yamashita T, Fujitani M, Hata K, Mimura F, Yamagishi S (2005) Diverse functions of the p75 neurotrophin receptor. Anat Sci Int 80:37-41.

Yan Q, Johnson EM, Jr. (1988) An immunohistochemical study of the nerve growth factor receptor in developing rats. J Neurosci 8:3481-3498.

Yan Q, Matheson C, Sun J, Radeke MJ, Feinstein SC, Miller JA (1994) Distribution of intracerebral ventricularly administered neurotrophins in rat brain and its correlation with trk receptor expression. Exp Neurol 127:23-36.

Yanpallewar SU, Barrick CA, Buckley H, Becker J, Tessarollo L (2012) Deletion of the BDNF truncated receptor TrkB.T1 delays disease onset in a mouse model of amyotrophic lateral sclerosis. PLoS One 7:e39946.

Yasuhara T, Borlongan CV, Date I (2006) Ex vivo gene therapy: transplantation of neurotrophic factor-secreting cells for cerebral ischemia. Front Biosci 11:760-775.

Yeaman C, Le Gall AH, Baldwin AN, Monlauzeur L, Le Bivic A, Rodriguez-Boulan E (1997) The O-glycosylated stalk domain is required for apical sorting of neurotrophin receptors in polarized MDCK cells. J Cell Biol 139:929-940.

Yeiser EC, Rutkoski NJ, Naito A, Inoue J, Carter BD (2004) Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice. J Neurosci 24:10521-10529.

Yin Y, Edelman GM, Vanderklish PW (2002) The brain-derived neurotrophic factor enhances synthesis of Arc in synaptoneurosomes. Proc Natl Acad Sci U S A 99:2368-2373.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV (1998) Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 18:3273-3281.

Yoshii A, Constantine-Paton M (2007) BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. Nat Neurosci 10:702-711.

Yoshii A, Constantine-Paton M (2010) Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Dev Neurobiol 70:304-322.

Yoshii A, Murata Y, Kim J, Zhang C, Shokat KM, Constantine-Paton M (2011) TrkB and protein kinase Mzeta regulate synaptic localization of PSD-95 in developing cortex. J Neurosci 31:11894-11904.

Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J, Suhara T, Trojanowski JQ, Lee VM (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53:337-351.

Yu H, Wang DD, Wang Y, Liu T, Lee FS, Chen ZY (2012) Variant brain-derived neurotrophic factor Val66Met polymorphism alters vulnerability to stress and response to antidepressants. J Neurosci 32:4092-4101.

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259-263.

Yu Z, Zhou D, Bruce-Keller AJ, Kindy MS, Mattson MP (1999) Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. J Neurosci 19:8856-8865.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802-809.

Yuan J, Lipinski M, Degterev A (2003a) Diversity in the mechanisms of neuronal cell death. Neuron 40:401-413.

Yuan XB, Jin M, Xu X, Song YQ, Wu CP, Poo MM, Duan S (2003b) Signalling and crosstalk of Rho GTPases in mediating axon guidance. Nat Cell Biol 5:38-45.

Yuste R, Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu Rev Neurosci 24:1071-1089.

Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J 9:3545-3550.

Zafra F, Lindholm D, Castren E, Hartikka J, Thoenen H (1992) Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. J Neurosci 12:4793-4799.

Zajac MS, Pang TY, Wong N, Weinrich B, Leang LS, Craig JM, Saffery R, Hannan AJ (2010) Wheel running and environmental enrichment differentially modify exon-specific BDNF expression in the hippocampus of wild-type and pre-motor symptomatic male and female Huntington's disease mice. Hippocampus 20:621-636.

Zakharenko SS, Patterson SL, Dragatsis I, Zeitlin SO, Siegelbaum SA, Kandel ER, Morozov A (2003) Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. Neuron 39:975-990.

Zala D, Hinckelmann MV, Yu H, Lyra da Cunha MM, Liot G, Cordelieres FP, Marco S, Saudou F (2013) Vesicular glycolysis provides on-board energy for fast axonal transport. Cell 152:479-491.

Zampieri N, Xu CF, Neubert TA, Chao MV (2005) Cleavage of p75 neurotrophin receptor by alpha-secretase and gamma-secretase requires specific receptor domains. J Biol Chem 280:14563-14571.

Zeron MM, Chen N, Moshaver A, Lee AT, Wellington CL, Hayden MR, Raymond LA (2001) Mutant huntingtin enhances excitotoxic cell death. Mol Cell Neurosci 17:41-53.

Zhai J, Zhou W, Li J, Hayworth CR, Zhang L, Misawa H, Klein R, Scherer SS, Balice-Gordon RJ, Kalb RG (2011) The in vivo contribution of motor neuron TrkB receptors to mutant SOD1 motor neuron disease. Hum Mol Genet 20:4116-4131.

Zhang J, Yan H, Wu YP, Li C, Zhang GY (2011) Activation of GluR6-containing kainate receptors induces ubiquitin-dependent Bcl-2 degradation via denitrosylation in the rat hippocampus after kainate treatment. J Biol Chem 286:7669-7680.

Zhang L, Zhang ZG, Zhang RL, Lu M, Adams J, Elliott PJ, Chopp M (2001) Postischemic (6-Hour) treatment with recombinant human tissue plasminogen activator and proteasome inhibitor PS-519 reduces infarction in a rat model of embolic focal cerebral ischemia. Stroke 32:2926-2931.

Zhang Y, Pardridge WM (2001) Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. Stroke 32:1378-1384.

Zhang YH, Chi XX, Nicol GD (2008) Brain-derived neurotrophic factor enhances the excitability of rat sensory neurons through activation of the p75 neurotrophin receptor and the sphingomyelin pathway. J Physiol 586:3113-3127.

Zheng WH, Quirion R (2004) Comparative signaling pathways of insulin-like growth factor-1 and brain-derived neurotrophic factor in hippocampal neurons and the role of the PI3 kinase pathway in cell survival. J Neurochem 89:844-852.

Zhou FQ, Zhou J, Dedhar S, Wu YH, Snider WD (2004) NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. Neuron 42:897-912.

Zhou M, Baudry M (2006) Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. J Neurosci 26:2956-2963.

Zhou N, Gordon GR, Feighan D, MacVicar BA (2010) Transient swelling, acidification, and mitochondrial depolarization occurs in neurons but not astrocytes during spreading depression. Cereb Cortex 20:2614-2624.

Zhu X, Jiang M, Birnbaumer L (1998) Receptor-activated Ca2+ influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells. Evidence for a non-capacitative Ca2+ entry. J Biol Chem 273:133-142.

Zirrgiebel U, Ohga Y, Carter B, Berninger B, Inagaki N, Thoenen H, Lindholm D (1995) Characterization of TrkB receptor-mediated signaling pathways in rat cerebellar granule neurons: involvement of protein kinase C in neuronal survival. J Neurochem 65:2241-2250.

Zuccato C, Ciammola A, Rigamonti D, Leavitt BR, Goffredo D, Conti L, MacDonald ME, Friedlander RM, Silani V, Hayden MR, Timmusk T, Sipione S, Cattaneo E (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. Science 293:493-498.

Zundel W, Swiersz LM, Giaccia A (2000) Caveolin 1-mediated regulation of receptor tyrosine kinase-associated phosphatidylinositol 3-kinase activity by ceramide. Mol Cell Biol 20:1507-1514.

Zwilling D et al. (2011) Kynurenine 3-monooxygenase inhibition in blood ameliorates neurodegeneration. Cell 145:863-874.