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Interaction of opioid and Notch signaling cascades. Implications in dopaminergic differentiation after morphine administration.

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Interaction of opioid and Notch signaling cascades. Implications in dopaminergic differentiation after morphine administration

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CERTIFICAN:

Que el trabajo titulado **“Interaction of opioid and Notch signaling cascades. Implications in dopaminergic differentiation after morphine administration”**, que presenta el doctorando D. Adrián García Concejo, ha sido realizado bajo su dirección en el Instituto de Neurociencias de Castilla y León, de la Universidad de Salamanca, y reúne a su juicio, originalidad y contenidos suficientes para que sea presentado ante el tribunal correspondiente y optar al Título de Doctor por la Universidad de Salamanca.

Y para que así conste, y a los efectos oportunos, expiden el presente certificado.

Salamanca a 28 de Junio de 2017.

Fdo. Prof. Raquel E. Rodríguez Rodríguez

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“Sometimes science is more art than science. A lot of people don’t get that”

Rick Sanchez, Earth dimension C-137

Abbreviations used in this work

AC: Adenylate cyclase

AMPS: Ammonium persulfate

CaM: Calmoduline

CaMKII: Calmoduline kinase II

CaMKIV: Calmoduline kinase IV

cAMP: Cyclic adenosine-monophosphate-3',5'

ChIP: Chromatin immunoprecipitation

CNS: Central Nervous System

CREB: cAMP response element binding

CSL: Hairless supressor

Ct: Cycle threshold

DA: Dopamine/Dopaminergic

DAPI: 4,6-diamin-2-phenilindole

DAPT: N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

DMSO: dimetil sulphoxide

DSL: Delta-Serrate-Lag2

EGF: Epidermal growth factor

ES: embryonic stem

FOXA2: forkhead box protein A2

gDNA: Genomic DNA

GFP: Green Fluorescent Protein

H3K4Me3: trimethylated lysine 4 of histone 3

Hes1: hairy and enhancer of split-1

HpF: hours post fertilization

IHC: Immunohistochemistry

IKB: inhibitor of $\kappa\beta$

IKK β : inhibitor of $\kappa\beta$ kinase

IP3: inositol 1,4,5-trisphosphate

IP-WB: immunoprecipitation-western blot

ISH: *In situ* hybridization

LC: *Locus Coeruleus*

MAP: Mitogen activated protein

Mb: *Mindbomb* mutants

MBNL1: Muscleblind Like Splicing regulator 1

MeCP2: methyl CpG binding protein 2

MEK1/2: Mitogen-activated protein kinase kinase

miRNA: MicroRNA

Mis: mismatch

Mo: Morphant

MPTP: 1-metil-4-phenil-1,2,3,6-tetrahidropyridine

mRNA: Messenger RNA

Nacc: *Nucleus accumbens*

NECD: Notch extracellular domain

NFDM: Non-fat dried milk

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells

NGN2: neurogenin2

NICD: Notch intracellular domain

NSS: Neural stem spheres

NURR1: nuclear receptor related 1, also Nr4a2a.

Nx: Naloxone

OPRD1/2: Delta opioid receptor 1/2

OPRM1: Mu opioid receptor 1

OPRK1: Kappa opioid receptor 1

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

pCREB: phospho CREB

PFA: Paraformaldehyde

PI3K: Phosphatidil inositol 3 kinase

PITX3: Paired-like homeodomain transcription factor 3

PKA: Protein kinase A

PKC: Protein kinase C

PMSF: Phenylmethylsulfonyl fluoride

Pre-miRNA: precursor miRNA

Pri-miRNA: primary miRNA

PTU: Phenylthiourea

RE1: repression complex REST binding site

REST: RE1-Silencing transcription factor

RISC: RNA-induced silencing complex

RT: Room temperature

SDS: Sodium dodecil sulphate

SIRT1: sirtuin1

SRSF2: Serine and arginine rich splicing factor 2

SRSF3: Serine and arginine rich splicing factor 3

TBS: Tris-Buffered Saline

TEMED: Tetramethylethylenediamine

Tg: Transgenic

TH: Tyrosine hydroxylase

Tm: Melting temperature

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

U0126: 1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate

UTR: Untranslated region

VTA: Ventral tegmental área

Wt: Wild type

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Introduction

This work presents new insights into the molecular pathway triggered after morphine administration in zebrafish embryos. The changes in miR-212, miR-132 and miR-29a regulate opioid and Notch signaling activity, which alter the differentiation of dopaminergic neurons. These findings lead to a better understanding of the addictive properties of morphine.

Morphine

Opioids, primarily used for pain relief, are still nowadays the preferred analgesics for the treatment of chronic pain. Among them, morphine is the most used molecule, although it is well known for inducing the appearance of tolerance and addiction after prolonged administration. This opiate binds to the mu opioid receptor (Oprm1) with the highest affinity and, therefore, the effects of this drug, especially in the central nervous system (CNS), are mediated by the activation of this receptor (Pert et al., 1987). Opioid receptors are G-protein coupled receptors and the signaling cascade triggered by them induces the activity of several transcription factors, including cAMP Response Element Binding (CREB) protein (Impey et al., 2004; Ma et al., 2001), a transcription factor involved in many molecular pathways by controlling the transcription of different genes. Morphine also alters calcium and potassium signaling and controls PKA activity by the inhibition of adenylate cyclase (AC); in this way it also induces changes in gene expression in the target cell (Kumar et al., 2010). It is also known that morphine regulates nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation through IP3/Akt pathway, although the molecular mechanism implicated in this situation still remains uncharacterized (Chen et al., 2006).

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The endogenous opioid system has been characterized in zebrafish (Barrallo et al., 2000; Rodriguez et al., 2000; Alvarez et al., 2006; Pinal-Seoane et al., 2006). Zebrafish express a mu opioid receptor (Oprm1), two delta opioid receptor duplicates (Oprd1 and Oprd2), a kappa opioid receptor (Oprk1) and an opioid receptor-like.

The opioid-induced drug addiction pathway has been suggested to involve the midbrain dopaminergic neurons located within ventral tegmental area and the nucleus accumbens (NAc). The alteration of dopamine levels in this region produces neuronal sensitization or desensitization, depending on the drug used. It has also been established that morphine increases dopamine levels through Oprm1 in the NAc, which may mediate the reinforcing effects of morphine (Gianoulakis, 2004). In relation to these observations, the endogenous opioid peptides, such as enkephalins or dynorphin, are up-regulated in the NAc after exposure to morphine and modulate dopamine release in the midbrain (Gieryk et al., 2010).

In addition, morphine is able to induce changes in the miRNA expression profile of the cell (Hwang et al., 2012). The modifications in the normal expression of miRNAs in zebrafish embryos could explain some of the molecular changes that induce tolerance, one of the most characteristic processes that are triggered by addiction. Therefore, the studies on the probable opioid regulation triggered by morphine leading to alterations of miRNA expression profile and the dysregulation of several transcription factors could explain the changes in dopaminergic activity in zebrafish, which could terminally provide insights into the embryonic development during chronic exposure to the drug. The analysis of the molecular signaling cascade triggered by morphine may lead to unveil new molecular targets that could be used to palliate the development of tolerance and addiction caused by the administration of opiate drugs.

miRNAs

Since their discovery, microRNAs (miRNAs) have emerged as outstanding promising tools in the modulation of the expression of many genes (Bartel, 2004). miRNAs are small non coding RNA transcripts that regulate gene expression at the post-transcriptional level. miRNAs are generated as a primary miRNA (pri-miRNA) by RNA polymerase II (Slezak-Prochazka et al., 2010). At least 30% of the miRNAs on the cell are organized in clusters (Megraw et al., 2007), i.e. polycistronic pri-miRNAs that after posterior processing lead to different mature mRNAs. The mechanism by which the splicing induces the mature RNA of clustered miRNAs is still unknown. Moreover, the relative quantities of each mature miRNA generated as a unique pri-miRNA are not equal (Mohammed et al., 2014), indicating that the process of splicing is not only a maturation process but also a regulatory mechanism that controls the expression of each of the mature miRNAs. After the pri-miRNA has been transcribed, it is cleaved by several processing enzymes such as Dicer, leading to a shorter hairpin-like structured named precursor miRNA (pre-miRNA). After this processing, pre-miRNAs are transported into the cytoplasm by exportin-5 and they suffer another splicing by the processing enzyme Drosha. After this process, two mature miRNAs are generated. In general terms, only one of these two miRNAs exert its function, although several cases have been described in which both molecules may have their own regulatory roles. These molecules control gene expression by binding to complementary sequences in the 3' untranslated region (3' UTR) of their target mRNA transcripts to facilitate their degradation and/or inhibit their translation (Bartel, 2009), although binding to other regions have been described (Doench and Sharp, 2004; Greco and Rameshwar, 2007). miRNAs are involved in many developmental processes, such as differentiation and proliferation and their dysregulation has been linked to a wide variety of diseases (Sidhu

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et al., 2015; Song et al., 2015). miRNAs, such as miR-let7d and miR-133b (Sanchez-Simon et al., 2010a; Lopez-Bellido et al., 2012; Garcia-Perez et al., 2015), have been described to be regulated by morphine and play a role in the behavioral effects of this drug.

miR-212 and miR-132

miR-212 and miR-132 form a cluster, although their functions may not be fully correlated (Kumarswamy et al., 2014; Wanet et al., 2012). Previous studies reported that the expression of miR-212 was increased in the dorsal striatum of rats with extended but not restricted daily access to intravenous cocaine self-administration (Vo et al., 2005; Im et al., 2010). Moreover, CREB activation has been shown to regulate miR-212 expression after the exposure to cocaine in murine models (Hollander et al., 2010). Although these miRNAs may control the same subset of genes due to their similarity in their seed region (which has a 100% homology (Wanet et al., 2012)), miR-132 has been specifically related to dopaminergic differentiation through the modulation of the levels of Nurr1 (Yang et al., 2012). miR-212/132 expression is also regulated by the RE1-Silencing Transcription factor (REST) which is known to act as a corepressor of transcription by its binding to RE1 sites together with methyl CpG binding protein 2 (MeCP2). The CpG islands within these sites must be methylated in order to obtain an effective binding of MeCP2 (Namiyama et al., 2008).

miR-29a

miR-29 family includes miR-29a, miR-29b-1, and miR-29c. Mature miR-29s are highly conserved in human, mouse, rat and zebrafish. They also share identical sequences at nucleotide positions 2–7, indicating that they may regulate related target genes and for this reason predicted target genes for the miR-29 family members are usually overlapped. Recent studies have identified several critical cis-elements in the proximal region of miR-29 gene promoters, including three NF- κ B binding sites at –561, –110, and +134 in the human miR-29b-1/a promoter (Mott et al., 2010).

In addition to the presence of these binding sites, miR-29 expression has been shown to be regulated by various transcriptional regulators and signaling pathways. miR-29a transcription is regulated by Wnt signaling (Kapinas et al., 2010), which may indicate a critical role for this miRNA in early development. NF- κ B plays a central role in the regulation of myoblasts proliferation and differentiation in part by regulating the YY1-miR-29 negative regulatory circuit. Developmental changes and cell type-specific distribution of miR-29 expression, such as that reported in the murine lung (Cushing et al., 2011), further support the presence of dynamic regulation of the expression of miR-29.

miR-29a has also been shown to regulate the expression of ADAM12 expression by binding to the 3'UTR region of the mRNA (Li et al., 2011). This metalloproteinase is required for the correct cleavage and activation of Notch, which may reinforce the statement that this miRNA plays a key role in controlling the early gene expression and the differentiation pattern of the organism.

Notch signaling pathway

The Notch pathway mediates juxtacrine communication, playing a critical role in cell-cell recognition. Furthermore, Notch signaling controls the differentiation of cells that are in contact. The Notch locus was first cloned in *Drosophila* encoding a type I transmembrane protein (Wharton et al., 1985). Notch receptors possess epidermal growth factor (EGF) repeats that mediate interaction with their canonical activators. The ligands for the receptor have been characterized: Delta and Serrate, in the Delta-Serrate-Lag2 (DSL) family. Both the receptors and the ligands are single transmembrane proteins and, therefore, to trigger the signaling cascade, cell-cell contact is required (Andersson et al., 2011; Greenwald and Kovall, 2013). The role of Notch in the development of multicellular species has been extensively studied (Dumortier et al., 2005; Radtke et al., 2005; Aster, 2014). Notch signaling relies on cellular interaction. Hence, Notch affects stem cell maintenance, cell fate choice, cell differentiation, lineage progression and apoptosis (Bray, 2006; Hori et al., 2013). Despite its multiple roles and versatility, the Notch pathway is relatively simple and is found to be conserved across species (Bray, 2006; Kopan and Ilagan, 2009). In mammals, there are four Notch receptors (NOTCH1-4) and five DSL ligands (JAG1-2 and Delta-like 1-3-4). During its maturation, Notch undergoes ligand-independent cleavage by a furin-like convertase in the trans-Golgi (Fiuza and Arias, 2007; Hori et al., 2013). This first cleavage (the S1) results in the production of a heterodimeric receptor comprised of a transmembrane/intracellular fragment non-covalently bound to the Notch extracellular domain (NECD). Notch is thus presented to the cell surface as a heterodimer. The second cleavage exposes the third cleavage site, S3, and is considered a rate-limiting step for the third and last cleavage (Brou et al., 2000; Mumm et al., 2000). Upon cleavage at the S3 site by a γ -secretase complex, the Notch intracellular domain (NICD)

is released (Schroeter et al., 1998) and translocates into the nucleus to activate transcription of target genes. Notch signaling does not require the use of second messengers. The activity is exclusively driven by nuclear concentration of NICD (Struhl and Adachi, 1998; Ehebauer et al., 2006). In the nucleus, NICD binds a bi-functional transcription factor Hairless suppressor (CSL) and a variety of other coactivators involved in the transcriptional activation of Notch target gene expression (Kopan and Ilagan, 2009; Hori et al., 2013). During this process, some kinases may phosphorylate NICD (Wu et al., 2000; Kitagawa et al., 2001; Nam et al., 2003), a crucial step in the regulation of NICD stability and activity (Espinosa et al., 2003).

Notch signaling has also been related to the differentiation of dopaminergic neurons (Tio et al., 2011; Trujillo-Paredes et al., 2016). The dysregulation of this pathway could affect the differentiation process and, therefore, control some aspects of the addictive development after morphine intake.

Dopaminergic neurons

Dopaminergic neurons (DA neurons) have been widely studied due to their value in cell replacement therapy, especially for Parkinson's disease (Collier et al., 2003; Lindvall et al., 2001; Perrier et al., 2003). The use of dopaminergic neurons for in vitro research on disease mechanisms, such as neuronal degeneration in Parkinson's disease is of extreme importance due to the lack of information about this pathology. Moreover, the role of central dopaminergic systems in the acute effects of drugs of abuse has been known since long ago (Wise, 1987; Di Chiara and Imperato, 1988). Hence, the better understanding of the role of dopaminergic neurons in development and maintenance of

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drug abuse is of extreme interest for the possible development of new therapies to solve this problem (Jentsch et al., 2000; Pierce and Bari, 2001; Volkow et al., 2002).

The midbrain dopaminergic neurons in the zebrafish embryo are organized into seven different groups (Figure 1).

Figure 1.- Localization of the seven dopaminergic groups of the zebrafish embryo at 96 hpf. The numbers indicate each subset of dopaminergic neurons in the zebrafish brain. LC, locus coeruleus. Modified from Schweitzer and Driever, 2009.

The rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH) is the most widely used marker to identify differentiated mesencephalic dopaminergic neurons (mDA neurons). However, the expression of other common mDA genes, such as the expression of the dopamine transporter SLC6A3 (formerly known as DAT), the vesicular monoamine transporter SLC18A2 (formerly known as VMAT2) and dopa decarboxylase (DDC) can also be used to characterize terminal differentiation. The expression of the mDA neuron-specific paired-like homeodomain transcription factor 3 (PITX3), and the nuclear receptor related 1 protein (NURR1) are required to terminally

induce mDA neuron identity. NURR1 induces TH expression, which eventually leads to differentiation into dopaminergic neurons. NURR1 has been demonstrated to induce differentiation in CNS precursor cells *in vitro* but they require additional factors to reach full maturity and dopaminergic differentiation (Kim et al., 2003). In contrast, the requirement of PITX3 expression is not fully characterized, and this factor has been related to dopaminergic survivability (Smits, 2006). In the last decade, a more progressively detailed picture of the signaling network of secreted molecules, cell surface receptors and transcription factors that control regionalization, specification, and differentiation of mDA progenitors has emerged. mDA neuron development has been studied extensively in chick and in rodents.

The opioid-induced drug addiction pathway has been suggested to involve the midbrain dopaminergic neurons located within the ventral tegmental area (VTA) and the nucleus accumbens (Nacc) (Kosten et al., 2002). The alteration of dopamine levels in this nucleus induces neuronal sensitization or desensitization. It has also been established that morphine increases dopamine level through Oprm1 in the NAc, which may be the pathway mediating the reinforcing effects of morphine (Gianoulakis, 2009).

Therefore, the studies on the regulation of opioid and Notch signaling, mediated by several miRNAs or by alterations on several dopaminergic transcription factors could provide new insights on the embryonic development during chronic exposure to morphine, which could terminally lead to alterations in the CNS after drug intake in both gestating and adult individuals.

Hypothesis and Objectives

Morphine is not only the most used analgesic at present, but it is also a molecule of important interest because of its ability to induce addiction by activating the reward mechanisms of the brain. The addictive properties of several other drugs have been related to the dysregulation of several non-coding RNAs, such as miR-212 or miR-132. The alteration of miRNA expression could affect downstream targets involved in developmental processes. Among the neuronal subtypes of the brain, dopaminergic neurons are involved in the reward system. Notch is also involved in the development of the CNS. Taking these facts into consideration, we propose the following three step hypothesis:

- a) Morphine modulates dopaminergic differentiation through the alteration of the transcription factors that induce the correct differentiation of dopaminergic neurons
- b) There is a possible cross-talk between opioid and Notch pathway in the regulation of the differentiation of dopaminergic neurons.
- c) These two signaling cascades may be regulated by several transcription factors such as CREB or NF- κ B, or by alterations of miR-212, miR-132 or miR-29a.

To validate this three-step hypothesis, we propose the following objectives:

- 1.- To determine whether morphine is exerting changes in the expression of miR-212 and miR-132, and to find out the roles of miR-212 and miR-132 as putative targets of opioid signaling. Also, to analyze if there is a putative relation between the pri-miR-212/132 and the differential expression of the mature forms miR-212 and miR-132.
- 2.- To analyze if the dysregulation of miR-212 and miR-132 is modifying mu opioid receptor expression and to determine the implication of Notch pathway in this process through the dysregulation of these two miRNAs.

Hypothesis and objectives

3.- To determine whether these alterations in miR-29a expression are mediated through NK-k β signaling, therefore regulating Notch cleavage through ADAM12, as previously described.

4.- To analyze the alteration of the levels of the transcription factors NGN2, FOXA2, SIRT1, NURR1, PITX3 after

a) The activation of the opioid pathway by morphine administration

b) The disruption of Notch signaling using DAPT, and whether this influence dopaminergic neuron differentiation, measured by the levels of expression of TH.

Also, it is of extreme interest to determine whether miR-212, miR-132 and miR-29a are playing a critical role by targeting downstream targets of this differentiation process, such as the transcription factors previously mentioned, and if this is mediated by opioid and Notch signaling.

5.- To analyze the influence of the possible alterations in the miRNAs and transcription factors expression in neuronal protection against oxidative insult mediated by MPTP.

The changes observed in the expression of transcription factors such as NGN2, PITX3 and NURR1 may induce neuroprotective effects specifically in dopaminergic neurons.

Material and methods

Experimental animals

Experiments were performed using the wild-type AB zebrafish line, the transgenic (tg) [-3.9*nestin:GFP*] fish (Lam et al., 2009) or *mindbomb* mutants (kindly provided by Dr. Patrick Blader, from the Université Paul Sabatier, Toulouse, France). Zebrafish were bred and raised in the Fish Facilities of our Lab. following standard protocols (Westerfield, 2000). In all experiments, adequate measures were taken to minimize pain or discomfort and animals were handled according to the guidelines of the European Communities Council Directive 2010/63/UE, to the current Spanish legislation for the use and care of animals RD 53/2013 and to the Guide for the Care and Use of Laboratory animals as adopted and promulgated by the U.S. National Institutes of Health. For ISH experiments, embryos were fixed in 4% paraformaldehyde, and for RNA extraction embryos were frozen in liquid nitrogen. These experiments were approved by the University of Salamanca Ethics Committee.

Drug Treatment and inhibitors

Zebrafish embryos were exposed to 10 nM or 10µM morphine-sulphate in E3 embryo buffer from 5 hours post fertilization (hpf) (50% epiboly) to 24/48 hpf. Fresh morphine solution was re-added at 24 hpf. A control group (E3 only) was used in parallel for each experiment. Morphine was provided by the Spanish Ministry of Health. The Naloxone dose used on these assays was 10µM. Concentrations used for each inhibitor were: U0126 (Promega) 20µM, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7, Sigma) 10µM, Forskolin (Sigma) 20µM, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) 20µM and Resveratrol 20µM. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) doses used in the apoptosis assays were 40µM.

Material and methods

Morpholino microinjection

The morpholino antisense (MO) oligomers used to knockdown *oprm1*, miR-212 and miR-132 were purchased from Gene Tools, LLC. The MO was diluted in sterilized water to a stock concentration of 1 mM. In addition to the three MO experimental groups (untreated, 10 nM morphine, and 10 μ M morphine), each experiment included a control MO group injected with morpholino that exhibits no binding target or biological activity (control morpholino). Morpholino was injected into the yolk at the one cell stage according to the published protocols (Nasevicius and Ekker, 2000). The concentrations of target MO and control MO used were 0.33 and 1 μ M, respectively (3 nl were injected into each embryo). The sequences of the morpholinos used are compiled in Table 1.

Gene	Morpholino Sequence
<i>oprm1</i>	AATGTTGCCAGTGTTTTCCATCATG
<i>miR-212</i>	AGCCATGACTGTAGACTGTTACTGT
<i>miR-132</i>	ACAGTAACAATCTAATGCCACGGTC

Table 1. Sequence of the morpholinos used to knock-down the genes studied in this work.

RNA Extraction and qPCR

Total RNA, including miRNA, was extracted using Trizol (Life Technologies), following the manufacturer's protocol. To study the levels of expression of the miRNAs of interest, a previous polyadenylation reaction was performed using Poly-A polymerase (New England Biolabs) and a retrotranscription was carried out using M-MuLV retrotranscriptase (New England Biolabs), as described in the manufacturer's

protocol. The retrotranscription of *oprm1* samples were carried out using the High Capacity RT Kit (Applied Biosystems). The absolute quantification of the PCR products was accomplished with a standard curve using the SYBR-Green method, and specifically designed oligonucleotides as described by Balcells et al. (2011) when miRNAs were detected. SYBR-Green was included in a 2× Master Mix (Life Technologies, SYBR Green PCR Master Mix). The final volume of each reaction was 20 µl, distributed as follows: 10 µl of Master Mix, 1.2 µl of each oligonucleotide, 7.4 µl of distilled water, and 1 µl of cDNA in a concentration of 25 ng/µl. A standard curve was constructed for each experiment by serial dilutions of cDNA. The amplification reaction were performed in an ABI 7300 qPCR Thermal Cycler (Applied Biosystems), with the following conditions: 15 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 70°C. qPCR was performed by triplicate, and each experiment was repeated with three different samples. The sequences of the primers used are collected in the Table 2.

Gene	Primer F	Primer R
<i>oprm1</i>	ACGAGCTGTGCAAGATTGTG	CCGATTGCAGATGAAAGAT
<i>miR-212</i>	AGTAAACAGTCTACAGCCATG	TCCATTTTTTTTTTTTTTTTTTGTCTG
<i>miR-132</i>	CGCAGTAACAGTCTACAGTC	CCAGTTTTTTTTTTTTTTTTTTTGCCAT
<i>primiR-212/132</i>	TCTGGCAACCTTCCACAG	TGGAGACAGCAGGAGGG
<i>miR-29a</i>	GTAGCACCATCTGAAATCG	GGTCCAGTTTTTTTTTTTTTTTGTTA

Table 2. Sequence of the primers used in qPCR amplification.

Material and methods

Total protein extraction, immunoprecipitation and western blot from zebrafish embryos

Total proteins were extracted from zebrafish embryos at 24 and 48 hpf using cold Ringer solution and 300 μ l of protein extraction buffer (10 mM tris pH 7.4, 2% triton X 100, 1 mM PMSF, 1 μ l/ml protease inhibitors [Sigma]). Embryos were aspirated using a syringe and centrifuged 10 minutes at 10000g at 4°C. The supernatant containing proteins was frozen at -80°C. Proteins were quantified by Bradford method. 350 μ g of total protein were used for the immunoprecipitation of each group and incubated with 25 μ l of Oprm1 antibody (Spanish patent license: P201330193) conjugated with protein A agarose beads. Beads were washed with lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS, 1x protease inhibitors) and centrifuged at 14000 rpm. Pellets were resuspended in 25 μ l of 4X loading buffer (10% SDS, 600 μ l of glycerol, 150 μ l of 2-mercaptoethanol, 1M Tris-HCl pH: 6.8 and 30 μ l of Bromophenol blue). Samples were boiled for 5 minutes and centrifuged at 14000 rpm. Supernatants were loaded in 10% polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked with 5% milk in TBS. Oprm1 primary antibody was used at 0.25 μ g/ml to detect the immunoprecipitated protein. After incubation, membranes were washed with TBS containing 0.1% Tween 20 and incubated for 1 hour with the goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology) diluted 1:5000. For NF- κ B western blot, the primary antibody (Abcam, ab106129) dilution used was 1:750, incubated over night at room temperature, and the secondary antibody dilution was anti-Rabbit 1:10000 for 1 hour. Blots were developed with ECL (Amersham). Experiments were performed in triplicates and quantified with ImageJ software. Actin (1:1000, thermo) was used as loading control gene.

Western blot from cultured cells

Proteins from NSS and neuronal precursors were extracted using RIPA buffer (150 mM NaCl, 1% triton X-100, 50 mM tris pH 8.0) and adding 1mM PMSF, 50 mM NaF and 1 µl/ml protease inhibitors (Sigma). Cells were maintained in this medium for 30 minutes at 4°C with occasional vortex and centrifuged at 13000 g for 10 minutes.

Protein	Membrane	Blocking reagent	Primary antibody dilution	Secondary antibody dilution (in tTBS)
OPRM1 ab63256	Nitrocellulose	3% NFDM	1:100	Anti-Ms 1:3000
NICD ab8925	PVDF	1% NFDM	1:500	Anti-Rb 1:500
NURR1 ab41917	PVDF	5% NFDM	1:1000	Anti-Ms 1:3000
PITX3 ab30734	Nitrocellulose	5% NFDM	1:250	Anti-Rb 1:10000
NGN2 PA5- 11892	Nitrocellulose	5% NFDM	1:1000	Anti-Rb 1:10000
TH AB152	PVDF	5% BSA	1:1000	Anti-Rb 1:1000

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FOXA2 PA5-19518	Nitrocellulose	5% NFDM	1:1000	Anti-Rb 1:2000
SIRT1 ab110304	Nitrocellulose	5% NFDM	1:1000	Anti-Ms 1:5000

Table 3. Summary of the conditions of each western blot performed on the proteins extracted from cultured cells. NFDM: non-fat dried milk. BSA: bovine serum albumine.

30µg of total protein were loaded in 8% polyacrylamide gels, and transferred into nitrocellulose or PVDF membranes. The blocking of the membrane was carried out with non-fat dried milk (NFDM) or BSA. The kind of membrane, the quantity of each primary antibody (all from Abcam except for NGN2 and FOXA2 antibody, purchased from Thermo-Scientific and TH, purchased from Millipore) and secondary antibody (Santacruz Biotechnology) are included in Table 3. Blots were developed with ECL (Amersham). Experiments were performed in triplicates and quantified with ImageJ software. Actin (1:1000, thermo) was used as loading control.

Whole-mount in Situ Hybridization (WISH) and immunohistochemistry (IHC)

Embryos at 24 hpf and 48 hpf were dechorionated, fixed with 4% paraformaldehyde (PFA) in phosphate saline buffer (PBS) overnight at 4°C, washed twice in PBS for 5 min at room temperature (RT), and stored in absolute methanol at -20°C until use. the probes used for this experiment were designed as described by He et al. (2011) and the hybridization was carried out as described by Sanchez-Simon et al. (2010b). Briefly, a

permeabilization step using methanol was performed. Proteinase k treatment was carried out to improve the probe penetration and a blockage step was done with NGS. Probes were incubated overnight. The immunohistochemistry against TH was performed as described by Barreto-Valer et al. (2013). Briefly, embryos were fixed in 4% (w/v) PFA (Sigma) in phosphate-buffered saline (PBS) overnight at 4 °C and stored in methanol (Sigma) at – 20 °C for at least 1 day. After rehydration, a blocking step was carried out with 5% (v/v) goat serum (Sigma) in PBS. The concentration of TH antibody (Millipore, AB152) was 1:1000, incubated at 4°C overnight, and the secondary antibody was 1:250, incubated one hour simultaneously with DAPI as a nuclei marker (1:1000).

Luciferase assay

For chemiluminescence detection we used the Dual-Glo Luciferase Assay (Promega). The sequences were cloned into PmirGlo (Promega) using the protocol for digestion, ligation and transformation described in GeneClip™ U1 Hairpin Cloning Systems (Promega). HEK293 cells were transfected with a wild-type and mutated construct using Lipofectamine 2000 (de Chevigny et al., 2012). Briefly, 300ng of reporter plasmid were diluted in 25 µl of DMEM in combination with 20 nM or 50nM of miR-212 (small, chemically modified double stranded RNAs that mimic endogenous miRNAs by up-regulation of miRNA activity) or 50nM of miR-1 mimics (Ambion) when required. This solution was combined with diluted Lipofectamine (1,5 µl/well) and the transfection was carried out for 48 hours at 37°C in a 5% CO₂ incubator. Luminescence was measured during 10 seconds as recommended by the manufacturer using a luminometer (Appliskan, Thermo Scientific). The ratio between luminescence from firefly and the control (Renilla) was used to check if the miRNA was binding to the sequence cloned into the plasmid. The mean for each group (n = 8) was calculated

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and normalized with the wells transfected only with the empty plasmid. The primers annealed and sub- cloned into the vector are compiled in Table 4.

	Primer Sequence
wtF	CTAGCTAGGTACCTAGTTAGTTTGAGGAAAGACTGTTGG
wtR	TCGACCAACAGTCCTTCCTCAAACCTAACTAGGTACCTAG
misF	TAGCTAGG TACCTAGTTAGTTTGAGGAAATCAGTGTGG
misR	TCGACCACACTGATTCCTCAAACCTAACTAGGTACCTAG

Table 4. Sequence of the primers annealed and cloned into pmiRGlo vector prior to HEK293 transfection.

Primary neuron cultures from zebrafish embryos

Brains from 42 hpf zebrafish embryos were placed in 1x E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO₄, 150 µM KH₂PO₄, 50µM Na₂HPO₄, 1 mM CaCl₂, 0.7 mM NaHCO₃, 0.5 mg/l methylene blue) with 1x tricaine as described by Chen et al. (2013). After dissociation, cells were grown in Dulbecco's modified medium up to 48 hours. Morphine (1 nM) was added after plating. 48 hours later neurons were fixed in 4% paraformaldehyde (PFA) (w/v).

Mouse embryonic stem cells

Cells were differentiated from R1/E embryonic stem (ES) cells stage (ATCC SCRC-1036), into neuronal stem spheres (NSS) and into neuronal precursors following the protocol established by Lau et al., (2006). Briefly, R1 mouse ES cells were cultured in knock-out Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10%

fetal bovine serum (FBS, Gibco), 100 mM MEM non-essential amino acids, 2 mM L-glutamine (Gibco) and 100mM β -mercaptoethanol (Sigma). ES cells were grown on 0.1% gelatin-coated dishes in presence of leukemia inhibitory factor (LIF, 1000 U/ml, Gibco). To produce neuronal stem spheres (NSS), ES cells were washed in 1x PBS, trypsinized until detachment at 37°C and resuspended in knock-out DMEM without the addition of LIF but supplemented with EGF (20 ng/ml), bFGF (10 ng/ml) and L-ascorbic acid (100 μ M, all from Sigma). The cells were then transferred to a bacteriological dish and grown in suspension. NSSs were collected 4 days after and trypsinized for 10 minutes at 37°C in a water bath. The cells were reconstituted in proliferation medium (1:1 DMEM/F12 supplemented with 10% FBS, 2 mM L-glutamine, 20 nM progesterone, 100 mM putrescine, 25 mg/ml insulin, 50 mg/ml transferrin, 30 nM sodium selenite [all from Sigma]) and plated on gelatine-coated dishes in presence of laminin and fibronectin to differentiate into neuronal precursors. Medium was changed daily and, four days after plating, cell medium was replaced with differentiation medium (Neurobasal medium supplemented with 10% FBS, 2% B27-supplement (all from Gibco) and L-ascorbic acid (100 mM, from Sigma)). Medium was replaced each two days, and cells were trypsinized and harvested or fixated in function of the experiment to be performed.

SH-SY5Y cell culture

The neuroblastoma SH-SY5Y cell line was cultured in DMEM (Dulbecco's modified Eagle's medium):HAM F-12 (1:1) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Gibco, Life Technologies), at 37 °C in humidified atmosphere containing 5% CO₂ in a Forma

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incubator. Cells were trypsinized or fixated with 4% PFA in function of the ongoing experiment.

Time-lapse imaging

Time-lapse pictures were taken with a live-cell microscopy (Cell Observer, Zeiss). Primary neuron cultures were separated in four different experimental conditions: untreated, 10nM morphine, 10μM DAPT and coadministration of morphine and DAPT. Cells were recorded for 8 hours.

Chromatin immunoprecipitation

ChIP-qPCR experiments were performed following the protocol established by Bogdanovic et al. (2013) and adapted with protocols from Lindeman et al. (2009) and Aday et al. (2011). Sonication times were adjusted to our experimental conditions using up to 10 minutes per sample with 30 seconds on/off cycles. 7,5 μl of anti-pCREB antibody (44-298G, Invitrogen) were used per sample. DNA shearing was corroborated by electrophoresis and only fragments of 400 Kb were used. qPCR was performed as described by Bogdanovic et al., (2013) and with two different sets of primers designed for the analyzed region (Table 5). Non-immunoprecipitated DNA (input DNA) was used to normalize Cts as follows: $\Delta Ct_{\text{normalized ChIP}} = Ct_{\text{ChIP}} - [Ct_{\text{Input}} - \text{Log}_2(\text{Input Dilution Factor})]$. Experiments were performed in triplicates.

Gene	Primer F	Primer R
<i>miR-Prom1</i>	TTCAGGGTCACAACAAGCAA	GGTTATGCAAGCCTAATTCAGT
<i>miR-Prom2</i>	AAGCAACACTTTAGTTTTTCCTC	TGCTGGTTGTGATGTGCAT

Table 5. Sequence of the primers used to amplify the CRE region of the miR-212/132 promoter in the ChIP-qPCR experiments.

DNA methylation analysis

Genomic DNA was obtained with the Genomic DNA purification kit following the manufacturer's instructions. Bisulfite conversion was performed with the EZ DNA methylation kit (Zymo Research) incubating for 30 minutes at 42° with the dilution buffer. The sequence corresponding to the RE1 site of the miR-212/132 promoter was bisulfite converted and amplified by nested PCR. The oligonucleotides were designed with the bioinformatic tool Bisulfite Primer Seeker (Table 6). The fragment was purified from PCR product (PCR purification kit [Promega]), subcloned into pGEM-T® (Promega) plasmid and transformed into Top10F' cells. Positive colonies were sequenced by the bioinformatics service of the University of Salamanca and analyzed with BiQanalyzer software (Bock et al. 2005).

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Gene	Primer F	Primer R
<i>miR-212/132</i> <i>promoter</i>	ATTTTGTGAAYGTTTGAGTAGTG AAATTATTTTG	AAATACATTCRCAATAA ACAAAAATCTCAAATAT C
<i>miR-212/132</i> <i>nested</i>	TTAGGGTTATAATAAGTAATATT TTAGTT	TTCCCCAACTCTATATTA ATTACAATA

Table 6. Sequence of the primers used to amplify the RE1 site region of the miR-212/132 promoter in the methylation analysis experiments. Y corresponds to pyrimidines and R to purines.

TUNEL assay

To determine the percentage of apoptotic cells the Click-iT® Plus TUNEL Assay kit was used (Thermo-Fischer) following the manufacturer's protocol. Briefly, SH-SY5Y cells were grown on coverslips and fixated with 4% PFA for 10 minutes. The fixative was retired and cells were washed thrice with cold PBS. Cells were then treated with the permeabilization reagent (0.25% Triton® X-100 in PBS) for 20 minutes completely covering the coverslips and then washed twice with deionized water. To induce the TdT reaction cells were treated with 100 µM of TdT Reaction Buffer (Component A) for 10 minutes, which was then replaced with TdT reaction mixture (47µl TdT reaction buffer 1X, 1µl EdUTP 50x, 2µl TdT enzyme 15U/µl) for an hour at 37°C. After the incubation, cells were washed thrice with PBS. Then, 50µL of Click-It® Plus TUNEL reaction cocktail was added to the coverslips and was incubated for 30 minutes at 37°C protected from light. Cells were then washed with 3% BSA in PBS for 5 minutes. Finally, cells were incubated with 1:1000 DAPI to mark cell nuclei and pictures were taken using a live cell microscope (Zeiss).

Bioinformatical analysis

The comparative analysis of the intergenic region of the cluster miR-212/132 was carried out using Clustal Omega server (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The determination for the existence of splicing enzymes was performed with <http://www.altanalyze.org/>. The 3D modeling of this region of the zebrafish miR-212/132 pri-miRNA was carried out with <http://genesilico.pl/SimRNAweb>, and the most stable structures were given for each region.

To determine the putative regulatory miRNAs on *oprm1* mRNA of zebrafish, the Microcosm database was used (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>). The thermodynamic of the binding between miR-212 and *oprm1* 3'UTR was analyzed using the mFold RNA server (<http://unafold.rna.albany.edu/?q=DINAMelt/Two-state-melting>).

The analysis of the binding sites CRE and RE1 of miR-212/132 promoter was performed using Genomatix software (<https://www.genomatix.de/solutions/genomatix-genome-analyzer.html>).

Statistical analysis

qPCR ct values were first included in REST-384 v2 software to calculate the relative expression of groups respect to control embryos. In western blot, the relative area of each band was calculated using ImageJ compared to the control group. Data were analyzed by one-way analysis of variance (one-way ANOVA). Tukey's post hoc test was then performed for multiple comparisons. Differences were considered significant at $p < 0.05$. Results are shown as means $\pm$ S.E.M. All statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, Inc).

Results

miR-212 and miR-132 have been related to the development of the central nervous system (CNS) (Wanet et al., 2012), and are codified in the same genetic cluster. miRNAs codified in this way are transcribed as a polycistronic pri-miRNA that must undergo posterior processing by several splicing enzymes, some of them still unknown, to generate two pre-miRNA. It has been described that clustered microRNAs have similar functions (Wang et al., 2011). The first eight nucleotides of a miRNA (known as the seed region) are crucial on the selective target recognition. miRNAs codified in clusters often have the same seed region and this fact may explain how many of these molecules share their targets and, therefore, their functions. In order to determine the relevance of miR-212 and miR-132 during zebrafish development, we studied their temporal expression in 5, 8, 16, 24 and 48 hpf embryos (Fig 2A and 2B). It is remarkable that, at the five stages analyzed, the levels of miR-132 were higher than the levels of miR-212, despite being codified as a unique pri-miRNA. Although both are highly expressed between 5 and 16 hpf, miR-212 expression reaches its maximum at 8 hpf. At 16 hpf, the expression of this miRNA reaches the levels observed at 5 hpf and then decreases to a minimum at 48 hpf. In contrast, we have observed a progressive increase of miR-132 from 5 hpf to 16 hpf. At 24 hpf, the levels of miR-132 reaches the lowest quantification of the five stages. However, the levels at 48 hpf are again enhanced, suggesting the importance of this miRNA at this particular stage when compared to miR-212. To study a possible relation of these miRNAs that could lead to a differential expression, we knocked-down miR-212 and miR-132 in order to analyze the dependence of each other in their expression profiles (Fig 2C and 2D). Silencing of miR-212 induced a decrease in the expression of miR-132 at several stages (5, 8 and 16 hpf) (Fig 2D). In addition, the levels of miR-212 were also downregulated in miR-132 morphants (Fig 2C).

Results

Figure 2. miR-212/132 temporal expression profile and their interdependent expression. miR-212 (A) and miR-132 (B) temporal expression was measured by qPCR. miR-212 expression levels in miR-132 morphant embryos (C), and miR-132 expression levels in miR-212 morphant embryos (D) were also quantified by qPCR. The results show differences in the temporal expression of the clustered microRNAs where miR-132 levels are always higher than the levels of miR-212. Figures C and D show a downregulation of one of the miRNAs when the other is knocked-down, indicating a possible regulation between the expression of these molecules in the context of their cluster. Results are shown as the number of copies per 2µg of cDNA (A and B) or the fold change respect to control levels at 5, 8 and 16 hpf. (C and D) Data are expressed as mean ± SEM, N=3 Control group is shown as the dotted line. Statistical significance; **, p < 0,01. ***, p < 0.001. Mo: morphant

Therefore, miR-212 and miR-132 expression is misbalanced, despite being transcribed as the same primary miRNA. These results indicate that during the splicing process miR-132 expression is enhanced over miR-212. To clarify the mechanism by which a bicistronic molecule induces different levels of the two mature miRNAs, we analyzed the intergenic sequences between miR-212 and miR-132 in four different species: *Homo sapiens*, *Mus musculus*, *Ratus norvegicus* and *Danio rerio*. For this purpose, a multiple alignment (ClustalW) of that region between both miRNAs that is present in the transcribed form was performed to look for conserved regions among the different species. This alignment showed two highly conserved regions, each one located on the extremes of the analyzed sequences (Fig 3A). An *in silico* analysis of putative splicing

proteins (<http://www.altanalyze.org/>), conserved within evolution among different organisms, showed that these sequences were predicted to interact with

A

Figure 3.- The intergenic region between miR-212 and miR-132 shows conserved sequences that may be key in the maturation of miRNA. CLUSTALW alignment of the intergenic region between miR-212 and miR-132 (A) and tridimensional modeling of the mentioned area (B). The alignment showed two highly conserved sequences that were predicted as binding sites for several splicing proteins such as SRSF2, SRSF3 and MBNL1 (yellow and orange sequences) (A). 3D modeling showed that the conserved regions (red circle) were always predicted to be located perpendicular to the Z axis of the molecules (black line) (B). This region could be the responsible for the differential expression of the two mature miRNAs from the same bicistronic pre-miRNA. Yellow, highly conserved sequences, orange, slight variations of the sequence.

three well known splicing proteins implicated in miRNA maturation: Serine and Arginine Rich Splicing Factor 2 (SRSF2), Serine and Arginine Rich Splicing Factor 3 (SRSF3) and Muscleblind Like Splicing Regulator 1 (MBNL1). The secondary

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structures of RNA are crucial during splicing, due to the need of the spliceosome complex to form and cleave the RNA sequence (Shepard and Hertel, 2008). Hence, to determine the accessibility of the miRNAs intergenic region to the splicing proteins, which could lead to the formation of a possible RNA-protein complex, a 3D modeling of the secondary structure of this sequence was performed (<http://genesilico.pl/SimRNAweb>) (Figure 3B). For the zebrafish intergenic region modeled, the most stable conformations were given and the consensus sequences (Red circles) were always located perpendicular to the Z axis (the axis between the two pre-miRNAs) of the molecule (black line) towards the surface, indicating high accessibility to the spliceosome complex.

Morphine modifies the levels of expression of pri-miR-212/ 132 and of miR-212

As previously described, the differential levels of both studied miRNAs pointed to differences between their functions. These different effects for these miRNAs have already been described, as miR-132 is closely related to the differentiation of neural precursors and to the regulation of several cellular processes such as circadian cycle (Wanet et al., 2012). On the other hand, miR-212 has been associated to drug addiction (Im et al., 2010) and it is believed to play a critical role in the molecular changes that lead to the development of tolerance after drug intake. Due to the involvement of morphine in the addictive processes and its importance in the present work, the expression of miR-212 was analyzed by qPCR after the administration of two different doses of morphine (10nM and 10μM) from 5 hpf to 24 hpf or 48hpf (Fig. 4). We also analyzed pri-miRNA expression to find a possible correlation between this molecule and the mature form, miR-212. In addition, we knocked-down *oprm1* in order to

determine a possible role of the receptor itself on the effects triggered by morphine. At 24 hpf (Fig. 4A), an increase in the levels of miR-212 was observed for both concentrations of morphine, although it was higher at 10 μ M. In contrast, both doses

Figure 4. Effects of morphine and *oprm1* knock-down in miR-212 and pri-miR-212/132 expression. qPCR analysis of miR-212 expression levels in control and *oprm1* morphant embryos after morphine (10 nM and 10 μ M) treatment (A, 24hpf; B, 48hpf) and pri-miR-212/132 expression (C, 24hpf; D, 48 hpf). Morphine upregulates miR-212 and pri-miR-212/132 at 24 hpf. At 48 hpf, miR-212 was downregulated whereas no changes were observed for the primary miRNA. The knock-down of *oprm1* did not induce changes in miR-212 in the untreated embryos, but reversed morphine effects at 24 hpf indicating that it is necessary for morphine effects at this stage. However, the knock-down of *oprm1* together with morphine exposure still triggered the same downregulation at 48 hpf. The results after *oprm1* knockdown showed that this receptor is required for morphine effects on these molecules, but only at 24 hpf. Results are shown as the fold change in respect to control embryos (dotted line) at 24 and 48 hpf. Data are expressed as mean $\pm$ SEM, N=3. Statistical significance; *, $p < 0,05$; **, $p < 0,01$, ***, $p < 0.001$. C-: Negative control Mo: morphant.

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downregulated this miRNA at 48 hpf (Fig. 4B). After knocking-down the mu opioid receptor, the up-regulation induced at 24 hpf by morphine administration was not observed (Fig. 4A). However, the decrease induced by morphine in miR-212 expression was not reversed after exposing *oprml* morphants to morphine at 48 hpf (Fig. 4B). Also, as shown in Fig. 4A and 4B, the levels of miR-212 were not modified in the control *oprml* morphants. The pri-miR-212/132 expression at 24 and 48 hpf (Fig. 4C and 4D) showed a correlation between its expression and miR-212, except for untreated and 10 nM morphine treated *oprml* morphants at 24 hpf and in all the groups at 48 hpf, where a strong upregulation was observed (Fig. 4D).

Morphine modifies miR-212 expression in the midbrain and hindbrain of zebrafish embryos

The changes observed after morphine administration in pri-miR-212/132 and miR-212 expression are likely to influence several differentiation processes within the zebrafish brain. To determine the areas where the changes observed by qPCR were more evident, an ISH probe against the pri-miR- 212/132 (Fig. 5A–5F) was obtained. This experiment indicated the areas of the brain where morphine was exerting the changes on the expression of these molecules. These miRNAs are not widely expressed at 24 hpf, and its expression is mostly located towards the preoptic region of the embryo (Fig. 5, Black arrows). However, these miRNAs are expressed in midbrain and hindbrain at 48hpf (Fig. 5, white arrows). The upregulation triggered by morphine on these areas did not totally correlate with the up and downregulation obtained in the qPCR experiments. At 24 hpf, a downregulation of the expression of pri-miR-212/132 after morphine exposure is observed in the preoptic region, whereas an upregulation was induced by 10nM

morphine at 48 hpf, in contrast to the decrease observed in the midbrain of zebrafish embryos exposed to 10 μ M morphine.

Figure 5. Morphine induces changes in the expression of primiR-212/132 in the zebrafish brain. Pattern of expression obtained by ISH in control (A, D) and morphine treated embryos (10 nM (B, E) and 10 μ M (C, F)) (left, 24hpf; right, 48hpf). The arrows show the regions in the encephalon where the changes observed are more relevant (Black arrows: preoptic area; White arrows: Midbrain and hindbrain). The upregulation triggered by morphine on these areas did not totally correlate with the up and downregulation obtained in the qPCR experiments.

Results

miR-212 regulates *oprm1* mRNA expression by binding to its 3'UTR

Our results showed that miR-212 expression is regulated after morphine administration mediated by Oprm1 activation. Hence, it was of interest to determine if there is a regulation between this miRNA and mu opioid receptor. Using miRNA predicted target databases such as Microcosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) we found a putative regulatory binding site in the 3'UTR of *oprm1*, as well as an additional site within the second exon of the mRNA. A deeper analysis of this binding site was carried out using RNA fold server (<http://unafold.rna.albany.edu/?q=DINAMelt/Two-state-melting>), to determine the thermodynamics of this binding process between the miRNA and the mRNA. The free Gibbs energy between the sequence in the 3'UTR and miR-212 was lower than -10/12KJ/mol, considered the threshold for an effective regulatory binding (Peterson et al., 2014). To experimentally prove the functionality of the predicted binding we performed a luciferase assay. In this method the predicted regulatory region is cloned in a plasmid besides the luciferase gene and transfected into HEK293 cells. miRNA mimics (small, chemically modified double-stranded RNAs that mimic endogenous miRNAs by the up-regulation of the miRNA activity) were cotransfected with the plasmids and, therefore, an increase in the miRNA of interest reduces the expression of the luciferase, resulting in a decrease in luminescence. We confirmed that the binding site for miR-212 within *oprm1* 3' UTR was actively repressing mu opioid receptor (Fig. 6), as we observed a significant decrease in the luminescence of the group transfected with the unmutated 3' UTR binding site (wild type, wt) (Fig. 6, left). The luminescence on the mutated predicted binding site was however not reduced (Fig. 6, right), indicating that the binding of the miRNA with the 3'UTR of *oprm1* was physiologically functional. Moreover, the increase on the concentration of miRNA mimics reduced the

luciferase activity, but only when cotransfected with the wt plasmid, corroborating the results.

Figure 6. miR-212 reduces *oprm1* expression by binding to the 3'UTR of the *oprm1* mRNA. Results from luciferase assay of the binding site for miR-212 in the 3'UTR of *oprm1*. Results are shown as the fold change respect to control. The groups wt and mis were both cotransfected with 20nM or 50nM miR-212 mimics. Higher mimic concentration induced a higher reduction of luminiscence. miR-212 regulates *oprm1* expression, as observed in the significant changes when the unmutated plasmid is cotransfected with an active form of miRNA 212. Data are expressed as mean $\pm$ SEM, n = 5. Statistical significance; ***, p < 0,001. wt, unmutated plasmid; mis, mutated plasmid; 20 and 50 indicates miRNA mimics nanomolar concentration.

Morphine modifies the levels of expression of *oprm1* through miR-212

As previously shown, miR-212 expression is affected after morphine administration, and miR-212 is actively repressing *oprm1* expression. Due to that effect, we analyzed how the levels of *oprm1* were actually affected by miR-212 and morphine. The expression of *oprm1* was analyzed by qPCR (Fig. 7). After morphine treatment, mu opioid receptor was strongly up-regulated at 24 hpf (Fig. 7A). In contrast, we observed

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a decrease in the levels of *oprm1* at 48 hpf after morphine administration (Fig. 7B). However, the knock-down of miR-212 induced an increase in the expression of the receptor, much higher than the observed when miR-212 was present at both 24 and 48 hpf. Finally, to determine whether these changes observed in the mRNA levels were

Figure 7. Morphine and miR-212 regulate Oprm1 expression. qPCR analysis of *oprm1* expression levels in control and miR-212 morphant embryos after morphine (10 nM and 10 μM) treatment (A, 24hpf; B, 48hpf). Immunoprecipitation-Western Blot of Oprm1 (C, 24hpf; D, 48 hpf). E, F: quantification of the results from C and D, respectively. Morphine regulates *oprm1* expression, inducing an upregulation at 24 hpf, but a downregulation at 48 hpf. The results after miR-212 knockdown show that this miRNA could be exerting regulatory effects after morphine administration. Results are shown as the fold change respect to control (dotted line) at 24 and 48 hpf. Data are expressed as mean ± SEM, n=3. Statistical significance; *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$. C-: Negative control Mo: morphant.

correlated with the protein levels, we performed an immunoprecipitation-western blot (IP-WB, Fig 7C-F). The results obtained with this technique did not totally correlate with the qPCR experiments, since miR-212 morphants after the 10 μ M dose showed a downregulation in the protein levels.

miR-212 and *oprm1* expression is regulated by MAPK, calmoduline and PKA

Oprm1 signaling is composed by several molecular pathways involved in the modulation of gene expression. Among these cascades, the signaling triggered by the Mitogen Activated Pathway Kinases (MAPK) is believed to be one of the most relevant triggered by morphine administration. However, mu opioid receptor activation induces changes in ionic concentration, such as calcium, and exerts the early inhibition of the adenylate cyclase (AC). To analyze in detail the signaling cascade triggered by the activation of Oprm1, the levels of miR-212 were studied after the coadministration of morphine and U0126, an inhibitor of MEK1/2 at 24 and 48 hpf (Fig. 8). The upregulation of miR-212 expression after morphine exposure at 24 hpf did not return to normal levels when the MAP Kinase pathway was blocked (Fig. 8A). In addition, when MAPK pathway was disrupted both doses of morphine induced a decrease of miR-212 levels compared to the control group. These results show that this pathway is necessary to mediate morphine upregulation of miR-212 and it also seems to be regulating the physiological levels of this miRNA expression. To assess the implication of calcium channels in opioid signaling and in miR-212 expression, we inhibited calmoduline (CaM) and, therefore, calmoduline kinase II (CaMKII) using the inhibitor

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Figure 8. MAPK signaling controls miR-212 overexpression after morphine administration. miR-212 expression levels measured by qPCR after morphine administration and inhibition of MEK1/2 (U0126, M), CaM/CaMKII inhibitor (W7, W) and PKA hyper activation (Forskolin, F) (A, 24hpf; B, 48hpf). MAPK pathway is required as an inducer of morphine modulation of miR-212. Moreover, calcium signaling and PKA are not mediating on morphine early response, but control miR-212 expression. Results are shown as the fold change respect to control (dotted line). The + sign represent the combination of drugs added to each condition, whereas – sign represent their absence. Data are expressed as mean $\pm$ SEM, n = 3. Statistical significance; *, p < 0,05; **, p < 0,01; ***, p < 0,001.

W7. As observed in figure 9A, calcium signaling inhibition induces an overexpression of miR-212 in control, 10nM and 10μM groups, even when coadministrated with morphine. Finally, with the use of forskolin, an activator of PKA, we observed a strong upregulation of this miRNA in the three groups (Fig. 8A). In contrast, at 48 hpf, miR-212 levels remained decreased after inhibiting MEK1/2 (Fig. 8B). Even though this downregulation persisted, we observed a different effect between 10nM and 10μM morphine. Calmodulin kinase disruption induced a decrease independent of morphine administration, an opposite effect to that observed at 24 hpf. Similarly, Forskolin

induced the same changes observed with W7 administration. However, the inhibition of the MAP kinase pathway with U0126 and CaM/CaMKII with W7 after 10nM morphine administration did not induce the strong downregulation observed in the embryos treated with 10nM morphine (Fig. 9B). The mild downregulation observed in the 10 μ M group is potentiated by disrupting MEK and calcium signaling. PKA over-activation using forskolin mimicked the decrease of 10 μ M control group, even when coadministrated with morphine.

The results from figure 8 showed that the three pathways studied were mediating miR-212 modulation by morphine through the mu opioid receptor. To analyze whether this signaling cascades were inducing the changes observed in *oprm1* changes after morphine exposure, we measured by qPCR the expression of the receptor using the same drugs as in the previous experiment (Fig. 9). At 24 hpf, mu opioid receptor overexpression induced by morphine was reversed when MAPK pathway was disrupted (Fig. 9A) as well as after inhibiting CAMK pathway. In addition, Forskolin prevents the induction of the expression of *oprm1*. However, the use of any of the three drugs did not induce any change in *oprm1* itself as compared to the control group. At 48 hpf, the downregulation in *oprm1* expression levels is partially reverted after MEK inhibition, especially after the 10 μ M dose exposure. Calcium pathway inhibition induced a reverse of the effects of morphine with the exception of 10 μ M dose. Finally, the activation of PKA through the administration of forskolin showed a mild decrease, in the presence or absence of morphine (Fig. 9B).

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Figure 9. MAPK, calcium signaling and PKA are necessary for *oprm1* overexpression triggered by morphine at 24 hpf. *oprm1* expression levels measured by qPCR after morphine administration and inhibition of MEK1/2 (U0126, M), CaM/CaMKII inhibitor (W7, W) and PKA hyper activation (Forskolin, F) (A, 24hpf; B, 48hpf). MAPK pathway is required as an inductor of morphine modulation of *oprm1*. Moreover, calcium signaling and PKA are also mediating morphine early response. At 48 hpf, the three pathways seem to regulate the downregulation observed after morphine administration. Results are shown as the fold change respect to control (dotted line). The + sign represent the combination of drugs added to each condition, where – sign represent the absence of it. Data are expressed as mean $\pm$ SEM, n = 3. Statistical significance; *, p < 0,05; **, p < 0,01; ***, p < 0,001.

The expression of miR-212/132 cluster is controlled by activated CREB

Both MAPK pathway and PKA activation can induce CREB (cAMP response element-binding) phosphorylation and activation. This leucine zipper transcription factor is involved in the induction and repression of multiple genes and it is known to act as an inductor of morphine response (Cao et al., 2010). To determine whether the regulation triggered by morphine of this miRNA was induced by these specific transcription

factors, a bioinformatic analysis of the promoter was performed using Genomatix software (<https://www.genomatix.de/solutions/genomatix-genome-analyzer.html>). The sequence of miR-212/132 zebrafish promoter showed a -2,3Kb upstream region with two predicted CRE sites (i.e., putative activated CREB binding sites, dark green in the Figure 10), flanking a RE1 site (binding site to repression complex REST, orange in the Figure 10). MeCP2 (methyl CpG binding protein 2) is a known modulator of miR-212/132 expression, due to the binding of this factor to a Repression complex REST binding site (RE1) site found in the promoter of mice (Hollander et al., 2010). The binding of MeCP2 to this RE1 site is induced by a heavy methylation pattern of the CpG islands. In order to analyze the functionality of the CRE sites of this region of the promoter, we performed a ChIP-qPCR using an antibody against phosphorylated and activated CREB (pCREB) (Fig. 11). The results show an increase in the binding of activated CREB after the administration of 10 nM morphine when compared to the control levels at 24 hpf. However, the exposure to morphine until 48 hpf induced a decrease in the binding of pCREB to the promoter, pointing to a repression mediated by the RE1 site and activated MeCP2.

miR-212/132 Promoter 2573bp

Figure 10. miR-212/132 promoter may be controlled by CREB and MeCP2 through CRE and RE1 binding sites present in the promoter. Fragment of the miR-212/132 promoter structure and sequence corresponding to the putative CRE and RE1 sites. CRE sites are responsible for CREB activity, as this protein binding to this region induces the expression of several genes. RE1 sites, repressive sites susceptible of being methylated, are related to inhibition of transcription through the binding of repression complex formed by a group of proteins, such as MeCP2.

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Figure 11. CRE sites on miR-212/132 promoter are actively controlling morphine effects on these molecules. ChIP-qPCR for phospho CREB (pCREB) binding to CRE site in miR-212/132 promoter. Results show that morphine increases binding at 24 hpf. However, a decrease is observed at 48 hpf, probably due to MeCP2 binding to RE1 site. Data is represented as the mean $\pm$ SEM. ** $p < 0,01$; $n=3$.

Notch intracellular domain regulates miR-212/132 expression

The expression of miR-212 and the changes exerted after morphine administration suggested an involvement of this miRNA in the early stages of development. Moreover, miR-132 has been already been described as a modulator of several differentiation processes. Notch signaling pathway is one of the most relevant cascades that control the division and specification of multiple cellular types, especially during CNS development (Wanet et al., 2012). In addition, Notch intracellular domain (NICD), released after a two-step cleavage of the receptor, is a known modulator of transcription through the interaction with several transcription factors, being Hairless Suppressor (CSL) the most relevant. In addition, NICD modulates CREB activity controlling the binding to CRE sites in the promoters (Hallaq et al., 2015). Hence, to determine the implication of Notch signaling in miR-212/132 expression and thus in the regulation of the early

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development of the CNS, zebrafish embryos were exposed to DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a pharmacological inhibitor of Notch activation), and DAPT together with morphine. The binding of pCREB to miR-212/132 promoter was analyzed by qPCR (Fig. 12). The disruption of Notch signaling induced a decrease in pCREB binding to miR-212/132 promoter. Moreover, the coadministration of morphine and DAPT at 24 hpf reversed the effects observed when only morphine was present, indicating that Notch signaling prevent the upregulation of these miRNAs previously observed.

Figure 12. Notch signaling modulates morphine effects on miR-212/132 expression. ChIP-qPCR for phospho CREB (pCREB) binding to CRE sites in miR-212/132 promoter at 24hpf. DMSO corresponds to the control group as DAPT cannot be dissolved in water. DAPT reduces pCREB binding to the promoter, even in the presence of morphine. Data is represented as mean $\pm$ SEM. n=3

Morphine administration does not modify the methylation pattern of RE1 site of miR-212/132 promoter

The reduction of pCREB binding to the CRE sites of miR-212/132 promoter at 48 hpf and the results obtained with the inhibitor of calcium signaling W7 suggested that MeCP2 could be acting as a repressor of the expression of both miRNAs. Since RE1 binding sites are susceptible of being methylated, it was of interest to analyze whether morphine could be exerting changes in the pattern of methylation of the RE1 site found within the miR-212/132 promoter. The bioinformatic analysis of the studied sequence showed four CpG islands, three of them located in the RE1 site. To measure the methylation of these cytosines within these CpG islands, a bisulfite conversion analysis of this region was performed. After this conversion, the cytosines that are not methylated will turn into uracil, and posterior sequencing will allow differentiating the islands that are methylated from the ones that are not. Total gDNA was converted by bisulfite treatment, and this region was amplified by nested PCR, cloned into PGEM®-T vector and sequenced. At both 24 and 48 hpf, these cytosines were methylated (Fig. 13). After morphine administration, the pattern of methylation of this region was not modified and every cytosine in the CpG islands was still methylated at both stages.

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Figure 13. Morphine does not modify the methylation pattern of RE1 site on miR-212/132 promoter. Methylation pattern on miR-212/132 promoter of the sequence of RE1 site. Each square represent a cytosine susceptible of being methylated. As observed in control conditions, at 24 and 48 hpf, all the cytosines are methylated (shown in yellow) (Figure A and C). In neither cases, the administration of morphine induced a significant change in the total percentage of methylation (blue, unmethylated cytosines), indicating that the changes in methylation of the RE1 site are not responsible of morphine direct regulation of miR-212/132 expression. n=6. Grey squares represent failures during sequencing.

Temporal expression of miR-29a during zebrafish development

Our results have shown a regulation of pCREB binding to miR-212/132 promoter after DAPT administration to zebrafish embryos. At the same time, the regulation of Notch pathway is mediated by several factors: the expression of the receptors Delta and Jagged on the adjacent cells, which mediate the first step of cleavage of the receptor, and the action of the γ -secretase complex that lead to the processing of the intracellular domain.

The expression of these proteases is controlled by several non-coding RNAs, such as miR-29a. This miRNA is a known repressor of Notch activity, mediated by the modulation of ADAM12 expression (a metalloproteinase needed for Notch cleavage and the consequent release of NICD) (Li et al., 2011). To determine the importance of Notch signaling in miR-212/132 expression, we first analyzed miR-29a expression through zebrafish development, to find any possible correlation with these three miRNAs during the early stages of neuronal differentiation.

For that purpose, we performed a qPCR in 5, 8, 16, 24 and 48 hpf embryos (Figure 14). Results showed a higher expression at 5, 16 and 48 hpf. However, during 8 and 24 hpf, the expression of this miRNA was clearly reduced. It is remarkable that the expression of this miRNA is lower than the observed for miR-212 and miR-132.

Figure 14. The levels of expression of miR-29a during zebrafish development follow a cyclic pattern of up and down expression. Temporal expression of miR-29a during zebrafish development at 5, 8, 16, 24 and 48 hpf. Results show that this miRNA is highly expressed during the early stages of development, especially during 5 and 16 hpf. However, an increase at 48 hpf is again observed, indicating a putative important role for this miRNA during development. These results correlate with the fact that Notch signaling, one of the downstream targets of miR-29a, is necessary in early specification of cell fate. Data are expressed as absolute quantity of miRNA-29a per 2µg of cDNA. Data are expressed as mean±SEM, n=3.

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Opioid and Notch signaling modulates NF- κ B phosphorylation and control miR-29a expression

NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) is known for acting as a repressor of the expression of miR-29a (Zhou et al., 2010). That same group has also described that miR-29a promoter has several binding sites for this transcription factor, that is known to form a repressor complex to prevent miR-29a expression. In addition, NF- κ B is a transcription factor known to control several process involved in cellular stress and in the molecular mechanism after drug intake (Nennig and Schank, 2017). Since Notch signaling control miR-212/132 expression through the modulation of pCREB binding to their promoter, we postulated that morphine could be regulating Notch signaling through the activation of NF- κ B and the expression of miR-29a. To determine this putative regulation, we analyzed phosphorylated NF- κ B (active form) expression by western blot in 24 hpf zebrafish embryos treated with 10 nM morphine. To determine the possible implication of MAPK pathway in NF- κ B activation, we also exposed the embryos to the inhibitor of MAPK pathway, U0126. To analyze a putative convergence of the two pathways mediated by NF- κ B, we also used *mindbomb* mutants (a kind gift from Dr. Patrick Blader, from the Université Paul Sabatier, Toulouse). This mutant fish line is commonly used to study Notch signaling, since the cleavage of the receptor is disrupted and the fish lacks NICD signaling.

Morphine induced a slight increase of phospho NF- κ B (Fig. 15). MAPK pathway inhibition also upregulated the levels of this protein, and the results were not reversed after the coadministration of morphine. In *mindbomb* mutants, the levels of NF- κ B were increased and the administration of morphine exacerbated that increase. The disruption of Notch signaling and MAPK pathway reversed the increase of NF- κ B observed in the untreated *mindbomb* embryos. As expected, the coadministration of morphine with

U0126 did not induce any change in NF- κ B expression, pointing to a direct relation between MAPK signaling and NF- κ B activation.

Figure 15. Morphine and Notch signaling regulate NF- κ B phosphorylation and activation. Western Blot quantification against phosphorylated NF- κ B in 24 hpf AB and *mindbomb* zebrafish embryos. Morphine induces the activation of NF- κ B, and so does the inhibition of MAPK pathway with the inhibitor U0126 in wild type embryos (AB). In *mindbomb* mutants (Mb), where Notch signaling is disrupted, the administration of morphine partially reverses the effects, but not when MAPK pathway is disrupted with U0126. Western Blot data are normalized against actin. Data are represented as mean $\pm$ SEM, n=4. * p < 0,05. Morph: Morphine.

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To assess the influence of the NF- κ B activation in miR-29a expression, we analyzed the levels of this molecule by qPCR on 24 hpf zebrafish embryos after the administration of morphine, U0126 or DAPT (Fig. 16). The results obtained correlated with the western blot of NF- κ B. The groups where this transcription factor was overactivated showed a decrease of miR-29a levels. Interestingly, DAPT administration induced a huge increase in comparison to the control group. This increase caused by DAPT was reversed by morphine, indicating a putative crosstalk between these pathways in the control of miR-29a expression.

Figure 16. Morphine and Notch signaling regulate miR-29a expression. The levels of miR-29a were measured by qPCR in 24 hpf zebrafish embryos. Morphine and MAPK inhibition (with U0126) reduces the expression of miR-29a. On the other hand, the disruption of Notch signaling with DAPT dramatically increases the expression of this miRNA, even when coadministered with morphine. Data are represented as mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$ $n=3$. MD, Morphine 10nM + DAPT; MU, Morphine 10nM + U0126.

Opioid and Notch cascade share a reciprocal regulation

Our results suggested that morphine could be modulating Notch signaling through miR-29a expression mediated by NF- κ B activation, and also that Notch signaling could be controlling opioid pathway through miR-212/132 modulation. In order to determine this possible reciprocal regulation between opioid and Notch signaling, we performed a western blot analysis of the mu opioid receptor and NICD in SH-SY5Y cells in presence of morphine (to activate the mu opioid receptor and to assess opioid signaling effect), DAPT (to prevent Notch cleavage and to evaluate the effects of this pathway), naloxone (as a reverse agonist of OPRM1 to reverse morphine effects) or U0126 (to inhibit MAPK pathway) to determine the molecular signaling involved in such processes (Fig. 17). This cell type has been commonly used as a simplified model to study morphine response in a neuronal like phenotype (Xie et al., 2010). SH-SY5Y cells express both mu and delta opioid receptor and Notch1 receptor, allowing us to determine whether a cross-talk between these two pathways exists. Results showed an increase in OPRM1 levels after morphine exposure (as observed in 24 hpf zebrafish embryos) and also after DAPT administration, probably due to an increase of pCREB activity induced by a decrease in the repression exerted by NICD on pCREB (Fig. 17A). U0126 administration induced the upregulation of the receptor while the coexposure with morphine reverted the higher upregulation observed in morphine exposed cells. Interestingly, DAPT coadministered with morphine reversed the effects of the only administration of morphine or DAPT reducing the levels of OPRM1 and pointing to a cross regulation between NICD and pCREB. This interaction could prevent the induction of mu opioid receptor expression after morphine administration (Fig. 17A). NICD levels were increased after morphine exposure (Fig. 17B). U0126 administration did not change the levels of the protein, although its co-administration with morphine

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downregulated NICD levels. In all the cases studied, DAPT exposure reduced NICD levels as expected.

Figure 17. Opioid and Notch signaling are reciprocally regulated. OPRM1 and NICD levels determined by western blot quantification after the addition of several drugs to SH-SY5Y cells. DAPT upregulates OPRM1. Morphine also upregulates OPRM1, as previously described (Garcia-Concejo et al., 2016). MAPK inhibition increased OPRM1 expression. Naloxone did not induce any effect but reversed morphine changes (A). Morphine induces the upregulation of activated notch (NICD). These results were not observed when morphine was coadministered with DAPT (B). These results suggest that both signaling cascades are related to each other, probably mediated by NICD interaction with pCREB binding to CRE sites or by the activity of the miRNAs studied. Results are expressed as the fold change Mean $\pm$ SEM, n=4. Nx, naloxone; MN, morphine 10nM + Naloxone; MU, morphine 10nM + U0126; DM, DAPT + U0126. *, p<0,05, **, p<0,01.

Opioid and Notch signaling control neuronal differentiation and aggregation

The effects of morphine and Notch signaling have been widely described in the context of development (Louvi and Artavanis-Tsakonas, 2006; Attarian et al., 2014). Nestin, a protein that constitutes intermediate filaments in neural precursors is commonly used to mark neuronal precursors (Suzuki, 2010). To determine whether any changes were observed during development of neural precursors, the effects of morphine and DAPT were studied in a primary culture obtained from Nestin:GFP zebrafish embryos (Fig. 18 and 19). The GFP positive cells represent neural precursors, since 3,9kb of the zebrafish Nestin promoter was cloned into a GFP plasmid (Fig. 18A, black arrows) (Lam et al., 2009). The culture was recorded for 8 hours using time lapse microscopy. After this time, the expression of GFP decayed indicating a differentiation of the cells.

Morphine induced an increase in cell aggregation respect to control (Fig. 18A and 18B). Moreover, the expression of GFP was maintained after the recording, indicating a delayed differentiation of neural precursors. Also, morphine treated cells were prone to aggregate to other cellular groups, as observed figure 19B, in the cells marked with white arrows. DAPT treated cells (Figure 19A), however, did not show the same increase in aggregation as seen in figure 18B. However, the delay in GFP expression was still present. Interestingly, in those cells where morphine and DAPT were coadministrated (Figure 19B), it was observed not only a reduced cell aggregation and cell-cell interaction (Figure 19B), but also a reduced expression of GFP. We cannot

Figure 18. Morphine induces changes in cell aggregation and delay neuronal precursors differentiation. Photograms of a primary neuron culture from transgenic zebrafish nestin:GFP *time lapse* experiment for 8 hours in a control group (A) and exposed to 10nM morphine (B). Results show a prolonged GFP expression after morphine exposure (Figure B). Morphine exerted a strong effect on neuronal precursor aggregation. Moreover, GFP expression was delayed and lasted during the 8 hours of the experiment. Black arrow heads indicate GFP positive cells, while white arrow heads indicate mature neurons.

Figure 19. DAPT and morphine induces changes in cell aggregation and delay neuronal precursors differentiation Photograms of a primary neuron culture from transgenic zebrafish *nestin:GFP* *time lapse* experiment for 8 hours in a DAPT group (A), DAPT + Morphine 10nM (B). Results show a prolonged nestin expression after DAPT exposure (Figure A), although the increase in cell aggregation was not observed. Morphine coadministered with DAPT not only reduced neuronal precursor aggregation, as it also reduced GFP expression indicating that differentiation was promoted.

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confirm that morphine and DAPT coadministration is inducing the death of those cells, but these results pointed that these drugs promoted cell differentiation, probably due to changes in Notch signaling modulated by the decrease of cell-cell interaction.

Morphine and Notch signaling control dopaminergic differentiation

Our results indicated that morphine was able to induce the upregulation of miR-212 as well as alter Notch signaling by upregulating NICD through miR-29a. Besides, opioid and Notch signaling seem to interact affecting neural precursors and mature neurons. Hence, we hypothesized that this effect on neuron maturation together with the changes observed in miR-212 may be involved in the addictive processes by regulating dopaminergic differentiation. Among all the cell types derived from the neural precursors, dopaminergic neurons are closely related to addiction. These neurons receive their name because of the use of dopamine as its characteristic neurotransmitter. The terminal enzyme responsible for dopamine synthesis is tyrosine hydroxylase (TH), and it is usually used as a marker for this particular subset of neurons. The effects observed after morphine and DAPT administration could be affecting specifically the dopaminergic differentiation, therefore explaining the early effects of these two pathways in dopaminergic development. The factors that are implicated in this process could lead to a better understanding of the effects of addictive drugs such as morphine during embryonic development. To assess the effects observed in the zebrafish primary neuron culture that could lead to an altered dopaminergic differentiation, we used a mouse embryonic cell line (R1 ES) which can be differentiated into dopaminergic neurons. During this process, the cells were exposed to morphine, DMSO and DAPT (Figure 20). The protocol of differentiation here used induces several steps from

undifferentiated stem cells (first stage), to the second stage or neural stem spheres (NSS). After these two stages, the line can be differentiated into neural precursors (third stage) and, finally, into dopaminergic neurons (fourth stage). The results obtained in figures 18 and 19 were mimicked in this model. Morphine induced an increase in cell number and in neuronal aggregation (Figure 20, M). The dopaminergic TH positive neurons were mostly located in the form of clusters (arrow), where peripheral cells were not expressing the protein. On the other hand, DAPT decreased neuronal aggregation (Figure 20, D) compared to DMSO treated cells (Figure 20, CD).

To determine if opioid or Notch signaling were affecting the percentage of cell differentiation into dopaminergic neurons, we quantified several fields of the cell culture exposed to morphine, DMSO and DAPT from the first stage of the differentiation process (Figure 21). The results showed a decrease of total dopaminergic neurons after morphine administration (21, M group). DAPT treatment, however, did not affect the total number of TH positive cells (21, D group). Therefore, DAPT reduced cell-cell interaction, although those cells expressed TH more consistently despite the lack of yuxtacrine cell communication because of a reduction in cell aggregation.

Figure 20. Morphine and DAPT control cell aggregation on R1 ES cultured cells differentiated into dopaminergic neurons. TH immunohistochemistry of R1 ES cells proliferation and aggregation changes during dopaminergic differentiation of ES R1 cells. Morphine induced an increase of cell proliferation compared to control group. The clustered cells showed an increase of TH positive cells. DAPT reduced cell aggregation when compared to DMSO group. TH: green, DAPI: blue, Phalloidin: red. CM, control, M, morphine, CD, DMSO, D, DAPT. Arrows indicate the most representative cell among the groups studied.

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Figure 21. Morphine reduces the total number of differentiated cells during the protocol of differentiation into dopaminergic neurons. Percentage of differentiation of R1 ES cells into dopaminergic neurons (TH positive cells). This percentage was calculated as the rate of TH positive cells per total cell number (stained with DAPI). Morphine reduced the differentiation of R1 ES cells, while DAPT had no effect on the total number of TH positive cells. For each immunocytochemistry 20 fields were counted to determine the percentage of differentiation. Each experiment was performed by triplicate. Data are expressed as Mean±SEM. **, $p < 0.01$. CM, control group; M, 10nM morphine, CD, control DMSO; D, DAPT.

Opioid and Notch signaling are involved in the modulation of dopaminergic markers expression during R1 ES cell differentiation

Dopaminergic neurons are of extreme interest as they are responsible for the addictive properties of drugs. In addition, they also control pain, which made them suitable to study morphine modulation of dopaminergic differentiation. This issue is of importance due to the unique analgesic and addictive properties of morphine. Since morphine and Notch signaling were involved in the differentiation of TH positive cells we analyzed other transcription factors to prove a real relation between these cascades. The process of dopaminergic differentiation is indeed a very complex mechanism, in which many

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transcription factors are involved (Wilson and Edlund, 2001). Also, the expression of all of these factors must occur in a particular order (Gerrard et al., 2005), so they can induce the expression of the consequent genes in a deterministic way. Hence, in order to exert the changes observed in differentiation of ES R1 cells, morphine and DAPT could be affecting the expression of several neuronal and dopaminergic transcription factors during the different stages of the differentiation. To analyze this possibility, cells were cultured in four groups: Control group, 10nM treated cells, 0.1% DMSO and 10 μ M DAPT. These cells were harvested at the end of each stage and western blot was performed to measure the levels of FOXA2, NGN2 and SIRT1, early inducers and modulators of dopaminergic differentiation, therefore analyzed at the first and second stages of development (Figures 22 and 23). PITX3, NURR1 and TH, considered to be the terminal transcription factors involved in differentiation, were also measured at the third and fourth stage of development, together with SIRT1, which is still expressed (Figures 24 and 25).

Morphine increased the expression of SIRT1 in the two first stages of differentiation, from embryonic stem cells to neural stem cells (Figs 22A and 23A), while FOXA2 was only significantly modified in the first stage (Figure 22B and 23B). The increase in SIRT1 was mimicked after DAPT administration in the first stage. Neurogenin2 (NGN2) was expressed on neural stem cells, but was not observed in early embryonic stem cells, and its levels were increased after morphine but not after DAPT administration (Figure 23C).

Figure 22. Western Blot quantification of several dopaminergic markers involved in dopaminergic differentiation in the first stage of development of R1 ES cells (embryonic stem cells). The results show an increase of SIRT1 after morphine administration (A). Morphine also increases FoxA2, a transcription factor needed for the correct development of neuronal precursors (B). DAPT also induces an increase of FOXA2 in the first stage of development (A). NGN1 is not expressed at this stage. Experiments were performed by triplicate. All results were normalized against actin (loading control). Data are represented as mean $\pm$ SEM, N=3. *, p<0,05.

Neural Stem Spheres (Stage 2)

Figure 23. Western Blot quantification of several dopaminergic markers involved in dopaminergic differentiation in the second stage of development of R1 ES cells (neural stem spheres). The results show an increase of SIRT1 after morphine administration (A). Morphine also increases FoxA2 (B). DAPT does not modify the expression of SIRT1 (A), but increases the expression of FOXA2 (B). NGN1 is expressed at this stage, and morphine induces an overexpression of this protein. Experiments were performed by triplicate. All results were normalized against actin (loading control). Data are represented as mean $\pm$ SEM, N=3. *, p<0,05; **, p<0,01.

In the third stage of the differentiation process, morphine and DAPT still induced an upregulation of SIRT1 (Figure 24A), but its expression ceased at the fourth stage (Figure 25A). Neural precursors expressed NURR1 and morphine seems to reduce its expression while DAPT administration increases it, when compared to DMSO treated cells (Fig 24B). At the fourth stage, this effect is reversed. In this case, morphine and DAPT reverse NURR1 expression back to the control levels (Figure 25B). PITX3 is also expressed in neural precursors (Figure 24C), but we were not able to detect this protein in mature neurons (Figure 25C). After morphine administration, an increase in the third stage was observed. However, these results were the opposite after DAPT exposure together with morphine. In this case, a decrease in the levels of PITX3 was observed. TH levels were not modified after morphine or DAPT administration (Figure 25D).

Morphine effects on dopaminergic transcription factors are mediated by miRNAs

As previously seen, morphine modulates the expression of miR-212, miR-132 and miR-29a. Moreover, we have already demonstrated that these three molecules are controlling the activity of opioid and Notch signaling. Dopaminergic differentiation of R1 ES cells showed differences in the total number of TH positive neurons after morphine and DAPT administration and we have shown that the transcription factors are modified and therefore involved in these mechanisms. To find the link between these pathways we proposed that the miRNAs we had analyzed as candidate modulators could be directly modulating the expression of these transcription factors. To assess the effect of the overexpression of these molecules, we performed a western blot against putative downstream targets of this miRNAs on SH-SY5Y cells which allowed us to transfect

Neural Precursors (Stage 3)

Figure 24. Western Blot quantification of several dopaminergic markers involved in dopaminergic differentiation in the third stage of development of R1 ES cells (neural precursors). Morphine and DAPT administration increases SIRT1 expression (A), DAPT seems to increase NURR1 expression (B) and also reduces the levels of expression of PITX3, respect to the control DMSO group (C). Morphine on the other hand, seems to reduce NURR1 expression (B) and increases PITX3 expression (C). The cells were not expressing TH yet (D). Experiments were performed by triplicate. All results were normalized against actin (loading control). Data are represented as mean $\pm$ SEM, N=3.

Figure 25. Western Blot quantification of several dopaminergic markers involved in dopaminergic differentiation in the fourth stage of development of R1 ES cells (dopaminergic neurons). These cells do not express SIRT1 (A) and PITX3 (C) at this stage. DAPT seems to decrease NURR1 expression (B). Morphine on the other hand, seems to increase NURR1 expression (B). The cells express TH at this point, but neither of the molecules induce any changes in its expression (D). Experiments were performed by triplicate. All results were normalized against actin (loading control). Data are represented as mean $\pm$ SEM, N=3.

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miRNA mimics to check their effect on these factors on a dopaminergic like cell (Figure 26). These cells express the transcription factors that were studied during the differentiation of the mouse cell line with the exception of PITX3. miR-132 overexpression induced an increase in NGN2 expression, but no significant changes were observed in presence of morphine (Figure 26A). miR-29a overexpression induced a decrease in NGN2, but only after the exposure to morphine. SIRT1 levels were upregulated by miR-212 and also after miR-132 overexpression but these miRNAs mimics did not induce changes when morphine was also added (Figure 26B). TH expression was upregulated after morphine administration and after the overexpression of miR-212, but the miR-212 mimic transfection without morphine exposure did not exert any significant change (Figure 26C). SH-SY5Y cells do not express PITX3. However, the exogenous expression of miR-29a induced the expression of this gene (Figure 26D). Moreover, this increase was potentiated by morphine.

Opioid and Notch signaling control dopaminergic differentiation in zebrafish embryos

Once that we had observed in a simplified model that morphine and DAPT induced changes in the expression of several dopaminergic factors and were involved in TH expression we analyzed whether similar effects were occurring in zebrafish development. Our results showed that morphine was exerting a crucial role during dopaminergic development, but that also meant that the endogenous opioids, activating mu opioid receptor in control embryos, could be inducing or affecting the correct temporal expression of these transcription factors. On the other hand, the use of *mindbomb* mutants allowed us to study a stable *in vivo* model of development lacking

Figure 26. miR-212, miR-132 and miR-212 control the expression of several dopaminergic transcription factors. Dopaminergic markers expression after miRNA 212, 132 or miR29a mimic transfection coadministered with morphine in SH-SY5Y cells. These results show that miR-132 controls NGN2 (A). miR-132 overexpression also induces SIRT1 overexpression (B). In addition, miR-212 overexpression together with morphine administration upregulates TH expression, but not with the only increase in the miRNA levels (C). miR-29a slightly downregulates NGN2 when coadministered with morphine (A). Furthermore, miR-29a overexpression induced the expression of PITX3, a dopaminergic marker not expressed in SH-SY5Y cells (D). Data is represented as mean $\pm$ SEM or relative area. N=3. Morph or M, 10nM morphine.

Results

Notch signaling. To analyze the effect of the knock-down of mu opioid receptor and thus the endogenous opioid involvement in dopaminergic differentiation, we used an *oprml* morpholino. The analysis was carried out in 36 hpf embryos, due to a low level of expression of the dopaminergic factors at 24 hpf. *Th*, *nurr1* and *pitx3* expression was analyzed in the zebrafish midbrain using *in situ* hybridization, due to the lack of antibodies against these late dopaminergic transcription factors (Fig 27). The results revealed an increase of *Th* expression in dopaminergic groups A1 and A2 (first dopaminergic groups of zebrafish midbrain), as well as an increase in the number of *th* positive neurons in A4-A6 groups (posterior dopaminergic groups, oriented to the caudal area of the brain, arrow) (Figure 27A and 27A'). *Nurr1* and *pitx3* levels were decreased compared to control group (Figure 27B-27B' and 27C-27C'). In *mindbomb* mutants, *th* expression was clearly increased (Figure 27D and 27D'). In addition, the dopaminergic groups A4, A5 and A6 were not detected, but a huge increase in the number of A3 dopaminergic neurons was observed (Figure 27D', arrow). These findings correlate with those obtained for *nurr1* and *pitx3* (Figure 27E' and 27F'). In those pictures a displacement of the expression of the transcription factors appeared since the cellular populations migrated towards the midline respect to control groups (Figure 27D, 27E and 27F). In addition, the posterior dopaminergic groups of zebrafish forebrain were highly overexpressed in *mindbomb* mutants (Figure 27D and 27D', arrows), indicating that the factors involved could be affecting several other neuronal populations other than the diencephalic groups.

Figure 27. Opioid and Notch signaling implications in dopaminergic differentiation. The pictures correspond to ISH pictures against the mRNA of the three genes of 36 hpf zebrafish embryos. AB strain fishes (A, B, C, D, E, F) Oprm1 KD: Oprm1 knock-down morpholino (A', B' and C'). *Mindbomb*, notch signaling defective mutants (D', E' and F'). As observed in the pictures, opioid signaling silencing induced a downregulation of *nurr1* and *pitx3*, as well as *th* expression. On the other hand, notch disruption alters laterality of dopaminergic differentiation, inducing the expression of the dopaminergic markers and *th* neurons along the midline.

Results

Morphine and Notch neuroprotective effects against MPTP induced apoptosis in SH-SY5Y cells

MPTP is a known toxin that induces apoptosis in dopaminergic neurons. This molecule has been used to induce a pharmacological model of Parkinson disease (PD). The changes observed in the transcription factors triggered by opioid and Notch pathway suggested that the dopaminergic neurons treated with these drugs could present different resistance to MPTP, due to changes in several transcription factors such as NGN2, NURR1 or PITX3. SH-SY5Y cells were used again as a simplified model to assess dopaminergic neuron susceptibility to 40 μ M MPTP after an exposure of 12 hours (Fig. 28 and 29).

Figure 28. Morphine and DAPT reduces MPTP induced apoptosis on SH-SY5Y cells. TUNEL assay in SH-SY5Y after apoptosis induced by MPTP. A) Control B) Positive apoptosis control C) MPTP control D) DMSO E) 10nM Morphine F) DAPT G) Resveratrol H) U0126 I) Percentage of TUNEL positive cells. Morphine reduces the apoptotic effect of MPTP. DAPT and U0126 also reduce the number of apoptotic cells. Resveratrol did not induce any significant change. 40 fields were counted to measure the apoptotic percentage. Resv, Resveratrol. Arrows indicate TUNEL (apoptotic) positive cells. Red, TUNEL positive cells; Blue, DAPI. n=3

To determine the pathways implicated on the apoptotic cascade triggered by MPTP, cells were also exposed to several drugs: Morphine, DAPT, Naloxone, U0126 and Resveratrol (pharmacological activator of SIRT1) and combinations with morphine

Figure 29. Morphine and MAPK signaling disruption induce neuroprotection to MPTP. TUNEL assay in SH-SY5Y after apoptosis induced by MPTP. A) Control B) Positive apoptosis control C) MPTP control D) Morphine + DAPT E) Morphine + U0126 F) Morphine + Naloxone G) Morphine + Resveratrol H) DAPT + Resveratrol I) TUNEL positive cells. Morphine coadministrated with U0126 induced a greater neuroprotective effect than the two molecules separated. Resveratrol did not induce any significant change, although its administration with DAPT reversed the effects of Notch signaling disruption. 40 fields were counted to measure the apoptotic percentage. MD, morphine + DAPT; MU, morphine + U0126; MN, morphine + naloxone; MR, morphine + resveratrol; DR, DAPT + Resveratrol. Arrows indicate TUNEL (apoptotic) positive cells. Red, TUNEL positive cells; Blue, DAPI. n=3

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or DAPT. Morphine, DAPT and U0126 reduced the apoptotic effects of MPTP (Fig. 28). Moreover, morphine coadministration with U0126 or Resveratrol (Fig. 29) induced a greater neuroprotective effect than the observed with the administration of the drugs separately (Fig. 28). Resveratrol exposure did not have a significant neuroprotective effect (Fig. 28), except when coadministered with DAPT, pointing to a regulation of SIRT1 on Notch signaling (Fig. 29). As expected, naloxone reversed the neuroprotective effects of morphine (Fig. 29).

Susceptibility of zebrafish dopaminergic cells to MPTP

Zebrafish embryos have been described to be susceptible to MPTP. In order to analyze the neuroprotective effects observed in the SH-SY5Y cells, the embryos were exposed to MPTP from 24 to 48 hpf (Figures 30-32). After this time, several dopaminergic groups were not observed, specifically A2, A3 and A5/6. Morphine increased TH expression in several dopaminergic groups, but MPTP effects were still observed in A5/6 groups (Figure 30). DAPT increase TH expression and also countered the effects triggered by the toxin (Figure 30). Naloxone and resveratrol reduced the number of TH positive neurons (Figure 31). However, only naloxone exerted neuroprotective effects (Figures 31). MAPK pathway inhibition triggered by U0126 drastically reduced the number of TH positive neurons to groups A1 and A2 (Figure 31). Interestingly, these neurons were resistant to MPTP (Figure 31). DMSO did not affect TH neuronal groups and did not exert any effect against MPTP (Figure 30 and 31). Morphine and DAPT coadministration induced a separation between the different dopaminergic groups (Figure 32), but strikingly MPTP depleted all neuronal groups.

Figure 30. Neuroprotective effects against MPTP in 48 hpf zebrafish embryos. Immunohistochemistry against TH. MPTP induced the specific apoptosis of midbrain dopaminergic neurons of zebrafish. Morphine and DAPT demonstrated to be an effective neuroprotective agent, although specifically in the anterior dopaminergic groups (A1 and A2) and not on the posterior (A4-A6). . Blue: DAPI Red: TH

Figure 31. Neuroprotective effects against MPTP in 48 hpf zebrafish embryos. Immunohistochemistry against TH MN, morphine + naloxone. MPTP induced the specific apoptosis of midbrain dopaminergic neurons of zebrafish. Resveratrol did not show any neuroprotective effect against MPTP. Naloxone, however, did show an effect in contrasting MPTP effects. U0126 disrupted the dopaminergic distribution, indicating that this pathway is required to the correct dopaminergic differentiation. Blue: DAPI Red: TH

Figure 32. Neuroprotective effects against MPTP in 48 hpf zebrafish embryos. Immunohistochemistry against TH . DM, DAPT + Morphine; DR, DAPT + Resveratrol; MR, Morphine + Resveratrol; MU, Morphine + U0126. MPTP induced the specific apoptosis of midbrain dopaminergic neurons of zebrafish. Morphine and DAPT coadministration induced a separation between the different dopaminergic groups, but did not protect any of the groups from MPTP. Morphine + Resveratrol did not show any neuroprotective effects. The disruption of dopaminergic patterning by U0126 was

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Morphine and naloxone increased the number of TH positive neurons in A1 and A2 groups (Figure 31). As observed in morphine and DAPT group, MPTP administration almost induced a total elimination of dopaminergic cells (Figure 32). Morphine coadministration with U0126 induced an increase in A1 and A2 groups, although the posterior groups were still present. These cells were still resistant to MPTP (Figure 32). Morphine and resveratrol increased TH neurons in A1, A2 and A4 groups (Figure 32). These cells were affected by MPTP (Figure 32). DAPT and resveratrol mimicked the effects observed in figure C (Figure 32). However, these drugs did not show the neuroprotective effects triggered by the only administration of DAPT (Figure 30).

Discussion

Morphine regulates pri-miR-212/132 and miR-212 expression

miR-212 and miR-132 are two highly evolutionary conserved miRNAs codified in the same genetic cluster (Wanet et al., 2012). They also share a large homology between their sequences. The high homology of clustered miRNAs may indicate that these molecules are generated as a result of genomic duplications (Hertel et al., 2005). 30% of the miRNAs are located within a cluster (Axtell et al., 2011) and are either controlled by their own promoter or by a host gene promoter (Barroso-delJesus et al., 2008). miRNAs located in clusters are also generally believed to share a common primary transcript and hence, expected to be co-regulated and co-expressed (Altuvia et al., 2005; Kim et al., 2009). In fact, their seed regions, responsible for a great part of the specificity of the miRNA, have a 100% of homology; therefore, they may also exhibit overlapping functions. For this reason and, due to their relation with the CNS (Klein et al., 2007), it is of interest to determine how these miRNAs are physiologically regulated, whether morphine exerts alterations in the expression of the primary miRNA, modifying their transcription, and how the process of splicing is leading to a differential expression of the two mature molecules. Interestingly, from our analysis of the miR-212/132 cluster we conclude that these miRNAs may not share all their roles since the expression of miR-132 is higher than that observed for miR-212 and they both showed different expression peaks. miR-212 expression decreases during development, whereas miR-132 is highly expressed again at 48 hpf. This differential expression correlates with the processes in which these miRNAs are involved. miR-132 is related to somite and muscle differentiation, as previously described by Bijkerk et al. (2016). miR-212 has been directly related to addiction (Hollander et al., 2010). In addition, the expression of miR-132 seems to be favored at the expense of the expression of miR-212, suggesting a specific and important role

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for this miRNA. The downregulation observed in miR-132 levels after knocking-down miR-212 may indicate that miR-212 is regulating the expression of miR-132 in the cluster, and it may have evolutionarily emerged as a regulator for this miRNA (see figure 2) as similarly described in other clusters (Wayman et al., 2008). This phenomenon correlates with the findings made by several authors that have already described a different regulation of miRNAs that belong to the same cluster in some diseases (Wibrand et al., 2010; Chang et al., 2015; Zhang et al., 2015). Hence, if the expression of the individual members of a miRNA cluster is not concordant between each other, it would again provide indirect evidence for the possibility that some miRNAs can be transcribed and regulated as independent transcription units, as it occurs with miR-212 and miR-132 in the present work. However, the mechanism for this differential expression remains unclear (Mohammed et al., 2014). Interestingly, pri-miRNA-212/132, despite being transcribed as a unique molecule, induce different levels of the mature miRNAs after being spliced twice by different splicing proteins. We consider that the reason why the functions of the two molecules differ lays in this splicing process. The genomic organization of miRNA clusters may serve as a protection function against degradation, as all the molecules within the polycistronic pri-miRNA are believed to be more stable in this conformation, due to the numerous hairpins on the secondary structure that stabilize the RNA molecules (Mathelier and Carbone, 2013). Taking these facts into consideration, we hypothesized that the intergenic region of clustered miRNA may serve as a positive regulator of pre-miRNA splicing. The analysis of this intergenic region located between the sequences of miR-212 and miR-132 showed several consensus sequences between *Danio rerio*, *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*. The 3D conformation of RNA sequences have been described to be crucial during miRNA

splicing (Munroe and Duthie, 1986). In our analysis, we found that these consensus sequences are always located perpendicular to the z axis of the molecules (the axis between miR-212 and miR-132), indicating that those sites could be accessible to the spliceosome complex. In this sense, the predicted splicing proteins (MBNL1, SRSF2 and SRSF3) here found may be the responsible for the differential expression by directly binding to this highly accessible region. These proteins have been described to be putative regulators of miRNA splicing (Konieczny et al., 2014; Butkýtè et al., 2016). Together, these results indicate that this region between miR-212 and miR-132 (as seen in figure 3) could be exerting a splicing regulation that induces the differential expression of these two miRNAs.

miR-212 and mu opioid receptor reciprocal regulation

miR-212 is related to the addictive properties of drugs such as cocaine (Im et al., 2010), and it could be expected that a similar relation is observed after morphine exposure. Our results have shown that morphine induces the upregulation of miR-212 in zebrafish embryos at 24 hpf. This response is mediated by the activation of the mu opioid receptor, since *oprm1* morphants do not show any significant change after the administration of morphine (as seen in figure 4). In contrast, a decrease of this miRNA is observed at 48 hpf but, in this case, this effect is not reversed in the morphants. These results indicate that the decrease of miR-212 levels induced after a prolonged morphine administration from 5hpf to 48 hpf is mediated by a different pathway than that triggered by the activation of mu opioid receptor. Moreover, the changes observed in the levels of the pri-miR-212/132 expression at 24 hpf suggest that morphine is not only preventing the degradation of the miRNA but also effectively inducing its expression. Besides that, mu opioid receptor seems to be

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involved in the expression of the primary miRNA, since the knock-down of the receptor affects its expression, but the process of maturation is independent due to the uncorrelated expression of miR-212 with the primary miRNA levels in the *oprm1* morphants. Several authors have already observed similar findings as a result of a post-transcriptional regulation of clustered miRNAs (Ryazanski et al., 2011). On the other hand, the primary miRNA is not affected by morphine administration at 48 hpf. This result may be explained by a process of habituation, in which the levels of the pri-miR-212/132 are not affected by morphine. The mature form miR-212 is indeed affected despite this unalteration of the pri-miRNA pointing again to some sort of splicing regulation which could be mediated by Oprm1. The effect of knocking-down *oprm1* indicates that the receptor is still necessary for the physiological expression of this molecule at 48 hpf.

All these changes triggered by morphine were also analyzed by means of an ISH in which the pri-miR-212/132 was detected (Fig. 5). The results obtained highlighted the areas of the zebrafish embryo where these changes were more evident. Despite a mild expression at 24 hpf, the primary miRNA was located on the preorbital regions of the brain. However at 48 hpf, the expression was spread through the encephalon, with a higher expression around the midbrain. These results indicate that both miR-212 and miR-132 could be controlling the expression of several molecules necessary for the correct maturation the CNS, as previously described by Nakazawa et al. (2003).

Our findings show that morphine is upregulating pri-miR-212/132 and the levels of mature miR-212 and that *oprm1* is involved in these processes at 24 hpf. From all the predicted targets for miR-212, we found *oprm1* among them. This finding suggests that Oprm1 is not only controlling miR-212 expression at the transcriptional level of

the pri-miR-212/132 and its posterior splicing and maturation, but also that this miRNA has as a function regulating *oprm1* translation. This hypothesis is reinforced by the upregulation of *oprm1* levels at 24 hpf observed after the knock-down of miR-212 (Fig. 7), and was confirmed by means of a luciferase assay. Hence, the upregulation of miR-212 prevents *oprm1* mRNA translation, which will result in a decrease on the levels of Oprm1. However, we have observed a simultaneous increase of miR-212 and Oprm1 in the same stage. The temporality of these events must be considered here, since probably we are not observing the consequent downregulation of *oprm1* when miR-212 increase because these two alterations are taking place at the same point of development. However, if miR-212 is knocked-down since the beginning of zebrafish development, the levels of *oprm1* are highly increased. These results confirm that miR-212 has a regulatory function controlling *oprm1* translation, which means that it may be responsible for inducing the tolerance typically observed in the early stages of drug administration. Taken together, these results allow us to describe the control exerted on the expression of miR-212 (and also the primary miRNA) by morphine through Oprm1 activation and a feedback mechanism between *oprm1* and miR-212. These changes are also related to *oprm1* modulation after morphine administration. This receptor has been shown to induce its own expression in a feedforward mechanism after an acute morphine dose in zebrafish embryos at 24 hpf (Sanchez-Simon et al., 2010b). In contrast, we observed a decrease in the levels of *oprm1* mRNA at 48 hpf, probably due to a prolonged activation of the receptor or to a reduction in the transcription exerted on its promoter by some known repressors, such as MeCP2 (Formisano et al., 2007) or PU.1 (Hwang et al., 2004). However, we have observed an increase at the protein level after morphine administration. This effect correlates with the reduction on miR-212 levels

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observed after the exposure to morphine. This means that miR-212 is blocking the translation leading to the increase in the protein levels despite the effects observed in the mRNA.

In summary, these results indicate that morphine, through the activation of Oprm1, induces miR-212 overexpression. This miRNA reduces the translation of *oprm1* mRNA, controlling the expression of the receptor at the post-transcriptional level. The regulation could be also mediated by miR-132 and other pathways such as neurotrophins or other opioid receptors as previously described (Numakawa et al., 2011; Remenyi et al., 2010). These findings are resumed in figures 33 and 34.

At 24 hpf (Fig. 33), morphine affects the expression of pri-miR-212/132 as well as the mature form miR-212. These changes are mediated by Oprm1 activation, and this receptor is also responsible for controlling the physiological levels of both molecules, as observed after knocking-down the receptor and its own expression in a feedforward mechanism. Moreover, the upregulation observed after the administration of 10nM in the pri-miR-212/132 is not reversed after the knock-down of the receptor, indicating that some other pathway or receptor (such as Oprd1/2) could be contributing to these changes.

Figure 33. Morphine regulates miR-212 and *oprm1* expression through Oprm1 activation in zebrafish embryos at 24 hpf. The activation of Oprm1 by morphine induces an upregulation of the pri-miR-212/132 which, after posterior splicing and maturation, leads to an increase of the mature form miR-212. Besides, the activation of Oprm1 induces its own expression in a feedforward mechanism. miR-212 controls the upregulation of the receptor by targeting the 3'UTR of the mRNA. However, the mechanism by which 10nM morphine administration induces the overexpression of the primary miRNA may be mediated by other pathway, since the knock-down of the receptor does not reverse the upregulatory mechanism of morphine. Dotted lines indicate alternative pathways.

At 48 hpf (Fig. 34), morphine decreases miR-212 expression but does not have any effect on the pri-miR-212/132 levels. In addition, this decrease in miR-212 levels is not mediated by Oprm1, but the decrease on the levels of the receptor induces an upregulation of the primary miRNA. These findings may indicate that the receptor is required to control the expression of pri-miR-212/132, since the administration of morphine to *oprm1* morphants induces an upregulation of the primary miRNA not

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observed in the wild type embryos. However, there must be some other signaling cascades that decrease the levels of miR-212. These changes between the mature form and the transcribed molecule could be mediated by the activity of the splicing proteins within the cell.

Figure 34. Morphine regulates miR-212 and *oprm1* expression in zebrafish embryos at 48 hpf, although the mechanism does not involve Oprm1 activation. The knock-down of *oprm1* induces an increase of pri-miR-212/132 which is exacerbated after morphine administration. This indicates a putative regulation of the expression of the primary miRNA by other receptors, such as delta opioid receptors 1/2. However, this upregulation of the primary miRNA does not induce an increase on the levels of the mature form, indicating that the process of splicing must be regulated after morphine administration. Moreover, the increase on the levels of miR-212 at 24 hpf is now repressing *oprm1* levels. Dotted lines indicate alternative pathways.

Molecular mechanisms implicated in the changes exerted by morphine in miR-212 and *oprm1* expression

miR-212 expression is determined by several transcription factors involved in drug addiction. CREB has been shown to be one these factors in murines (Hollander et al.,

2010). In our study, the bioinformatic analysis of the miRNA-212/132 promoter of zebrafish revealed an interesting region where two CRE sites (i.e. CREB binding sites) were flanking a RE1 site (binding sites for RE1-silencing transcription factor or REST) (as seen in figure 10). RE1 sites are responsible for the repression of the transcription triggered by methyl CpG binding protein 2 (MeCP2). The induction of the activity of MeCP2 is mediated by calmodulin (CaM) and calmodulin kinase II (CaMKII), after phosphorylating the Ser421 of MeCP2 (Buchtal et al., 2012). This protein forms an epigenetic protein complex after binding to RE1 sites and, therefore, reduces the expression of miR-212 (Im et al., 2010). The CpG islands within these sites must be methylated in order to allow an effective binding of MeCP2 (Namiyama et al., 2008). We have found an increase of pCREB binding to miR-212/132 promoter after morphine administration at 24 hpf by ChIP-qPCR, maybe due to a putative increase of the phosphorylation of this protein or to its facilitated binding to this region. Moreover, the decrease observed at 48 hpf in miR-212 levels after morphine administration correlated with the decrease of pCREB binding to the promoter probably due to MeCP2 repression exerted on the RE1 site. Interestingly, the methylation of this region of the promoter was not affected by morphine administration at both stages (see figure 13). These results were not expected, although concurred with the proposed mechanism by which MeCP2 could be repressing miR-212 expression at 48 hpf since RE1 site is methylated. The alterations observed between 24 and 48 hpf may be mediated by changes in MeCP2 activation rather than changing the methylation pattern of the promoter.

The molecular mechanisms underlying Oprm1 activation is comprised by several pathways such as MAP Kinases, Calmodulin/Calmodulin Kinase II and Protein Kinase A (PKA). MAP kinase activation by Oprm1 is triggered by the action of the

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G α subunit of the G protein coupled to the receptor. This protein interacts with PKC, which induces the phosphorylation of RAS and Raf1, which activate the signaling cascade by subsequent phosphorylation of MEK1/2, ERK1/2 and MNK1/2. The functions of MAPK pathway end with the activation of several transcription factors, in which CREB is included. Besides MAPK pathway, calcium signaling is also controlled by Oprm1. The activation of mu opioid receptor is known to block calcium channels via the α subunit Gi (α Gi), modifying the levels of intracellular calcium (Bourinet et al., 1996). The decrease of intracellular calcium triggers a lower activation of calmodulin (CaM), with a posterior reduction in the activation of the several calmodulin kinases that are expressed (CaMK). CaM also induces MAP kinase activity mediated by PKC, RAS and Raf1 activation (Tebar et al., 2002) therefore triggering CREB phosphorylation. In addition, the $\beta\gamma$ subunits dissociation after Oprm1 activation induces the release of the intracellular deposits of calcium of the endoplasmic reticulum mediated by inositol triphosphate (IP₃) through phospholipase C (PLC) activation (Murthy and Makhoulouf, 1996). Finally, the G α subunit also inhibits the activity of the adenylate cyclase (AC) preventing the synthesis of cAMP, a second messenger necessary for the activation of the protein kinase A (PKA) which has been demonstrated to regulate morphine response (Nestler et al., 1996). For those reasons, the aim of this study was to analyze if the above described pathways, which are terminally related to CREB activation, were the responsible for the alterations triggered by morphine in the expression of miR-212 and *oprm1* observed in zebrafish embryos. To study these molecular pathways we selectively inhibited or promoted each of them (see figures 8 and 9).

Mechanism implicated in miR-212 and *oprm1* expression at 24 hpf

Using the inhibitor U0126, we have demonstrated that the upregulation of miR-212 induced at 24 hpf after the administration of morphine is mediated by the MAP kinase pathway (through the activation of MEK1/2), as the increase observed after morphine exposure was reversed by this drug. In fact, the levels of miR-212 are slightly downregulated compared to the control group. This indicates that MAP kinase signaling is necessary to maintain miR-212 physiological levels through the activation exerted by other pathways such as neurotrophins (Numakawa et al., 2011; Remenyi et al., 2010). The overexpression of *oprm1* after morphine exposure was also reversed after the addition of U0126 which confirms the implication of MAP kinase not only on its activity but also on its own expression.

When CaMKII activity was inhibited by W7, we observed a strong increase in the levels of miR-212 even when the embryos were not exposed to morphine. The inhibition of CaMKII may be inducing two complementary mechanisms within the cell. On the one hand, the $\beta\gamma$ subunits activation of PLC and posterior IP3 release induces the liberation of intracellular calcium stored in the endoplasmatic reticulum, with the subsequent activation of CaMKIV (Müller et al., 2011). This kinase induces the activation of MAP kinase pathway (Park et al., 1995) and the posterior phosphorylation of CREB, resulting in an overexpression of miR-212. On the other hand, the REST protein, binding to RE1 sites, and the consequent binding of MeCP2, which activation is CaMK-mediated, reduces the activity of miRNAs 212/132 promoter (Conaco et al., 2006) as well as *oprm1* (Formisano et al., 2007). Hence, the regulation exerted on these two genes after morphine exposure is also probably mediated through the binding of MeCP2 to the methylated RE1 sites here found within the miRNAs promoter. In summary, while CaM/CaMKII inhibition reduces

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MeCP2 phosphorylation and activation, it also increases CREB activity and miR-212 expression.

On the other hand, CAMKII pathway does not affect mu opioid receptor physiological expression, as its blockage does not involve any significant change in *oprm1* expression. However, this pathway is necessary for morphine overexpression of this receptor, as its inhibition reversed the effects of the drug. In this sense, the regulation exerted by miR-212 in the expression of *oprm1* may be the responsible for this effect, since miR-212 was upregulated after blocking this pathway.

PKA activation through the administration of forskolin induces CREB phosphorylation, therefore activating miR-212 expression. However, it did not modify *oprm1* levels. PKA activation by forskolin partially rescues the normal expression of these molecules. CREB phosphorylation mediated by PKA could provoke an induction of the expression of miR-212 and *oprm1*, but as MeCP2 is still activated and bound to the miRNAs promoter, the transcription is partially blocked, as has been described in the regulation of other genes of many cellular processes (Zhou et al., 2006).

Mechanisms implicated in miR-212 and *oprm1* expression at 48 hpf

However, at 48 hpf the pathways implicated in the changes exerted by morphine on miR-212 and *oprm1* expression seem to differ from the pathways described at 24 hpf. miR-212 levels were downregulated after morphine administration, especially after 10nM treatment. The inhibition of MAPK pathway or calcium signaling did not reverse the effects of morphine, probably due to a repression of the promoter that is

controlled by other pathways. PKA overactivation by forskolin partially reversed morphine effects, although the physiological levels were not retrieved. These results indicated that pCREB overactivation by an increase in PKA activity could counteract the effects of MeCP2 repression but, since this protein is still bound to the promoter due to its methylation degree, the effects observed after the only administration of morphine were not recovered. The expression of *oprm1* is also reduced after morphine administration at 48 hpf. However, the results showed that the inhibition of MAPK pathway reversed the effects of morphine, as well as calcium signaling inhibition after 10nM morphine administration. These findings correlate with the mechanism that we have proposed: the phosphorylation and activation of MeCP2 by CaMKIV mediated by intracellular calcium might be responsible for the binding of MeCP2 to *oprm1* promoter, and therefore it may be controlling the expression of the receptor. As observed with miR-212, PKA overactivation partially rescued *oprm1* expression, presumably by the increase in CREB phosphorylation and the subsequent increase in transcription.

Taking the above into consideration, we propose the following simplified activity scheme which relates the expression of mu opioid receptor and miR-212 (Fig 35): the activation of Oprm1 (light green) induces several intracellular changes; 1: activation of α Gi subunit inhibits adenylyl cyclase and reduces cAMP production, preventing the activity of Protein Kinase A (PKA, red); 2: In addition, α Gi subunit closes calcium channels (CaCh, blue); 3: Oprm1 activation of Protein Kinase C induces the activation of RAS and Raf1, which activate MAP Kinase pathway (MEK, ERK and MNK1/2, dark blue), inducing the phosphorylation of CREB (orange). In contrast, the miR-212 downregulation observed in all the experimental groups at 48 hpf (Figure 36) seems to be mediated by several factors, but not by Oprm1 activation.

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Moreover, the expression of miR-212 and *oprm1* is clearly reduced, indicating that the mechanism undergoing after morphine administration must be different. The regulatory mechanism involved in the responses observed is mediated by three processes: 1, cAMP overproduction after prolonged morphine administration induces PKA activation and thus induces *oprm1* expression (Conaco et al., 2006), 2, the reopening of calcium channels induces MeCP2 phosphorylation and activation through CaMKII, allowing the repression of miR-212/132 expression through the binding to the RE1 site of the promoter, reducing pCREB activity, as seen in our ChIP-qPCR analysis and 3, the release of intracellular calcium activates CaMKIV which can also control MeCP2 phosphorylation and activation.

Figure 35. Proposed mechanism for the regulation of pri-miR-212/132 expression through the activation of mu opioid receptor (Oprm1) by morphine at 24 hpf. The mechanism proposed at 24 hpf involves three different pathways: **1**, inhibition of adenylate cyclase mediated by α Gi that induces a decrease of cAMP and thus reduces PKA activity **2**, α Gi subunit closes calcium channels, therefore reducing MeCP2 phosphorylation and preventing the repression of the pri-miR-212/132 expression **3**, PKC activation induces MAP kinase pathway, phosphorylating CREB. The repression of calcium signaling prevents the activation of CAMKII and the phosphorylation of MeCP2, thus a repression of the transcription is not observed. CREB phosphorylation mediated by MAPK pathway induces the upregulation of miR-212/132, as observed in our ChIP-qPCR studies. Dotted lines implicate that these pathways are not active during the process.

Figure 36. Proposed mechanism for the regulation of pri-miR-212/132 expression through the activation of mu opioid receptor (Oprm1) by morphine at 48 hpf. The mechanism proposed at 48 hpf involves three different pathways: **1**, hyperactivation of PKA induces CREB phosphorylation, thus controlling *oprm1* expression. **2**, The re-opening of calcium channels induce MeCP2 phosphorylation through CaMKII and binds to miR-212/132 promoter R1 site, repressing the expression of the miRNAs, **3**, the release of intracellular calcium induces CaMKIV phosphorylation, enhancing the repressive effects of MeCP2 on the RE1 sites of *oprm1* and miR-212/132 promoters. Dotted lines indicate pathways that are not active during this process.

miR-29a regulation through NF- κ B signaling modulates Notch activation after morphine administration

Our study has proved that morphine controls miR-212 expression, and this molecule has been shown to control mu opioid receptor expression through direct regulation of *oprm1* mRNA. This regulation is mediated by the phosphorylation of CREB, a transcription factor that is modulated by several pathways. Several authors have proposed that Notch signaling cascade, via Notch intracellular domain (NICD) interacts with pCREB signaling in many cellular processes (Hallaq et al., 2014; Brai et al., 2015). These findings were corroborated by our ChIP-qPCR studies, which showed a decrease in pCREB binding to the miR-212/132 promoter after DAPT administration, indicating that Notch signaling actively controls pri-miR-212/132 expression (see figure 12) and through the modulation of miR-212 maturation is, therefore, regulating *Oprm1* expression. Moreover, morphine has been related to the alteration on the expression of many other miRNAs apart from miR-212 and miR-132 (Hwang et al., 2012). Among them, several authors pointed to a possible dysregulation of miR-29a after morphine administration in the immune system (Pilakka-Kanthikeel and Nair, 2015) and in the CNS (Kaur et al., 2012). This miRNA induces a feedforward mechanism by which miR-29a induces a downregulation of Disintegrin and metalloproteinase domain-containing protein 12 (ADAM12), a metalloproteinase involved in Notch cleavage mediated activation (Li et al., 2011). The downregulation on this protein, decreases Notch1 cleavage and induces a reduction of NICD levels. In addition, NICD, interacting together with CSL and NF- κ B, acts as a repressor of miR-29a expression. We hypothesized that morphine could modify the levels of this miRNA on zebrafish embryos, and therefore regulate Notch signaling. In this sense, the temporal expression of miR-29a

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on zebrafish embryos on the five stages of development was analyzed (5, 8, 16, 24 and 48 hpf) (as seen in figure 14). Since miR-29a is required for Notch1 cleavage and therefore it controls Notch pathway, the cyclic pattern in which miR-29a levels rise (5, 16 and 48 hpf) and decrease (8 and 24 hpf) periodically indicate that there are several stages in which this molecular signaling is more important. NICD also controls NF- κ B expression through Hairy and enhancer of split1 (Hes1) (Espinosa et al., 2010). This protein controls neurogenesis and it has been shown to be expressed following a cyclic pattern during certain stages of development (Dhanesh et al., 2016) as well as it occurs with miR-29a. Moreover, miR-29a controls Nuclear factor I A (Nfia) expression, a transcription factor repressor of Hes1 expression (Liu et al., 2015). Together these results point that miR-29a, presenting lower levels of expression than the other miRNAs studied (miR-212 and miR-132), is controlling the neurogenesis of specific populations by targeting Hes1 and NF- κ B.

We have also corroborated that morphine increases the phosphorylated NF- κ B levels and consequently alters miR-29a expression. Morphine induces changes in gene expression by affecting the activation of several transcription factors, but the mechanisms underlying NF- κ B activation remain unclear. Our western blot analysis showed that the disruption of MAPK signaling increased NF- κ B activation. However, the effects were not reversed when morphine was co-administrated together with the MAPK inhibitor. These results suggest that MAPK exert a direct repressive effect on NF- κ B activation and that the effects of morphine on this protein are probably related to an alteration in the MAPK pathway. In addition, western blot of proteins extracted from *mindbomb* mutants, in which Notch cleavage is decreased, showed an increase in the phospho-NF- κ B since NICD is a known regulator of NF- κ B activity (Schwarzer et al., 2012). We have also observed that morphine administration

induced an increase of phosphorylated NF- κ B in *mindbomb* mutants, mimicking the upregulation observed in the untreated mutants. Through its binding to the mu opioid receptor, morphine is responsible for the activation of the *inhibitor of κ B kinase* (IKKB) via PI3K/Akt (Ye et al., 2001). IKKB is involved in the regulation of NF- κ B activation through the phosphorylation of NF- κ B repressor *inhibitor of κ B* (IKB). Also, NICD and CSL induce IKKB activation. Hence, the upregulation of NF- κ B triggered by NICD absence indicates that the activity of IKKB may be still promoted despite the expected lower expression triggered by the lack of the complex formed by NICD and CSL. Taking the above into consideration, these findings may provide new insights into the role of NICD on miR-29a expression and the implication of this miRNA in the expression of Hes1, which regulates the expression of NF- κ B. This mechanism may explain the alternative pathway by which this upregulatory effect on NF- κ B expression is occurring. Morphine decreases miR-29a even when coadministrated with DAPT, explaining the recovery in the levels of NF- κ B after the exposure to this drug in both AB and *mindbomb* zebrafish embryos. This fact means that morphine is not only triggering a pathway that enhances NF- κ B levels in the mutants but that also its activity in non-mutated embryos is NICD-dependent. In contrast, a decrease of phosphorylated NF- κ B was observed in the mutant embryos treated with U0126 when compared with the untreated *mindbomb* mutants. Its coadministration with morphine did not induce any significant change when compared with the AB zebrafish embryos.

Morphine administration increased NICD levels in SH-SY5Y, indicating that the reduction of miR-29a levels, mediated by an increase of phosphorylated NF- κ B, could increase ADAM12 levels, thus enhancing the cleavage of Notch1. In addition, ChIP-qPCR has shown that DAPT administration partially blocked pCREB binding

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to miR-212/132 promoter. NICD modulates CRE-dependent transcription (Hallaq et al., 2015), and this could indicate that Notch signaling controls opioid pathway through modulation of miR-212, direct regulator of OPRM1 expression. These results also correlates with the increase in OPRM1 levels after DAPT administration, as the levels of miR-212 are reduced due to less pCREB binding to the promoter. After these findings, the following mechanism that explains Notch and opioid signaling interaction mediated by the three miRNAs studied (miR-212, miR-132 and miR-29a) and trough the modulation of CREB and NF- κ B (Figure 37) has been proposed. Morphine activates OPRM1 and, through PI3K/Akt pathway, induces IKK β activation and NF- κ B phosphorylation leading to the decrease of miR-29a levels. This reduction induces an increase in ADAM12 expression, and Notch cleavage is enhanced and NICD levels are raised. NICD, on the other hand, induces a decrease in pCREB binding of miR-212/132 promoter, decreasing the levels of this miRNAs and increasing OPRM1 expression (as seen in figure 17).

Figure 37. Opioid and Notch signaling cascades are related by the three miRNAs studied (miR-212, miR-132 and miR-29a) and through the modulation of CREB and NF-κB. Morphine activates OPRM1, and through PI3K/Akt pathway induces IKK activation and NF-κB phosphorylation decreasing miR-29a levels, although MAPK signaling, through an unknown intermediate reduces NF-κB phosphorylation (dotted lines and interrogation sign). The reduction of miR-29a induces an increase in ADAM12 expression, and Notch cleavage is increased raising NICD levels. NICD induces a decrease in pCREB binding of miR-212/132 promoter, decreasing the levels of these miRNAs and increasing OPRM1 levels.

Opioid and Notch signaling affect dopaminergic development

We have demonstrated that opioid and Notch signaling pathways regulate each other through the interaction of three miRNAs. These two pathways are also known to control cell proliferation, aggregation and differentiation (Reynolds-Kenneally and Mlodzik, 2005; Khurshid et al., 2010). In addition, miR-212 and miR-132 have been related to these same processes (Wada et al., 2010; Anand et al., 2010; Mehta et al., 2015). Besides that, miR-29a is implicated in cell proliferation, apoptosis and differentiation of several cell groups (Calin et al., 2005; Li et al., 2009; Winbanks et al., 2011). We have observed that the administration of morphine to the primary neuron culture of zebrafish induced morphological changes that not only could be related with cell morphology but with cell differentiation. These facts indicate that opioid interacting with Notch signaling could be controlling several of the processes that lead to the correct development of the zebrafish embryos. We observed an increase in cell aggregation after morphine administration, where clusters of neuronal precursors, expressing GFP, were found. The delayed differentiation indicates that morphine is affecting the maturation of neuronal precursors to neurons. DAPT also delayed cell differentiation, but the effects in aggregation were not comparable to the morphine group. Interestingly, morphine and DAPT coadministration increased neuronal differentiation, and reduced cell aggregation, meaning that Notch signaling is necessary to trigger the effects induced by morphine, as we have previously observed for the expression of NF- κ B.

Among all the neuronal populations derived from the neuronal precursors, the dopaminergic neurons have been studied due to the implication in the appearance of addiction and for their implication in Parkinson's Disease (PD). Our results in the

dopaminergic cultured cells indicate that the dopaminergic differentiation may be one of the regulated by the opioid and notch signaling. The modulation of dopaminergic markers after morphine administration has been already described (Garcia-Perez et al., 2013). In the same way, Notch signaling has also been described to be involved in dopaminergic differentiation, although the exact molecular pathway triggered by this receptor is still unclear (Mahler et al., 2010). As observed in figure 21, morphine reduced the total number of TH positive cells. DAPT, on the other hand, did not alter the total number of differentiated dopaminergic cells. However, the results observed in the primary cell culture were mimicked in the mature neurons obtained from R1 ES cells. Cell aggregation was increased in morphine treated cells, and interestingly, these clustered groups of cells expressed TH. Since morphine is inducing an increase in NICD levels, the delay in differentiation may be mediated by this protein imitating the activation of Notch cascade through yuxtacrine communication. Although DAPT decreased cell aggregation and proliferation, the isolated cells were expressing TH in contrast to morphine group. Since Notch signaling activation is mediated by cell-cell interaction, these events suggest that this signaling cascade may be modulating the induction of dopaminergic phenotype.

The alterations in cell maturation and in dopaminergic differentiation after morphine or DAPT administration suggested that several dopaminergic markers and transcription factors (such as NGN2, FOXA2, SIRT1, PITX3, NURR1 and TH) could be playing a critical role in allowing the changes triggered by opioid and Notch pathways. Hence, we analyzed whether the transcription factors implicated on dopaminergic development were altered after morphine or DAPT exposure (see figures 22 to 25). NGN2 is expressed on NSS (second stage of differentiation of R1 ES cells), but not on the embryonic stem cell stage. Moreover, morphine induced an

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upregulation of the levels of NGN2 at this stage. Morphine has been reported to induce changes in Droscha expression (Xu et al., 2015) and this protein is necessary to induce NGN2 expression (Knuckles et al., 2012), which may explain the overexpression here observed. DAPT administration did not induce any significant change on NGN2 expression compared to DMSO group, indicating that the effects exerted by Notch signaling were not mediated by this protein. FOXA2 was expressed at the first and second stage of R1 ES cells. DAPT induced an increase on FOXA2 levels on the embryonic cell line at the first stage. FOXA2 is an important intermediate transcription factor with several functions in dopaminergic development, from neuronal specification (Ang, 2009) to dopaminergic differentiation (Xi et al., 2012), acting as a known inducer of Lmx1a and Lmx1b, upstream inducers of the terminal transcription factors PITX3 and NURR1. This means, that Notch may be altering the dopaminergic differentiation through FOXA2.

At the third stage of differentiation (i.e. neuronal precursors), PITX3 expression is decreased after DAPT administration, but increased by morphine. However, NURR1 is decreased in the morphine group but slightly increased after DAPT administration. These results observed in the third stage could correlate with the delay in the differentiation observed in the primary neuronal culture of zebrafish embryos since these proteins are well-known terminal transcription factors involved in TH expression. On the other hand, in the latest stage of differentiation of the cells (mature neurons), morphine slightly upregulates NURR1 while DAPT downregulates its expression, although this decrease was not significant. However, the levels of TH are not modified in any of the groups. These results are in contrast with the IHC experiments, in which we appreciated changes in dopaminergic development. These results do not mean that these signaling cascades are not controlling the

differentiation process. In fact, the results of the IHC showed differences between the aggregation of the cells, indicating that the changes triggered on all the transcription factors could be affecting to a specific subset of dopaminergic neurons. The existence of different mature dopaminergic neurons that express different combinations of transcription factors have been previously described (Cave et al., 2010; Luk et al., 2013; Khan et al., 2017) reinforcing our findings.

Finally, SIRT1 expression was analyzed. This protein was expressed in the three first stages of development of R1 ES cells, but we were not able to detect its expression on mature dopaminergic neurons. Morphine and DAPT administration induced an upregulation of SIRT1 levels in all the stages studied, with the exception of NSS, in which DAPT did not affect the levels of the protein. Notch activation is known to be an intermediate of SIRT1 expression, through the modulation of chromatin remodeling via tri-methylation of histone 3 (H3K4Me3) a well-described epigenetic mark (Xie et al., 2012). These results show a feedback mechanism that control SIRT1 expression by Notch signaling disruption. This means that a decrease in NICD induces an increase in SIRT1 expression, and allows the further epigenetic regulation exerted by SIRT1 in the expression of other genes. These results indicate that SIRT1 may be responsible for several of the changes observed on the other transcription factors studied as previously described (Hisahara et al., 2008; Sugino et al., 2010). Hence, our results show that morphine and DAPT have similar effects in the early genes involved in dopaminergic differentiation, although the changes did not totally correlate.

In addition, we used miRNA mimics in SH-SY5Y cells to assess the implications of miR-212, miR-132 and miR-29a during the dopaminergic development (see figure 26). The findings here obtained could explain how opioid and Notch signaling are

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regulating the differentiation of these neurons via the modification of the levels of the miRNAs analyzed in this work. The western blot showed that miR-132 mimics induced an upregulation of SIRT1, and so did miR-212 to a lesser extent. These effects are not mediated by opioid pathway activation after morphine exposure, since morphine did not induce any alteration in the analyzed groups. Morphine decreases SIRT1 expression in miR-132 mimic group. Previous findings have already described that miR-132 downregulates SIRT1 expression through the binding to the 3'UTR of its mRNA (Zhang et al., 2014). However, the upregulation here observed suggests that there might be an alternative intermediate which could regulate SIRT1 expression. Our results have shown that miR-132 regulates NGN2 expression and this regulation is also found after morphine administration. This relation between this miRNA and NGN2 is a novel regulatory mechanism by which miR-132 is modulating the physiological expression of this molecule.

Interestingly, miR-29a was able to induce PITX3 expression in SY-SY5Y cells that are not constitutively expressing it. As morphine and DAPT are not able to induce PITX3 expression by itself, these results mean that miR-29a is inducing the repression of some genes that are indeed preventing SH cells to express PITX3, although the identity of these targets is unknown.

Morphine upregulates TH expression, in contrast to the overexpression of the three miRNAs which did not induce any significant change, hence the analyzed miRNAs are not involved in the physiological expression of TH. However, miR-132 and miR-29a mimics reversed the increase triggered by morphine since its coadministration with the mimic did not show the same upregulatory effect. This indicates that these molecules may be downregulating an upstream target that promotes TH expression thus regulating morphine response.

However, these findings are not excluding the fact that there are different subsets of dopaminergic neurons that can differ on their gene expression.

Up to this point, these findings suggested that opioid signaling and notch pathway are regulating dopaminergic differentiation modifying the expression of several transcription factors. Moreover, these pathways are regulating each other via miR-212, miR-132 and miR-29a, and these miRNAs are able to regulate several of the transcription factors implicated in dopaminergic differentiation. These results were obtained using simplified cellular models of dopaminergic differentiation. To correlate these findings with the development of zebrafish embryos an ISH experiment was performed due to the lack of antibodies to be used on this model (see figure 27). The microinjection of *oprm1* morpholino showed that endogenous opioids were required to the correct dopaminergic development. The levels of *th* were increased after the knock-down of *oprm1*. These results showed for the first time that endogenous opioids not only play an important role in reward mechanisms (Colasanti et al., 2012), but also in neuronal specification, since the expression of *nurr1* is increased and the expression of *pitx3* is reduced in *oprm1* morphants. Our studies show that opioid and notch signaling affect dopaminergic development. In addition, *Mindbomb* mutants and Notch signaling have been related to alteration in zebrafish dopaminergic development (Mahler et al., 2010). However, our results showed a pattern of expression of the transcription factors *nurr1* and *pitx3* towards the midline. These results indicate that Notch pathway is not only altering the level of expression of several of the transcription factors involved in dopaminergic development, but also specify the laterality and the area of the brain where these neurons differentiate. Several authors have described that Notch induces the left-right laterality of the transcription factors involved in this process. These pathways are also related to the

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asymmetry determination through Nodal expression (Krebs et al., 2003). Also, these factors have to be expressed on a certain area since their coexpression in the same region of the midbrain of zebrafish embryos terminally induces *th* expression (Figure 39).

Figure 38. Expression of dopaminergic transcription factors in AB zebrafish embryos (A) and in *mindbomb* mutants (B). The regions where these factors are colocalized induce the expression of *th* on the area of the midbrain (C). These results suggest that Notch pathway is required for the correct specification of dopaminergic neurons and determine the laterality and the regions where dopaminergic neurons are generated.

Hence, our results indicated that morphine and Notch signaling were controlling TH expression in a deterministic manner in several subsets of dopaminergic neurons. As previously described, morphine and Notch control CREB activity. CREB is a known

inductor of NURR1 expression. Besides that, Nurr1 phosphorylation mediated by MAPK pathway is required to the effective binding of NURR1 to *th* promoter (Sakurada et al., 1999). Morphine through the activation of MAPK induces NURR1 expression and also its phosphorylation and activation, promoting TH expression (Figure 39). However, NICD can induce NURR1 expression by reducing the binding to its promoter of several repressor complex mediated by CSL. These results indicate that NURR1 *per se* is not sufficient to induce TH expression, and this particular mechanism could explain the differences observed in different neuronal groups during differentiation. Moreover, SIRT1 can modulate NICD activity by its deacetylation (Guarani et al., 2011) and also actuate as a corepressor of NURR1 activity (Kim et al., 2013). Hence the regulation of SIRT1 by miR-212 and miR-132 may be controlling NURR1 expression and leading to the alterations found in TH levels.

Figure 39. Notch and opioid signaling interaction mediate TH expression through NURR1 expression. Morphine induces NURR1 expression and also its phosphorylation and activation, promoting TH expression. However, NICD can induce NURR1 expression by reducing the binding to its promoter of several repressor complex mediated by CSL. Moreover, SIRT1 can modulate NICD activity by its deacetylation (Guarani et al., 2011) and also actuate as a corepressor of Nurr1 activity (Kim et al., 2013). These results indicate that NURR1 *per se* is not sufficient to induce TH expression that could explain the differences observed in different neuronal groups during differentiation.

Opioid signaling and Notch pathway affect dopaminergic susceptibility to MPTP through the modulation of dopaminergic transcription factors

The changes triggered by opioid and notch signaling, as well as the dysregulation of miR-212, miR-132 and miR-29a induced several changes in the expression of several transcription factors implicated in dopaminergic differentiation. These pathways also modified the cell aggregation, even the areas of the brain where these neurons normally are generated. All these changes also pointed to different subsets of dopaminergic neurons that express slightly different transcription factors. This fact could affect also the susceptibility of these neurons to oxidative insult, as it pathologically occurs in Parkinson's Disease (PD). Some of these transcription factors are known for their neuroprotective effects by reducing the apoptotic effect that some specific toxins, such as MPTP, can exert on the dopaminergic cells (Meredith and Rademacher, 2012). PITX3 has been differentially observed in specific subsets of dopaminergic neurons, which confers them different vulnerabilities to neurodegeneration (Korotkova et al., 2005). Also, FOXA2 expression in mature neurons induces neuroprotection (Oh et al., 2015). NURR1 has also been related to neuroprotection of dopaminergic neurons, as it reduces NF- κ B expression thus reducing the inflammatory gene expression. In addition, NURR1 has been shown to interact with p53 suppressing its transcriptional activity, reducing the apoptosis triggered by several toxins. Nurr1 may serve as a therapeutical approach as a neuroprotector against Parkinson Disease specifically apoptotic effect of dopaminergic neurons (Dong et al., 2016). Moreover, FOXA2 interaction together with NURR1 is believed to be an important modulator of dopaminergic neurons survivability (Oh et al., 2015).

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The exposure of SH-SY5Y cells to morphine (see figures 28 and 29), DAPT and MAPK inhibitor U0126 significantly reduced the apoptotic effects of MPTP. Morphine and DAPT induced an increase of PITX3 and NURR1 on ES R1 cells, which could be implicated on the resistance of these cells to MPTP. SIRT1 has also been related to survival of dopaminergic neurons. However, resveratrol did not show any neuroprotective effects against MPTP, suggesting that the upregulation of SIRT1 triggered by morphine and DAPT are not related to the neuroprotective effects of these drugs.

Interestingly, from all the dopaminergic groups in the zebrafish midbrain some seem to be affected more than others, specifically A1, A3 and A4/5 groups (see figures 30-32). These findings suggest that the gene expression of these different groups of neurons although similar, is not identical, which confers them different properties and partial resistance to MPTP. Although the complete mechanism by which dopaminergic specification occurs is partially known, recent studies indicate that a certain subset of dopaminergic neurons does not require the same elements to induce dopaminergic differentiation (Fujimoto et al., 2012). These results, together with the laterality studies in *mindbomb* mutants, indicate that the dopaminergic development implicates different transcription factors that make those neurons different from each other.

Conclusions

The unique properties of morphine, which is involved the pain and reward mechanism of the brain simultaneously, make this molecule an interesting candidate to evaluate the processes triggered in the brain after drug intake. In this study, we show novel insights in the molecular pathway triggered by morphine that regulates the opioid system. We also defined a cross-talk between this pathway and Notch signaling. This interaction modulates dopaminergic differentiation, and could play a critical role in the development of addiction after chronic morphine intake. After the analysis of the results obtained in this work we can present the following conclusions:

1. Morphine controls the expression of miR-212 and miR-132 through the control of the transcription of pri-miR-212/132. The process of maturation of the primary miRNA induces a differential expression between both mature forms, which at the same time lead to different functions between them.
2. miR-212 controls opioid signaling by targeting the 3'UTR of the *oprm1* mRNA, therefore controlling the self-regulatory mechanism triggered by morphine and mu opioid receptor.
3. The upregulation triggered by morphine on the expression of these miRNAs depends on the developmental stage. The first responses triggered by morphine on the expression of these miRNAs are mediated by MAPK signaling and by the phosphorylation of CREB. On the other hand, the cascades implicated at 48 hpf are related to calcium signaling and the phosphorylation of MeCP2.
4. Notch signaling, via NICD, controls miR-212 and miR-132 expression through the binding of pCREB on the promoter of the miRNAs. Our findings corroborate that Notch signaling is altering opioid signaling cascade via alterations of *Oprm1* expression.

Conclusions

5. Morphine induces the phosphorylation and activation of NF- κ B on 24 hpf zebrafish embryos, therefore modulating the cleavage and activity of Notch signaling through miR-29a. miR-212 regulates *oprml* expression both physiologically and after morphine administration. These findings corroborate our hypothesis of a cross-talk between the two pathways mediated by miR-212/132 and miR-29a.
6. Opioid and Notch signaling control the expression of the dopaminergic transcription factors necessary for the correct differentiation of dopaminergic neurons.
7. The changes on the expression of the dopaminergic transcription factors and markers triggered by opioid and Notch signaling alter the susceptibility of dopaminergic neurons to oxidative insult by MPTP. However, several dopaminergic populations of zebrafish must present different characteristics that induce differential resistance to this toxin.

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ANNEX I

SPANISH SUMMARY OF THE DISSERTATION

Resumen en castellano de esta tesis doctoral

Interacción entre las cascadas de señalización Notch y opioide. Implicación en el desarrollo dopaminérgico tras la exposición a morfina.

Memoria presentada por

Adrián García Concejo

Para optar al título de Doctor

Por la Universidad de Salamanca

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Introducción

El análisis de la cascada de señalización molecular activada por la morfina puede permitir el descubrimiento de nuevas dianas que puedan ser usadas para paliar los efectos secundarios derivados del uso de opioides. Los receptores opioides han sido ampliamente estudiados, y su regulación después de su activación podrían explicar las bases moleculares de la adicción, en especial la del receptor opioide mu (Oprm1), dado que es el receptor por el cual la morfina muestra su máxima afinidad (Pert y cols. 1973). Los receptores opioides son receptores acoplados a proteínas G que inducen la activación de la proteína de unión a elementos en respuesta a AMPc (CREB) (Impey y cols. 2004; Ma y cols. 2001), un factor de transcripción envuelto en numerosos procesos moleculares.

Los miRNAs son pequeñas moléculas de RNA no codificante que tienen como función la regulación de la expresión génica a nivel post-transcripcional. Estas moléculas controlan la expresión génica mediante la unión a secuencias complementarias en la región no traducida del extremo 3' del RNAm (3' UTR), e inducen la degradación y/o inhiben la traducción (Bartel y cols. 2009). Los miRNAs están envueltos en numerosos procesos durante el desarrollo, tales como la diferenciación y la proliferación, y su desregulación se ha relacionado con una gran variedad de enfermedades (Shidu y cols. 2015; Song y cols. 2015). Otros miRNAs, como el miR-let7d y el miR-133b (Lopez-Bellido y cols. 2012; Garcia-Perez y cols. 2015) son regulados por la morfina y podrían tener un papel en los efectos comportamentales de esta molécula.

Los miR-212 y miR-132 forman un clúster, aunque sus funciones podrían no estar del todo correlacionadas (Kumarswamy y cols. 2014; Wanet y cols. 2012). Estudios previos (Vo y cols. 2005; Im y cols. 2010) han descrito que la expresión del miR-212 se veía incrementada en el núcleo estriado dorsal en ratas que tenían acceso extendido y no restringido a cocaína administrada por vía intravenosa. Además, la activación de CREB induce la regulación de la expresión del miR-212 después de la ingesta de cocaína en modelos murinos (Hollander y cols. 2010). Por otra parte, el miR-29^a ha sido descrito como un regulador de la actividad de la vía de señalización de Notch (Li y cols. 2011). Este miRNA tiene como diana a la proteína ADAM12, una metaloproteasa necesaria para el corte y la activación del receptor Notch. Mediante este proceso, este miRNA puede reducir la actividad de esta ruta de señalización.

La cascada de señalización de Notch está envuelta en la proliferación celular y la diferenciación mediada por interacciones célula-célula (Irles y cols. 2016). Este receptor, después de ser activado por sus ligandos Delta o Jagged, es cortado dos veces consecutivas con la consecuente liberación de su fragmento intracelular (NICD) (Artavanis-Tsakonas, 1999). Este fragmento interacciona con varias proteínas que regulan la expresión génica.

Las neuronas dopaminérgicas, que usan como neurotransmisor dopamina gracias a su síntesis por la enzima tirosina hidroxilasa (TH) juegan un papel crucial en el proceso de recompensa, aunque un grupo heterogéneo de neuronas se ha relacionado tanto con los estímulos aversivos y placenteros (Anstrom y cols. 2009; Brischoux y cols. 2009). La modulación de los factores de transcripción involucrados en el desarrollo dopaminérgico podrían ser usados como diana terapéutica para prevenir el desarrollo de la tolerancia y la adicción, así como ser empleados en terapias para el tratamiento de la enfermedad de Parkinson.

Hipótesis y objetivos

La morfina no es solo el analgésico de uso más común, sino que es una molécula de gran interés debido a su capacidad de inducir la aparición de tolerancia y adicción mediante la activación de los mecanismos de recompensa del cerebro. Las propiedades adictivas de otras drogas han sido relacionadas con la desregulación de varios RNAs no codificantes, tales como el miR-212 o el miR-132. La alteración de la expresión de estos miRNAs podría afectar a sus dianas, implicadas directa o indirectamente con el desarrollo. Entre los distintos subtipos neuronales del cerebro, las neuronas dopaminérgicas están involucradas en el sistema de recompensa. Por este motivo, el desarrollo y diferenciación de estas neuronas es crucial. Teniendo en cuenta estos hechos, proponemos que la morfina modula la diferenciación de las neuronas dopaminérgicas mediante alteraciones en los factores de transcripción que inducen la correcta diferenciación de estas neuronas. La cascada de señalización de Notch también está envuelta en este proceso, y teniendo en cuenta las consideraciones anteriores, también proponemos la hipótesis de que ambas cascadas están relacionadas. Estas dos vías de señalización podrían estar reguladas por varios factores de transcripción, tales como CREB o NF- κ B, o mediante alteraciones en los miR-212, miR-132 o miR-29^a. Para validar esta hipótesis, proponemos los siguientes objetivos:

- 1.- Determinar si la morfina induce cambios en la expresión del miR-212 y el miR-132, y dilucidar las funciones de estos miRNAs en la cascada opioide
- 2.- Analizar una posible relación entre el pri-miR-212/132 (miRNA primario) y la expresión diferencial entre las formas maduras miR-212 y miR-132-
- 3.- Analizar si la desregulación del miR-212 y del miR-132 altera la expresión del receptor opioide mu. Además, se estudiará si la cascada de señalización de Notch está envuelta en el proceso mediante la desregulación de estos miRNAs.
- 4.- Medir los niveles de expresión del miR-29^a después de la administración de morfina, y determinar si estas alteraciones en la expresión del miR-29a están mediadas por NF- κ B, por lo tanto también induciendo cambios en la actividad de Notch a través de ADAM12. Estos resultados corroborarán la hipótesis de la relación entre la actividad de ambas vías.
- 5.- Analizar la alteración de los niveles de varios factores de transcripción (PITX3, FOXA2, SIRT1, NURR1 y NGN2) después de a) la activación de la vía opioide tras la exposición a morfina y b) la disrupción de la señalización de Notch empleando DAPT, y determinar si estos cambios inducen alteraciones en la diferenciación dopaminérgica.
- 6.- Determinar si el miR-212, el miR-132 y el miR-29a juegan un papel crucial alterando la expresión de varias de sus dianas involucradas en el proceso de diferenciación dopaminérgica.
- 7.- Analizar la influencia de las posibles alteraciones en la expresión de los miRNAs y los factores de transcripción inducen cambios en la neuroprotección de estas neuronas a la toxina MPTP. Los posibles cambios en la expresión de NGN2, PITX3 y NURR1 podrían estar mediando este proceso.

Material y métodos

Animales

Los peces cebra de la cepa AB fueron criados y crecidos en nuestras instalaciones siguiendo los protocolos estándar de cuidado (Westerfield y cols. 2000).

Tratamientos e inhibidores

Resumen en castellano

Los embriones de pez cebra se expusieron a sulfato de morfina 10nM o 10μM en medio E3 desde las 5 horas post fecundación (hpf) hasta las 24 o 48 hpf. Un grupo control sin exposición a la droga fue usado en todos los casos. La morfina fue cedida por el Ministerio de Sanidad de España. Las concentraciones usadas para cada inhibidor fueron las siguientes: U0126 (Promega) 20μM, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamida hidrocloreuro (W7, Sigma) 10μM y Forskolin (Sigma) 20μM. La concentración de naloxona usada fue de 10μM. La concentración usada de N-[N-(3,5-Difluorophenacetyl)-L-alanil]-S-fenilglicina t-butil éster (DAPT) fue de 10μM. La concentración de resveratrol fue de 50μM, mientras que la de MPTP fue de 40μM.

Extracción de RNA y qPCR

El RNA total, incluyendo los miRNAs fueron extraídos usando Trizol (Life Technologies), siguiendo las instrucciones del fabricante. Para estudiar los niveles de expresión de los miRNAs de interés se realizó una reacción previa de poliadenilación empleando poli-A polimerasa (New England Biolabs) tal y como se describe en el protocolo recomendado. La retrotranscripción de las muestras para el análisis de *oprm1* se realizó con el kit High Capacity (Applied Biosystems), mientras que la de los miRNAs se realizó con el kit de M-MuLV retrotranscriptase (New England Biolabs). La cuantificación de las muestras por qPCR se realizó usando una curva patrón con SYBR-Green (Applied Biosystems) en un aparato de qPCR ABI 7300 (Applied Biosystems) usando oligonucleótidos diseñados según describe Balcells y cols (2011).

Extracción de proteínas, inmunoprecipitación y western blot

La extracción de proteínas, la inmunoprecipitación y el western blot se realizaron tal y como describen Arévalo y colaboradores (2000). Las concentraciones de los anticuerpos empleados fueron las siguientes: Oprm1 pez cebra (0,25μg/ml), Mouse OPRM1 (1:100), Nurr1 (1:1000), NICD (1:500), Pitx3 (1:250), Ngn2 (1:2000), Th (1:1000), FoxA2 (1:1000) y SIRT1 (1:1000).

Micro inyección de morfolidos

Los morfolidos empleados para silenciar *oprm1*, miR-212 y miR-132 fueron adquiridos de Gene Tools. El morfolino fue microinyectado en el embrión en el estadio de una sola célula de acuerdo con los protocolos previos (Nasevicius y cols. 2000). Las secuencias de los morfolidos son las que siguen: *oprm1* MO, AATGTTGCCAGTGTTTTCCATCATG; *dre-miR-132* MO, ACAGTAACAATCTAATGCCACGGTC; *dre-miR-212* MO, AGCCATGACTGTAGACTGTTACTGT.

Whole-mount in Situ Hybridization (WISH)

Los embriones de 24, 36 o 48 hpf se decorionaron, se fijaron en paraformaldehído al 4% a 4°C durante toda la noche, se lavaron dos veces con PBS durante 5 minutos a temperatura ambiente y se almacenaron en metanol absoluto a -20°C hasta su uso. La hibridación se realizó como describe Sanchez-Simon y cols (2010) y las sondas se diseñaron tal y como se describe en He y cols. 2011.

Ensayo de luciferasa

Para la detección de la quimioluminiscencia se empleó el Dual-Glo Luciferase Assay (Promega). Las secuencias se clonaron en el plásmido PmirGlo (Promega) usando el protocolo de digestión, ligación y transformación descrito en GeneClip™ U1 Hairpin Cloning Systems (Promega). Las células HEK293 se transfectaron con el plásmido conteniendo la secuencia diana sin mutar (wild type) o mutada (Mismatch) usando Lipofectamina 2000 (de Chevigny y cols. 2012). Los primers empleados fueron los siguientes:

wtF,	CTAGCTAGGTACCTAGTTAGTTTGAGGAAAGACTGTTGG;	wtR,
TCGACCAACAGTCTTTCTCTCAAACCTAACTAGGTACCTAG;		misF,
CTAGCTAGGTACCTAGTTAGTTTGAGGAAATCAGTGTGG;		misR,
TCGACCACACTGATTTCTCTCAAACCTAACTAGGTACCTAG		

Cultivo neuronal primario

Los cerebros de embriones de pez cebra de 42 hpf se extrajeron como se describe en Chen y cols. (2013).

Inmuhistoquímica

El análisis de los cultivos primarios se realizó como describe Chen y cols. (2013). Las neuronas se fijaron con 4% PFA durante 20 minutos y se bloquearon con tampón de anticuerpo y 5% de suero de cabra. La concentración para el anticuerpo de Oprm1 fue de 1:250, mientras que el de TH fue de 1:1000 (Millipore, AB152). Como marcadores nucleares y del soma se usaron To-PRO (1:1000) y Alexa fluor 594 Faloidina (1:10, ThermoFisher), respectivamente. Las fotografías se tomaron con un microscopio de célula viva (Zeiss).

Time-lapse

Los experimentos se realizaron con un microscopio de célula viva (Zeiss). Los cultivos neuronales fueron grabados durante 8 horas.

Análisis estadístico

Los valores de ct obtenidos por qPCR se analizaron en el software REST-384 v” para calcular la expresión relativa de los grupos respecto a los controles. Los datos se analizaron mediante un análisis de varianza de una vía (ANOVA de una vía)- El test post hoc de tukey se realizó para múltiples comparaciones. Las diferencias fueron consideradas significativas para una $p < 0.05$. Los resultados se muestran como la media $\pm$ SEM. Todos los análisis estadísticos se realizaron con el software Prism 7 (GraphPad Software, Inc).

Hipótesis y objetivos

La expresión del miR-212 y el miR-132 es codependiente

El miR-212 y el miR-132 forman un clúster genético (Kumarswamy y cols. 2014; Wanet y cols. 2012). Ambos se expresan como un único pri-miRNA policistrónico, y tras el procesamiento posterior de varias enzimas se generan dos miRNAs maduros. Se ha descrito que los miRNAs codificados de esta forma suelen poseer funciones similares. De este modo, para determinar la relevancia del miR-212 y el miR-132 durante el desarrollo, estudiamos la expresión temporal de ambos miRNAs en varios estadios del desarrollo (a 5, 8, 16, 24 y 48 hpf) (Figuras 1A y 1B). Es de especial importancia destacar que la expresión del miR-132 siempre es superior a la encontrada para el miR-212. Sin embargo aún es desconocido el proceso por el cual un único pri-miRNA es procesado de manera diferencial afectando a las cantidades relativas de ambos miRNAs maduros. Aunque la expresión de ambos miRNAs es alta durante los primeros estadios del desarrollo, el pico de expresión del miR-212 se alcanza a las 8 hpf. A las 16 hpf, la expresión de este miRNA alcanza los niveles de expresión observados a las 5 hpf, y desciende hasta niveles mínimos a las 48 hpf. En contraste, se observó un ligero aumento del miR-132 hasta las 16 hpf. A las 24 hpf los niveles del miR-132 alcanzan el mínimo de los cinco estadios analizados, aunque a 48 hpf los niveles vuelven a aumentar, sugiriendo un papel importante de este miRNA en este particular estadio. Para estudiar una posible relación entre los niveles de los miRNAs, silenciamos el miR-212 y el miR-132 y medimos los niveles del otro miRNA para determinar la dependencia en la expresión de cada uno de ellos (fig 1C y 1D). Tras el silenciamiento del miR-212, y a varios estadios (5, 8 y 16 hpf) se observó una disminución de la expresión del miR-132 (Fig1C). Del mismo modo, se observó un descenso no significativo en los niveles de expresión del miR-212 en los morfantes del miR-132 (Fig 1D). Aunque

el miR-132 está más expresado que el miR-212 durante el desarrollo, este último es que ha sido relacionado con el proceso de adicción (Hollander y cols. 2010), y es probablemente crucial durante los primeros estadios del desarrollo.

Expresión diferencial de miRNAs localizados en clústeres

El proceso de maduración de los pre-miRNAs no se conoce completamente. Para ayudar a clarificar porque un único miRNA bicistronico induce una expresión de miRNAs maduros que no se correlacionan, analizamos la región intergénica localizada entre ambos miRNAs en cuatro especies: *Homo sapiens*, *Mus musculus*, *Ratus norvegicus* y *Danio rerio*. Con el objetivo de buscar regiones conservadas, se realizó un alineamiento entre todas las secuencias, y se observaron dos regiones con un elevado porcentaje de homología (Fig 2A). Un análisis bioinformático posterior de estas regiones mostró que tres proteínas relacionadas con el splicing y maduración de los miRNAs podrían reconocer a estas secuencias: SRSF2, SRSF3 y MBNL1. Después de este primer análisis realizamos un modelado 3D de esta región para la determinación de la accesibilidad de estas regiones frente a estas proteínas (Fig 2B). En todas las secuencias modeladas, las secuencias consenso encontradas se localizaban perpendiculares al eje Z de la molécula, hacia la superficie (mostrados en círculos rojos).

La morfina modifica la expresión del miR-212, del pri-miR-212/132 y de *oprm1*

Los niveles de expresión del miR-212 en embriones de pez cebra se midieron mediante qPCR después de la administración de dos diferentes dosis de morfina (10nM y 10µM) (Fig 3). A las 34 hpf (Fig 3A), se observó un aumento de los niveles del miR-212 después de la administración de ambas dosis de morfina, aunque el aumento fue significativamente mayor tras la dosis de 10µM. Por otra parte, el miR-212 estaba disminuido a las 48 hpf. Además, analizamos las implicaciones del receptor opioide mu en la expresión del miR-212. Después de su silenciamiento, la sobreexpresión del miR-212 no se observó tras la exposición a morfina (Fig 3A). Sin embargo, la reducción en la expresión de este miRNA a las 48 hpf no se vio modificada tras el silenciamiento de *oprm1*. Los niveles de expresión del miR-212 no variaron tras el silenciamiento del receptor opioide mu.

Los niveles del pri-miR-212/132 también fueron medidos tras la exposición a morfina (Fig 3C y 3D), observándose una correlación entre los cambios del miRNA maduro y del precursor del mismo, a excepción de los morfantes de *oprm1* expuestos a morfina 10µM. Además, realizamos los mimos

experimentos pero esta vez exponiendo los embriones desde las 20 a las 24 hpf, y los resultados obtenidos fueron los mismos. Estos experimentos probablemente indicaban que la respuesta que estábamos observando a las 24 hpf era consecuencia de una dosis aguda de morfina.

Del mismo modo que el miR-212 está siendo controlado por la actividad del receptor opioide mu, analizamos del mismo modo si el receptor opioide mu estaba siendo regulado por este miRNA (Fig 6A y 6B). Tras el tratamiento con morfina, el receptor opioide mostró una elevada sobreexpresión a las 24 hpf, mientras que se encontraba disminuido a las 48 hpf. Sin embargo, el silenciamiento del miR-212 indujo un aumento en la expresión del receptor, mucho mayor que la observada tras la exposición a morfina, tanto a 24 como a 48 hpf. Por último, para determinar si estos cambios se correlacionaban con cambios en los niveles de proteína, realizamos un experimento de inmunoprecipitación-western blot (Fig 6C y 6D). Los resultados obtenidos con esta técnica se correlacionaban con nuestros experimentos de qPCR (6E y 6F). Sin embargo, *oprm1* disminuyó su expresión en los morfantes del miR-212 expuestos a morfina 10µM a 24 y 48 hpf.

miR-212 regula la traducción de *oprm1* a través del extremo 3'UTR de su mRNA

Para determinar si la regulación observada del miR-212 sobre *oprm1* era directa y sin acción de ninguna molécula intermediaria, se realizó un análisis bioinformático de los posibles sitios de unión del receptor opioide mu para los miRNAs de pez cebra (Khun y cols. 2008). Tras el análisis se encontró un posible sitio de unión para el miR-212 en el extremo 3'UTR del RNAm de *oprm1*. A través de un ensayo de luciferasa comprobamos que este sitio era funcional, y que el miR-212 estaba controlando fisiológicamente la expresión del receptor opioide mu (Fig 5). Para ello, se transfectaron plásmidos que contenían la secuencia de interés (en este caso de *oprm1*) junto al gen de la luciferasa, junto con miRNA mimics, pequeñas moléculas sintéticas que inducen una sobreexpresión del miRNA de interés (en este caso del miR-212). La cotransfección de ambas moléculas indujo una disminución en la luminiscencia, indicando que el miRNA-212 estaba induciendo la represión en la traducción de la luciferasa. Por otra parte, la mutación de 6 nucleótidos de la secuencia clonada dejó de reducir la luminiscencia, indicando que la unión ya no era funcional.

La morfina modifica la expresión del pri-miR-212/132 en el SNC de los embriones de pez cebra

Los resultados observados mediante qPCR se corroboraron usando una sonda contra el pri-miR-212/132 (Fig 4A-4F) mediante la técnica de ISH. Estos miRNAs se expresan en el diencefalo y en el rombencéfalo de los embriones de pez cebra. Los cambios observados mediante qPCR se correlacionaron con los cambios observados en la ISH en los embriones expuestos a morfina.

La expresión del miR-212 está regulada por la vía de las MAPK, la calmodulina y PKA

Para analizar en detalle la cascada de señalización tras la activación del receptor opioide mu por la exposición a morfina, los niveles de expresión del miR-212 y *oprm1* se analizaron tras la inhibición de la vía de las MAPK gracias al uso del inhibidor U0126 (Fig 7 y fig 8). La sobreexpresión del miR-212 observada a las 24 hpf tras la exposición a morfina fue revertida a los niveles control (Fig 7A). Además, se inhibió la calmodulina (CaM) y la calmodulina quinasa II (CaMKII) usando el inhibidor W7 para determinar la implicación de la señalización por calcio en la expresión del miR-212. Como se observa en la figura 5A, la inhibición de la señalización por calcio induce una sobreexpresión del miR-212 en el control, y tras la exposición a morfina. Usando forskolin, un activador de la PKA, observamos un marcado aumento de la expresión del miRNA en los 3 grupos. Por el contrario, a 48 hpf, los niveles del miR-212 permanecen reducidos en los embriones expuestos a morfina 10nM (Fig 7B). La discreta disminución observada en el grupo expuesto a morfina 10μM esta potenciada por la disrupción de la vía de las MAP quinasas y la señalización por calcio. La sobreactivación de PKA, por otra parte, produjo el mismo descenso que en el grupo de morfina 10μM, incluso tras la coadministración con morfina. La sobreexpresión de *oprm1* después de la exposición a morfina esta mediada por MEK, y bloqueada por U0126 a las 24 hpf (Fig 8A). Además, la forskolina previene la inducción de la expresión de *oprm1*. A 48 hpf la disminución de *oprm1* es revertida parcialmente tras el uso de U0126 y la señalización de calcio, como también por la sobreactivación de la PKA (Fig 8B).

La metilación del promotor de los miR-212 y miR-132 no está modificada por la administración de morfina

El análisis bioinformático del promotor miR-212/132 mostró dos sitios CRE flanqueando un sitio RE1 (Fig 9). Dado que los sitios RE1 pueden encontrarse metilados. Para analizar si la morfina induce cambios en los patrones de metilación en los sitios RE1, se realizó una conversión por bisulfitos (Fig 10).

La región de interés del promotor fue clonada en un plásmido PGEM®-T y secuenciada. El análisis mostró cuatro islas CpG, tres de ellas dentro del sitio RE1 del promotor. A 24 y 48 hpf estas citosinas se encuentran metiladas. Sin embargo, la administración de morfina no indujo ningún cambio en el patrón de metilación de esta región.

La morfina y DAPT modifican la unión de CREB al promotor del miR-212/132

Para analizar si los sitios CRE eran funcionales, realizamos un estudio de ChIP-qPCR para la unión de la proteína CREB fosforilada a esta región (Fig 11). Los resultados corroboraron lo que fue observado en la qPCR del miR-212. La morfina aumentaba la unión de CREB al promotor a 24 hpf, mientras que a 48 hpf lo disminuía. Por otra parte, la unión de CREB a varios promotores está regulada por el dominio intracelular de Notch, NICD. Los resultados mostraron que la inhibición de esta vía usando DAPT reducía la unión de CREB al promotor (Fig 12), indicando una regulación entre ambas vías mediada por el miR-212.

Expresión del miR-29a durante el desarrollo del pez cebra

El miR-29a es un conocido represor de la actividad de Notch, mediado por la metaloproteinasa ADAM12 (Li y cols. 2011). Para determinar la importancia de la señalización de Notch en la expresión del miR-212 y el miR-132, se realizó una qPCR a 5, 8, 16, 24 y 48 hpf (Fig 13). Los resultados mostraron un marcado aumento en su expresión a las 5, a las 16 y a las 48 hpf, mientras que se observaron niveles menores a 8 y 24 hpf.

La morfina modula la fosforilación de NF- κ B

NF- κ B es un factor de transcripción conocido por su control de varios procesos relacionados con el estrés celular. Además, este factor actúa como represor de la expresión del miR-29a. Como la vía de señalización de Notch controla la expresión del miR-212 y el miR-132 reprimiendo la actividad de CREB en el promotor, postulamos que la morfina podría estar regulando la señalización por Notch a través de la expresión del miR-29a. Para determinar esta posible regulación, analizamos por western blot la forma activa de NF- κ B en embriones de 24 hpf. Para analizar la relación entre ambas vías, también incluimos en los experimentos a embriones *mindbomb*, en los que la señalización por Notch es inexistente (Fig 14).

La morfina indujo un incremento en la fosforilación de NF- κ B. La inhibición de la vía de las MAPK usando U0126 indujo un aumento similar al observado en el grupo de morfina. En los embriones mutantes los niveles de NF- κ B fosforilados también estaban aumentados, pero la administración de morfina los redujo. En los mutantes *mindbomb* tratadas con U0126 se indujo un aumento en la cantidad de proteína fosforilada comparado con los mutantes no tratados. Como se esperaba, la coadministración de morfina con U0126 no indujo ningún efecto en los embriones *mindbomb*. Los niveles de miR-29a fueron medidos (Fig 15), corroborando los resultados obtenidos en el western blot de NF- κ B.

Las cascadas de señalización opioide y la vía de señalización Notch controlan la diferenciación y la agregación celular

Los efectos de la morfina y la vía de señalización de Notch se han descrito ampliamente en el contexto del desarrollo. Hemos estudiado los efectos de la morfina y del DAPT en un cultivo primario obtenido de embriones de pez cebra Nestin:GFP. Las células que expresan GFP indican precursores neuronales (Fig 17A). El cultivo fue grabado durante 8 horas. Como se observa en la figura 17B, la morfina indujo la agregación de las células en cultivo. Las células tratadas con DAPT por otra parte no indujeron esos mismos cambios (Fig 18D) comparado con el grupo de células tratadas con DMSO (Fig 18C).

Para determinar el porcentaje de células diferenciadas a neuronas dopaminérgicas contamos varios campos de los cultivos tratados con morfina, DMSO y DAPT, y las células fueron marcadas con un anticuerpo anti TH (Fig 19). Los resultados mostraron una disminución en el número total de neuronas dopaminérgicas después de la administración de morfina (Fig 19B). Las células tratadas con DAPT sin embargo no se vieron afectadas (Fig 19D).

La morfina y el DAPT inducen cambios en los factores de transcripción de las células R1 ES

Después de observar que la morfina y el DAPT indujeron cambios en el número total de neuronas dopaminérgicas, analizamos si estas dos moléculas podrían estar afectando a la expresión de varios de los factores dopaminérgicos implicados en el proceso de diferenciación. Para ello, las células en cultivo se recogieron al final de cada uno de los estadios de diferenciación, y se realizó un western blot contra FOXA2, NGN2, SIRT1, PITX3, NURR1 y TH.

La morfina incremento los niveles de SIRT en los dos primeros estadios de la diferenciación (Fig 21A), así como los de FOXA2 (Fig 21B). Estos resultados se repitieron tras la administración de DAPT. En el

tercer estadio la morfina y el DAPT indujeron el mismo aumento observado en los niveles de SIRT1. Sin embargo, no se observó la expresión de esta proteína en neuronas maduras. La expresión de NGN2 fue detectada en el segundo estadio del desarrollo, y sus niveles se vieron incrementados tras la administración de morfina y DAPT (Fig 22C). NURR1 se vio sobreexpresado tras la exposición a morfina en el tercer estadio, al contrario que por exposición al DAPT. Los mismos resultados se observaron en el cuarto estadio de diferenciación. En el tercer y cuarto estadio, se detectó la expresión de PITX3 (Fig 22C). Después de la administración de morfina, se observó un incremento en su expresión, aunque estos resultados no se vieron repetidos tras la exposición a DAPT. En este caso, se observó un descenso en la expresión de PITX3. Los niveles de TH no se vieron alterados tras la administración de ninguna de las dos drogas (Fig 22D).

Los efectos de la morfina en los factores de transcripción dopaminérgicos esta mediada por los miRNAs

Tal y como fue descrito anteriormente, la morfina modula la expresión del miR-212, el miR-132 y el miR-29a. Para analizar la posible relación entre los cambios en los factores de transcripción observados por western blot junto con los cambios medidos en los niveles de los miRNAs, transfectamos células SH-SY5Y con miRNA mímicas (Fig 23). La sobreexpresión del miR-132 indujo un descenso en la expresión de NGN2, aunque no se observaron cambios con la coadministración de morfina (Fig 23A). La sobreexpresión del miR-29a indujo un descenso en NGN2, pero solo tras la exposición a morfina. Los niveles de SIRT1 no se vieron modificados por el miR-212, pero si por el miR-132. Del mismo modo que para NGN2, la sobreexpresión del miR-29a indujo un descenso en la expresión de SIRT1 junto con la administración de morfina. La expresión de TH se veía aumentada tras la administración de morfina y la sobreexpresión de los 3 miRNAs. Las células SH-SY5Y no expresan PITX3. Sin embargo, la sobreexpresión del miR-29a indujo su expresión, tanto en presencia como en ausencia de morfina (Fig 23D).

Las vías de señalización opioide y la vía Notch controlan la diferenciación dopaminérgica en embriones de pez cebra

Los resultados obtenidos indicaban que tanto la morfina como el DAPT controlaban la expresión de varios factores de transcripción involucrados en la expresión de TH. Para analizar las implicaciones de estas vías *in vivo*, se realizaron ISH contra *Th*, *Nurr1* y *Pitx3* (Fig 24). El silenciamiento del receptor

opioide mu usando un morfolino indujo una disminución de los 3 genes estudiados (Fig 24A-C). En los mutantes *mindbomb* los resultados mostraron que los genes estudiados habían migrado su expresión hacia la línea media del embrión.

Efectos neuroprotectores de la morfina y el DAPT contra el MPTP

El MPTP es una toxina que induce la apoptosis específicamente en neuronas dopaminérgicas. Esta molécula ha sido usada como un modelo farmacológico para el estudio de la enfermedad de Parkinson. Los cambios observados en los factores de transcripción por ambas moléculas podrían inducir cambios en la resistencia de estas células al MPTP.

Para ello, las células SH-SY5Y se expusieron a MPTP 40µM durante 12 horas (Fig 25 y 26). Para determinar las vías moleculares implicadas en la cascada apoptótica, las células fueron a su vez tratadas con morfina, DAPT, naloxona, U0126, resveratrol, y varias combinaciones. La morfina, DAPT y U0126 redujeron los efectos apoptóticos del MPTP. Además, la morfina coadministrada con U0126 o resveratrol indujo un mayor efecto neuroprotector que con las moléculas por separado. De hecho, el resveratrol por si solo no tuvo ningún efecto neuroprotector. Por otra parte, el resveratrol coadministrado con DAPT revirtió los efectos observados con este último.

Los embriones de pez cebra también son susceptibles al MPTP. Para analizar los mismos efectos neuroprotectores *in vivo*, se expusieron a los embriones de pez cebra a MPTP durante 24 horas (Fig 26-28). Después de este tiempo, varios grupos de neuronas dopaminérgicas dejaron de observarse en algunos casos, especialmente los grupos A2, A3 y A5/6. La morfina incremento la expresión de Th en varios grupos, pero los efectos del MPTP aún eran patentes en los grupos posteriores de células. El DAPT incremento la expresión de Th y también contrarresto los efectos del MPTP. Por otra parte, la naloxona y el resveratrol redujeron los niveles de expresión de Th en el diencefalo de los embriones de pez cebra. La inhibición de la vía de las MAPK indujo una disminución drástica de las neuronas dopaminérgicas de los grupos A1 y A2, aunque las neuronas restantes eran resistentes al MPTP. La coadministración de morfina y DAPT indujo una gran cantidad de neuronas dopaminérgicas, pero todas ellas fueron eliminadas tras la adición de MPTP. La morfina y la naloxona incrementaron en número de neuronas positivas para Th en los grupos A1 y A2. Del mismo modo que en el grupo de morfina y DAPT, la administración de MPTP a estos embriones elimino en su totalidad el número de neuronas Th positivas. La coadministración de morfina con U0126 indujo un aumento en los grupos A1 y A2, aunque los grupos posteriores aún seguían

presentes. Estas células, además, eran resistentes al MPTP. La morfina y al resveratrol indujeron un incremento en el número de neuronas Th positiva en los grupos A1, A2 y A4. Estas células aun así estaban afectadas por el MPTP. DAPT y resveratrol indujeron los mismos efectos que los observados en la figura C, aunque esta combinación no mostró efectos neuroprotectores.

Discusión

Nuestro análisis del clúster formado por los miRNA-212 y miR-132 mostró que la expresión del miR-132 es siempre superior que la observada para el miR-212. Además, la secuencia del miR-132 está más conservada durante la evolución que la del miR-212 (Luo y cols. 2014), indicando que la función de este miRNA podría ser esencial durante las primeras etapas del desarrollo. El miR-212 está relacionado con la diferenciación de los somitas (implicado en la formación de la musculatura) que se está desarrollando en este estadio (Bijkerk y cols. 2016). Varios autores han descrito la desregulación de varios miRNAs que pertenecen al mismo clúster en algunas enfermedades (Chang y cols. 2015; Wibrand y cols. 2010). Aunque el mecanismo para esta regulación ha sido estudiada, aun no se conoce (Mohammed y cols. 2015). La desregulación observada en los niveles del miR-132 después de silenciar el miR-212 parece indicar que el miR-212 está regulando la expresión de los miRNAs del clúster, y que ha emergido evolutivamente como un regulador del miR-132, que es importante durante el desarrollo (Wayman y cols. 2008). El proceso de maduración de los pre-miRNAs es todavía incierto. Los resultados observados en la figura 2 muestran que las posibles secuencias consenso podría ser empleado como un nuevo elemento de regulación de la expresión de los miRNA en clústeres.

La sobreexpresión observada a 24 hpf en la expresión del miR-212 después de la administración de morfina esta mediada por la activación del receptor opioide μ , dado que los morfantes de *oprm1* no muestran ningún cambio significativo (ver figura 3). Por otra parte, un descenso en este miRNA se observó a 48 hpf, aunque este resultado no es revertido en los morfantes. Los resultados indicaban que la sobreexpresión de este miRNA después de la exposición a morfina en los primeros estadios del desarrollo están mediados por el receptor opioide μ . Por otra parte, la disminución del miR-212 inducidos tras una exposición prolongada a morfina esta mediada por una vía diferente que la activada por el receptor opioide μ , tal y como se observó en los cambios en los pri-miR-212/132 en los embriones morfantes del receptor expuestos a morfina 10 μ M. Resultados previos de nuestro grupo demostraron una expresión diferencial del miR-132 y del miR-212 en embriones de 48 hpf (Jimenez-

Gonzalez y cols. 2016). Por otra parte, el silenciamiento del receptor opioide mu no modifico los niveles de expresión del miR-212, por lo que la expresión fisiológica de este miRNA no depende del receptor, pero es responsable de los cambios ejercidos por la morfina. De esta forma, la activación de *Oprm1* induce la sobreexpresión del miR-212. Por otra parte, observamos cambios en los niveles de los miRNAs primarios. Los resultados sugerían que la morfina no solo está previniendo la degradación del miRNA, sino que también induce su expresión. Sin embargo, la regulación de la expresión y maduración de estos miRNAs podría estar mediada por otros factores, induciendo una expresión diferencial de la expresión de estos miRNAs durante el desarrollo, como se observó en los morfantes de *oprm1* tratados con la dosis más alta de morfina. Algunos autores ya habían observado resultados como resultado de una regulación postrascriptcional de los clústeres de miRNAs (Ryazansky y cols. 2012). La localización del precursor, como se observa en la ISH se correlaciona con los resultados medidos por qPCR. Los resultados se resumen en las figuras 30 y 31.

Una sobreexpresión de los niveles de *oprm1* se observó a 24 hpf después de la administración de morfina. Resultados previos indicaban que la activación de este receptor induce su propia expresión en un mecanismo de feedforward después de una dosis aguda de morfina en embriones de pez cebra (Sanchez-Simon y cols. 2010). Por otra parte, una disminución de los niveles de *oprm1* se observó a 48 hpf, probablemente por la sobreactivación del receptor o por una reducción de su expresión a nivel del promotor por conocidos represores como MeCP2 (Formisano y cols. 2007) o PU.1 (Hwang y cols. 2004). Estos resultados se correlacionaron a nivel proteico, como se mostró en los experimentos de inmunoprecipitación. Los resultados sugerían que la reducción del miR-212 inducían un bloqueo en la traducción del RNAm. Además, el papel de los opioides endógenos en la expresión del receptor opioide mu ya han sido descritos. Estas moléculas ampliamente expresadas en el SNC están afectadas tras la exposición a morfina (Gonzalez-Nunez y cols. 2013). Después de silenciar el miR-212, observamos un aumento en la expresión de *oprm1*. Además, el descenso inducido por la morfina a 48 hpf se transformó en un aumento. Sin embargo, el descenso después de exponer los morfantes de *oprm1* a morfina 10µM podría estar debido a un aumento del miR-132. Esta expresión diferencial entre miR-212 y el miR-132 podría estar mediada por otro receptor distinto a *Oprm1*. Los resultados mostraron una nueva regulación por el clúster sobre la regulación postrascriptcional de *oprm1*, previniendo la traducción del RNAm. Al mismo tiempo, este mecanismo previene la consecuente sobreexpresión del receptor después de la administración de morfina. En resumen, estos resultados indican que la morfina, a través de la activación

del receptor opioide mu induce la sobreexpresión del miR-212. Este miRNA, junto con otros reguladores transcripcionales, reduce la traducción del RNAm de *oprm1*, controlando la expresión del receptor a nivel postrascriptional. Además, la regulación podría estar mediada por el miR-132, u otras vías como las neurotrofinas u otros receptores opioides.

Para estudiar los mecanismos moleculares tras la activación de *Oprm1*, inhibimos selectivamente varias rutas de señalización relacionadas con la actividad opioide, como las MAPK, la vía del calcio por Calmodulina y la proteína quinasa A (PKA). Hemos demostrado que la sobreexpresión del miR-212 a 24 hpf esta mediada por la actividad de las MAPK usando el inhibidor U0126. La sobreexpresión de *oprm1* también esta contrarrestada por la adición de U0126. Sin embargo, hemos observado una disminución de los niveles del miR-212 y *oprm1* a 48 hpf comparados con 24 hpf. Esta reducción no fue totalmente revertida por U0126, indicando que la respuesta no solo estaba medada por la vía de las MAP quinasas. Y por la activación de CREB. Este factor de transcripción se ha relacionado con la expresión del miR-212 mientras que la proteína REST, uniéndose a sitios RE1, se sabe que reduce la actividad del promotor de los miRNAs (Conaco y cols. 2006) y *oprm1* (Formisano y cols. 2007). Además, MeCP2 está envuelta en la reducción de la expresión del miRNA formando un complejo epigenetico (Im y cols. 2010). De este modo, la represión ejercida sobre estos dos genes esta mediada por MeCP2 y sitios RE1. Además, nuestros resultados mostraron que la señalización por calcio es necesaria para inducir la sobreexpresión de *oprm1* después de la administración de morfina, dado que el inhibidor W7 previno la sobreexpresión del mRNA.

Además, la inducción de la actividad de MeCP2 está mediada por calmodulina (CaM) y calmodulina quinasa II (CaMKII) (Buchtal y cols., 2012). Se sabe que la activación del receptor opioide mu bloquea los canales de calcio a través de la subunidad alfa Gi (α Gi), modificando los niveles de calcio intracelular (Bourinet y cols., 1996). CaM induce la activación de la MAP quinasa mediada por la activación de PKC, RAS y Raf1 (Tebar y cols., 2002) provocando por lo tanto la fosforilación de CREB. Además, la activación de las subunidades $\beta\gamma$ induce la liberación de los depósitos intracelulares de calcio del RE mediado por el trifosfato de inositol (IP3) a través de la activación de la fosfolipasa C (PLC) (Murthy y Makhoulf 1996). Además, se ha demostrado que la inhibición de la señalización de cAMP inhibe la respuesta de la morfina (Nestler y cols., 1996). Por lo tanto, mientras que la inhibición de CaM / CaMKII reduce la fosforilación y activación de MeCP2, también aumenta la actividad de CREB y la expresión de miR-212, como hemos mostrado después de usar el inhibidor W7. Por otro lado, la activación de PKA

mediada por forskolina puede inducir la fosforilación de CREB y por lo tanto activar miR-212 y la transcripción de *oprm1*. De hecho, los niveles de miR-212 se encuentran disminuidos en comparación con el grupo de control. Esto indica que la señalización de la MAP quinasa, activada por otras vías (tales como las neurotrofinas, tal como se describe por Numakawa (Numakawa y cols., 2011) y Remenyi (Remenyi y cols., 2010)) es necesaria para mantener los niveles fisiológicos miR-212. Si la actividad CaMKII es inhibida por W7, observamos un fuerte aumento en los niveles del miRNA. CaMKII fosforilación de Ser421 de MeCP2 inhibe la expresión de este miRNA bloqueando la transcripción a través de la unión a RE1 sitios (Im y cols., 2010). La pérdida de actividad en la vía del calcio induce una pérdida de fosforilación en MeCP2, y por lo tanto reduce su actividad. Por otra parte, esta inhibición epigenética no controla la expresión del receptor mu opioide, ya que la inhibición de la CaM / CaMKII no implica ningún cambio significativo. La activación de las subunidades $\beta\gamma$ de PLC y la liberación posterior de IP3 induce la liberación de calcio intracelular almacenado en el retículo endoplasmático y la activación de CaMKIV (Müller y cols., 2011). Esta quinasa induce la activación de la vía de MAP quinasa (Park y cols., 1995) y la fosforilación posterior de CREB, dando como resultado una sobreexpresión de miR-212 y *oprm1*. Además, el miR-212 se dirige al RNAm de *oprm1* bloqueando la traducción mediada por el complejo RISC (Kawamata y Tomari 2010) que impide la sobreexpresión adicional del receptor. Este mecanismo sirve como un control de retroalimentación de la expresión del receptor mu opioide: la morfina induce miR-212 y transcripción *oprm1*, aumentando sus niveles. Este miRNA, por otro lado, se dirige al RNAm de *oprm1* y reduce los niveles de la proteína.

Además, los cambios en el patrón de metilación de miR-212 y *oprm1* ya han sido estudiados (Ryazansky y cols., 2011; Sanchez-Simon y cols., 2010). MeCP2 está reprimiendo activamente su promotor que se une a sitios RE1 e impide la sobreexpresión observada después de la administración de morfina. Dado que la inhibición de CaM / CaMKII induce un aumento de la expresión de estos dos genes, nuestros resultados indican que la fosforilación de MeCP2 en la Ser421 (Buchtal y cols., 2012) es necesaria para la actividad de este factor epigenético. La activación de PKA por forskolina rescata parcialmente la expresión normal de estas moléculas. La fosforilación de CREB mediada por la PKA podría provocar una inducción de la expresión de miR-212 y *oprm1*, pero como MeCP2 sigue siendo activado y unido al promotor, la transcripción está parcialmente bloqueada, como se ha descrito en la regulación de otros genes de muchas células (Zhou y cols., 2006).

Está claro, teniendo en cuenta lo anterior, que existe una relación entre la actividad del receptor opioide mu y miR-212. Proponemos el siguiente mecanismo después de la administración de morfina (Fig. 23): la activación de Oprm1 (luz verde) induce varios cambios intracelulares; 1: la activación de la subunidad α Gi (verde oscuro) inhibe la adenilato ciclasa (AC, roja), interrumpiendo la producción de AMPc e impidiendo la actividad de la Proteína Quinasa A (PKA, amarillo); 2: Además, la subunidad α Gi cierra los canales de calcio (CC, azul claro); 3: La activación de Oprm1 de Proteína Kinasa C (PKC, verde) induce la activación de RAS y Raf1, que activan la vía de MAP quinasa (MEK, ERK y MNK1 / 2, naranja), induciendo la fosforilación de CREB (naranja); 4: Las subunidades $\beta\gamma$ inducen la liberación de IP3 y la liberación posterior de calcio intracelular, almacenada en el retículo endoplasmático (ER). Por el contrario, la downregulation observada en todos los grupos experimentales a 48 hpf parece estar mediada por varios factores, pero no por la activación de Oprm1. Además, la expresión de miR-212 y *oprm1* está claramente reducida, lo que indica que el mecanismo sometido a la administración de morfina debe ser diferente. El mecanismo de regulación implicado en las respuestas observadas está mediado por dos procesos; 5: los cambios en el patrón de metilación en miR-212 y *oprm1*, probablemente en sitios RE1, inducen la represión de la transcripción mediada por MeCP2 6: cAMP después de la administración prolongada de morfina induce la activación de PKA (Conaco y cols.2006). Estos resultados se resumen en las figuras 32 y 33.

NF- κ B es un factor de transcripción bien conocido que desempeña papeles críticos en varios procesos (Li y Verma 2002, Pomerantz y Baltimore 2002). La morfina induce cambios en la expresión génica al afectar la activación de varios de estos factores de transcripción, pero el mecanismo subyacente NF- κ B activación siguen siendo poco claras. En este sentido hemos demostrado que la morfina induce la fosforilación de NF- κ B (como se ve en la figura 12). La morfina es conocida por inducir la actividad de la quinasa IKK β a través de PI3K / Akt (Ye y cols., 2001). Además, la interrupción de la señalización de MAPK aumentó la activación de NF- κ B, pero los resultados no mostraron un efecto sinérgico cuando se administró morfina. Estos resultados sugieren por primera vez que la vía de las MAPK puede ejercer un efecto represivo sobre la activación de NF- κ B. NICD es un represor conocido de la actividad NF- κ B. En este sentido, el Western Blot de las proteínas extraídas de los mutantes mindbomb, mostró un aumento de NF- κ B fosforilación como se esperaba. Sin embargo, la administración de morfina indujo una disminución en los mutantes mindbomb, pero un aumento se observó tras la administración de U0126 a los embriones mutantes. Además, la morfina coadministrada con U0126 no mostró cambios significativos

en comparación con los embriones de pez cebra de la cepa AB. La interacción NICD con CLS induce la activación de IKK β , por lo tanto los resultados observados se correlacionan con hallazgos previos (Espinosa y cols., 2010).

Se ha demostrado que el miR-212 y miR-132 controlan la expresión del receptor opioide mu. Por otro lado, se ha descrito que miR-29a es un regulador directo de ADAM12, una de las metaloproteasas necesarias para la escisión de Notch y la activación (Li y cols., 2011). NF- κ B es un represor de la expresión de miR-29a. Como hemos demostrado, la morfina induce cambios en la fosforilación de NF- κ B y, por lo tanto, la morfina podría ejercer cambios en la activación de la vía Notch a través de la modulación de miR-29a, especialmente durante las primeras etapas de desarrollo como se muestra en los resultados y previamente descrita en la literatura (Shi y cols., 2006, Latimer y cols., 2006).

Como se observó en el Western blot contra NICD, la administración de morfina aumentó los niveles de NICD, lo que indica que la reducción de los niveles de miR-29a podría aumentar los niveles de ADAM12 y aumentar así la escisión de Notch1. Por otro lado, el DAPT indujo un aumento en los niveles de OPRM1. Además, hemos demostrado utilizando ChIP-qPCR que la administración de DAPT bloquea la unión de pCREB al promotor del miR-212/132. NICD reprime la transcripción dependiente de CRE (Hallaq y cols., 2015), y esto podría indicar que la señalización Notch controla la señalización opioide a través de la modulación de miR-212/132, ambos reguladores directos de la expresión de OPRM1.

Después de estos resultados, hemos propuesto el siguiente mecanismo que explica la interacción de señalización Notch y opioide mediada por los tres miRNAs estudiados (miR-212, miR-132 y miR-29a) y a través de la modulación de CREB y NF- κ B (Figura 24). La morfina activa OPRM1, y a través de PI3K / Akt se induce la activación de IKK y la fosforilación de NF- κ B y los niveles de miR-29a se reducen. Esta reducción induce un aumento en la expresión de ADAM12, y la escisión de Notch se incrementa y los niveles de NICD se incrementan. NICD, por otro lado, induce una disminución de PCREB vinculante de miR-212/132 promotor, la disminución de los niveles de este miRNAs y aumentar por esa razón OPRM1 niveles. Estos hechos se resumen en la figura 34.

Hemos demostrado que los opioides y la señalización Notch se regulan mutuamente a través de la vía de la interacción de tres miRNAs. Estas dos vías también son conocidas por controlar la proliferación celular, la agregación y la diferenciación (Khursid y cols., 2010, Reynolds-Kennewally y Mlodzik, Murata y Hayashi 2016). Para determinar los efectos y la sinergia de estas dos vías, se realizó un

experimento de time lapse de un cultivo primario obtenido de embriones de pez cebra Nestin: GFP (véase la figura 14). Los resultados mostraron un sorprendente aumento en la agregación celular, donde se observó clúster de precursores neuronales que expresan GFP. La diferenciación tardía indicó que la morfina podría estar afectando la diferenciación de los precursores neuronales a las neuronas maduras. El DAPT también retrasó la diferenciación celular, pero los efectos en la agregación no eran comparables con el grupo de la morfina. Curiosamente, la morfina y DAPT coadministrados aumentaron la diferenciación neuronal, y la redujeron la agregación celular. Estos resultados indicaron que la señalización opioide y Notch podrían estar controlando varios procesos de desarrollo tales como la diferenciación dopaminérgica. La modulación de marcadores dopaminérgicos después de la administración de morfina ya ha sido descrita (Garcia-Perez y cols., 2013). De la misma manera, también se ha descrito que la señalización Notch está implicada en la diferenciación dopaminérgica (Mahler y cols., 2010). Para determinar si estas dos vías determinan el destino neuronal durante el desarrollo, analizamos la expresión de diferentes factores de transcripción temprana y tardía durante el desarrollo de células madre embrionarias de ratón y el porcentaje total de células TH positivas al final del proceso de diferenciación. La morfina redujo el número total de células TH positivas. El DAPT, por otro lado, no alteró el número total de células dopaminérgicas diferenciadas. Sin embargo, los resultados observados en el cultivo de células primarias se imitaron en las neuronas maduras obtenidas de células R1 ES. La agregación celular se incrementó en las células tratadas con morfina, e interesantemente, fueron los grupos agrupados las células que expresaron TH. A pesar de que el DAPT disminuyó la agregación celular y la proliferación, las células aisladas estaban expresando TH en contraste con el grupo de la morfina.

Para determinar los factores de transcripción implicados en este proceso se analizaron por western blot varios factores de transcripción implicados en la diferenciación dopaminérgica. Los resultados mostraron que varios de estos factores se expresaron en las primeras etapas de desarrollo (como NGN2). Se ha descrito que la morfina induce cambios en la expresión de Drosha (Xu y cols., 2015) y que esta proteína es necesaria para inducir la expresión de NGN2 (Knuckles y cols., 2012). Además, se sabe que la activación Notch es un intermediario en los cambios inducidos por la expresión SIRT1 en la expresión génica (Xie y cols., 2012). Estos resultados muestran un mecanismo de retroalimentación de control de la expresión de SIRT1 por interrupción de señalización Notch. FOXA2 es también otro factor de transcripción intermedio con varias funciones en el desarrollo dopaminérgico, desde la especificación

neuronal (Ang 2009) hasta la diferenciación dopaminérgica (Xi y cols., 2012). Además, la expresión de FOXA2 en neuronas maduras induce neuroprotección (Sho y cols., 2015). Estos resultados mostraron que la morfina y DAPT tienen efectos similares en los primeros genes implicados en la diferenciación dopaminérgica, aunque los cambios no se correlacionan totalmente. Las alteraciones en los niveles de SIRT1 después de la administración de morfina y DAPT se mantuvieron en la tercera y cuarta etapa del desarrollo de las células R1 ES. Estos resultados indican que SIRT1 puede ser responsable de varios de los cambios ejercidos sobre los otros factores de transcripción estudiados, tal como se describió anteriormente de una manera general (Hisara y otros, Sugino y cols., 2010). PITX3 y NURR1, los últimos factores de transcripción implicados en la expresión de TH en las neuronas dopaminérgicas se reducen en los grupos de morfina y DAPT. Estos resultados observados en la tercera etapa podrían correlacionarse con el retraso en la diferenciación observada en el cultivo neuronal primario de embriones de pez cebra. La reducción de los factores de transcripción terminales podría explicarse como una diferenciación tardía de estas neuronas.

Los resultados observados en el modelo simplificado de diferenciación neuronal dopaminérgica se investigó más a fondo utilizando miRNA mimics. Hemos demostrado que ambas vías están regulando recíprocamente la expresión del miR-212, miR-132 y miR-29a. Los resultados obtenidos podrían explicar cómo la señalización opioide y de Notch está regulando la diferenciación de estas neuronas. Varios autores han descrito la implicación en la diferenciación dopaminérgica de estos miRNAs en algunos de los genes estudiados. miR-132 ha sido descrito como un regulador de la expresión SIRT1 (Zhang y cols., 2014) y NURR1 (Yang y cols.2012). Sin embargo, la regulación ejercida por este miRNA sobre NGN2 no se ha descrito hasta este estudio. Curiosamente, el miR-29a fue capaz de inducir la expresión de PITX3 en células SY-SY5Y que constitutivamente no lo expresan. Dado que la morfina y el DAPT no son capaces de inducir la expresión de PITX3 por sí misma, estos resultados significan que miR-29a está induciendo la represión de algún gen que de hecho impide que las células SH expresen PITX3, aunque la identidad de este intermediario es desconocida.

Estos resultados obtenidos en modelos simplificados utilizando cultivos celulares se imitaron en embriones de pez cebra. El silenciamiento de Oprm1 demuestra que los opioides endógenos son necesarios para el correcto desarrollo dopaminérgico. Estos resultados muestran por primera vez que los opioides endógenos no sólo juegan un papel importante en los mecanismos de recompensa (Colasanti y cols., 2012), sino también en la especificación dopaminérgica. Los mutantes *Mindbomb* y la señalización

de Notch, sin embargo, se han relacionado con la alteración en el desarrollo del dopaminérgico del pez cebra (Mahler y cols., 2010). Sin embargo, nuestros resultados muestran un patrón de expresión de los factores de transcripción *nurr1* y *pitx3* hacia la línea media. Estos resultados indican que la vía de Notch no sólo está alterando el nivel de expresión de varios de los factores de transcripción implicados en el desarrollo dopaminérgico, sino que también especifica la lateralidad y el área del cerebro donde esas neuronas se diferencian. Estos hechos se resumen en la figura 35.

La actividad de CREB mediada por la señalización de morfina y Notch y su unión al promotor de *th* a través de *Nurr1* esta inducida por fosforilación mediada por la vía MAPK (Sakurada y cols., 1999). La morfina induce la expresión de *NURR1* y también su fosforilación y activación, promoviendo la expresión de *TH*. Sin embargo, *NICD* puede inducir *NURR1* expresión mediante la reducción de la unión a su promotor de varios complejos represores mediados por *CSL*. Por otra parte, *SIRT1* puede modular la actividad de *NICD* por su desacetilación (Guarani y cols., 2011) y actuar también como un correpresor de la actividad de *Nurr1* (Kim y cols., 2013). Estos resultados indican que *NURR1 per se* no es suficiente para inducir la expresión de *TH*, lo que podría explicar las diferencias observadas en diferentes grupos neuronales durante la diferenciación. Además, la actividad de *NURR1* se ha implicado en la inhibición de *p53*, modulando así la apoptosis (Zhang y cols., 2009). Estos resultados se resumen en la figura 35.

Todos los cambios en los miRNAs y los factores transcripcionales implicados en la señalización opioide y Notch no sólo podrían afectar el patrón de diferenciación de estas neuronas, sino también inducir cambios en la susceptibilidad de estas células a la apoptosis inducida por el estrés oxidativo, como la producida por la toxina MPTP (Meredith y Rademacher, 2012). Para medir los posibles efectos neuroprotectores de estas moléculas, se realizó un ensayo TUNEL en células SH-SY5Y expuestas a MPTP (véase la figura 21). Las células tratadas con morfina, DAPT y U0126 fueron parcialmente resistentes a MPTP. Estos resultados indican que los cambios ejercidos, como la sobreexpresión de *PITX3*, *FOXA2* o *NURR1* (Sho y cols., 2015) observada en los western blot, mediadas por Notch y la señalización opioide sugieren que de alguna manera están protegiendo estas neuronas contra los efectos apoptóticos de MPTP.

Estos resultados fueron correlacionados en 48 hpf embriones de pez cebra. Curiosamente, de todos los grupos dopaminérgicos en el mesencéfalo del pez cebra varios grupos neuronales parecen ser afectados más que otros, específicamente A1, A3 y A4 / 5 grupos. Estos hallazgos sugieren que la expresión génica

de estos diferentes grupos de neuronas aunque similares, no es idéntica, lo que les confiere propiedades diferentes y resistencia parcial a MPTP.

Conclusiones

Las propiedades únicas de la morfina, que implica el dolor y el mecanismo de recompensa del cerebro simultáneamente, hacen de esta molécula un candidato interesante para evaluar los procesos desencadenados en el cerebro después de la ingesta de drogas de abuso. En este estudio, hemos presentado nuevas perspectivas en la vía molecular desencadenada por la morfina que regula el sistema opioide, y además, una interacción cruzada de esta vía con la señalización Notch. Esta interacción modula la diferenciación dopaminérgica, y podría jugar un papel crítico en el desarrollo de la adicción después de la ingesta crónica de morfina. Después del análisis de los resultados obtenidos en este trabajo podemos presentar las siguientes conclusiones:

1. La morfina controla la expresión de miR-212 y miR-132 a través del control de la transcripción de pri-miR-212/132. El proceso de maduración del miRNA primario conducirá a una expresión diferencial entre ambas formas maduras, lo que conduce a diferentes funciones entre ellas.
2. El miR-212 controla la señalización opioide uniéndose al 3'UTR del mRNA *oprm1*, controlando por lo tanto el mecanismo autorregulador desencadenado por la morfina y el receptor opioide μ .
3. La regulación positiva desencadenada por la morfina en la expresión de estos miRNAs es temporalmente dependiente, ya que las vías implicadas a 24 hpf y 48 hpf difieren entre sí. Las primeras respuestas desencadenadas por la morfina en la expresión de estos miRNAs están mediadas por la señalización MAPK, y controladas terminalmente por la fosforilación de CREB. Sin embargo, las cascadas implicadas a 48 hpf están relacionadas con la señalización del calcio y CaMKII / CaMKIV y la fosforilación de MeCP2.
4. La señalización de Notch, a través de NICD, controla la expresión del miR-212 y miR-132 a través de la unión de pCREB en el promotor de los miRNAs. Este proceso indica que la señalización Notch está alterando la cascada de señalización de opioides a través de alteraciones de la expresión de *Oprm1*.

5. La morfina induce la fosforilación y activación de NF- κ B en embriones de pez cebra de 24 hpf, modulando así la escisión y la actividad de la señalización Notch. Estos hallazgos corroboran la hipótesis de una interacción entre las dos vías mediadas por miR-212, miR-132 y miR-29a.

6. La señalización opioide y Notch controlan la expresión de los factores de transcripción dopaminérgicos necesarios para la correcta diferenciación de las neuronas dopaminérgicas. La morfina y la administración de DAPT inducen la regulación positiva de SIRT1, un posible intermediario sobre los cambios desencadenados por estos dos fármacos, y ambas moléculas alteran la expresión de NGN2, FOXA2, NURR1 y PITX3, que afecta de forma terminal a la expresión de TH en neuronas dopaminérgicas maduras.

7. Los cambios en la expresión de los factores de transcripción desencadenados después de la morfina o DAPT alteran la susceptibilidad de las neuronas dopaminérgicas al estrés oxidativo mediado por MPTP. Sin embargo, varias poblaciones dopaminérgicas deben presentar características diferentes que induzcan resistencia diferencial a esta toxina.

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Figuras citadas en este resumen

Figura 1. Perfil de expresión temporal miR-212/132 y su expresión interdependiente. La expresión temporal de miR-212 (A) y miR-132 (B) se midió mediante qPCR. Los niveles de expresión miR-212 en miR-132 embriones morfantes (C), y los niveles de expresión miR-132 en miR-212 embriones morfantes (D) también fueron cuantificados por qPCR. Los resultados muestran diferencias en la expresión temporal de los microRNAs del clúster. Los niveles de miR-132 son siempre mayores que los niveles de miR-212. Las Figuras C y D muestran una disminución de uno de los miRNAs cuando el otro es silenciado, lo que indica una posible regulación entre la expresión de estas moléculas en el contexto de su clúster. Los resultados se muestran como el número de copias por 2/µg de cDNA (A y B) o el cambio respecto a los niveles control a 5, 8 y 16 hpf. (C y D) Los datos se expresan como la media $\pm$ SEM. El grupo de control se muestra como la línea punteada. Significancia estadística; **, $p < 0,01$. ***, $p < 0,001$. Mo: morfante.

Figura 2.- La región intergénica entre miR-212 y miR-132 muestra secuencias conservadas que pueden ser clave en la maduración de los miRNAs primarios. La alineación mediante CLUSTALW de la región intergénica entre miR-212 y miR-132 (A) y modelado tridimensional de esa área (B). La alineación mostró dos secuencias altamente conservadas predichas como sitios de unión para varias proteínas de splicing como SRSF2, SRSF3 y MBNL1 (amarillo y naranja secuencias) (A). El modelado 3D mostró que las regiones conservadas (círculo rojo) siempre se encuentran perpendiculares al eje Z de las moléculas (línea negra) (B). Esta región podría ser la responsable de la expresión diferencial de los dos miRNAs maduro de la misma molécula de pri-miRNA. Amarillo, secuencias altamente conservadas, naranja, ligeras variaciones de la secuencia.

Figura 3. Efectos de la morfina y el silenciamiento de Oprm1 en la expresión del miR-212 y pri-miR-212/132. Análisis por qPCR de los niveles de expresión miR-212 en embriones control y morfantes de *oprm1* embriones después de la administración de morfina (10 nM y 10 μ M) (A, 24hpf, B, 48hpf) y pri-miR-212/132 (C, 24 hpf D, 48 hpf) . La morfina sobreexpresa el miR-212 y pri-miR-212/132 a 24 hpf. A 48 hpf, el miR-212 fue disminuido mientras que no se observaron cambios para el miRNA primario. El silenciamiento de *oprm1* no indujo cambios en miR-212 en los embriones no tratados, pero invirtió los efectos de la morfina a 24 hpf indicando que es necesario para los efectos de la morfina en esta etapa. Sin embargo, el silenciamiento de *oprm1* junto con la exposición a la morfina aun causaba la misma reducción a 48 hpf. Los resultados después del silenciamiento de *oprm1* mostró que este receptor es necesario para los efectos de la morfina en estas moléculas, pero sólo a 24 hpf. Los resultados se muestran como el cambio de expresión con respecto a los embriones control (línea punteada) a 24 y 48 hpf. Los datos se expresan como la media ± SEM. Significancia estadística; *, P <0,05; **, p <0,01, *, p <0,001. C-: Control negativo Mo: morfante.**

Figura 4. La morfina induce cambios en la expresión de pri-miR-212/132 en el cerebro de pez cebra. El patrón de expresión obtenido por ISH en control (A, D) y después de tratamiento con morfina (10 nM (B, E) y 10 μM (C, F)) (izquierda, 24hpf, derecha, 48hpf). Las flechas muestran las regiones en el mesencéfalo y en el rombencefalo donde los cambios observados son más relevantes. La regulación desencadenada por la morfina en estas áreas no se correlaciona totalmente con la subida y la bajada obtenida en los experimentos qPCR.

Figura 5. El miR-212 reduce la expresión de *oprml* uniéndose al 3'UTR del mRNA. Resultados del ensayo de luciferasa del sitio de unión para miR-212 en el 3'UTR de *oprml*. Los resultados se muestran como el cambio de veces respecto al control. Los grupos wt 212 y mis 212 fueron cotransfectados con mimics del miR-212 20 nM o 50 nM. Una mayor concentración indujo una mayor reducción de la luminiscencia. miR-212 regula la expresión de *oprml*, como se observa en los cambios cuando el plásmido no mutado se cotransfecta con una forma activa del miR-212. Los datos se expresan como media $\pm$ SEM, N = 5. Significancia estadística; ***, $p < 0,001$. wt, plásmido no mutado; Mis, plásmido mutado; 20 y 50 indica la concentración nanomolar del miRNA mimic.

Figura 6. Morfina y miR-212 controlan la expresión de Oprm1. Análisis de qPCR de los niveles de expresión de *oprm1* en los embriones morfantes control y miR-212 después del tratamiento con morfina (10 nM y 10 μ M) (A, 24 hp, B, 48 hpf). Inmunoprecipitation-Western Blot de Oprm1 (C y E, 24hpf; D y F, 48 hpf). La morfina regula la expresión de *oprm1*, induciendo una regulación positiva a 24 hpf, pero una disminución a 48 hpf. Los resultados después del silenciamiento del miR-212 parecía indicar que este miRNA podría estar ejerciendo efectos reguladores después de la administración de morfina. Los resultados se muestran como el cambio respecto al control (línea punteada) a 24 y 48 hpf. Los datos se expresan como media $\pm$ SEM. Significancia estadística; *, P <0,05; **, p <0,01; *, p <0,001. C-: Control negativo Mo: morfante.**

Figura 7. La señalización MAPK controla la sobreexpresión miR-212 después de la administración de morfina. Los niveles de expresión de miR-212 medidos por qPCR después de la administración de morfina e inhibición de MEK1 / 2 (U0126, M), inhibidor de CaM / CaMKII (W7, W) y hiperactivación de PKA (Forskolin, F) (A, 24hpf, B, 48hpf) . La vía MAPK se requiere como un inductor de la modulación de morfina de miR-212. Además, la señalización del calcio y la PKA no intervienen en la respuesta temprana de la morfina, pero controlan la expresión de miR-212. A 48 hpf, las tres vías parecen regular la regulación negativa observada después de la administración de morfina, especialmente la dosis más alta de morfina. Los resultados se muestran como el cambio de veces respecto al control (línea de puntos). El signo + representa la combinación de fármacos añadidos a cada condición, donde - signo representa la ausencia de ellos. Los datos se expresan como media $\pm$ SEM, N = 3. Significancia estadística; *, P <0,05; **, p <0,01; *, p <0,001.**

oprm1

Figura 8. La cascada de las MAPK, la señalización del calcio y la PKA son necesarias para la sobreexpresión de *oprm1* desencadenada por la morfina a 24 hpf. La figura muestra los niveles de expresión medidos por qPCR después de la administración de morfina e inhibición de MEK1 / 2 (U0126, M), CaM / CaMKII inhibidor (W7, W) y Hiperactivación de la PKA (Forskolin, F) (A, 24hpf; B, 48hpf). La vía de las MAPK se requiere como un inductor de la modulación de *oprm1* por la morfina. Además, la señalización del calcio y la PKA no intervienen en la respuesta temprana de la morfina. A 48 hpf, las tres vías parecen regular la regulación negativa observada después de la administración de morfina, especialmente la dosis más alta de morfina. Los resultados se muestran como el cambio de veces respecto al control (línea de puntos). El signo + representa la combinación de fármacos añadidos a cada condición, donde - signo representa la ausencia de ella. Los datos se expresan como media $\pm$ SEM, N = 3. Significancia estadística; *, P <0,05; **, p <0,01; ***, p <0,001.

miR-212/132 Promoter 2573bp

Figura 9. El promotor miR-212/132 puede ser controlado por CREB y MeCP2 a través de sitios de unión CRE y RE1 presentes en el promotor. Fragmento de la estructura del promotor miR-212/132 y secuencia correspondiente a los sitios CRE y RE1. Los sitios CRE son los responsables de la actividad de CREB, ya que la unión de esta proteína a esta región induce la expresión de varios genes. Los sitios RE1, sitios represores susceptibles de metilación, están relacionados con la inhibición de la transcripción a través de la unión del complejo de represión formado por un grupo de proteínas.

Figura 10. La morfina no modifica el patrón de metilación del sitio RE1 en el promotor miR-212/132. Patrón de metilación en el promotor miR-212/132 de la secuencia del sitio RE1. Cada cuadrado representa una citosina de una isla CpG susceptible de ser metilada. Como se observa, en ambas condiciones de control, 24 y 48 hpf, todas las citosinas están metiladas (mostradas en amarillo) (Figura A y C). En ninguno de los dos casos, la administración de morfina induce un cambio significativo en el porcentaje total de metilación (citosinas azules, no metiladas), lo que indica que los cambios en la metilación del sitio RE1 no son responsables de la regulación directa de la expresión de miR-212/132. N = 6. Los cuadrados grises representan fallos durante la secuenciación.

Figura 11. Los sitios CRE en el promotor miR-212/132 controlan activamente los efectos de la morfina sobre estas moléculas. ChIP-qPCR para CREB fosforilada (pCREB) unida a los sitios CRE en el promotor de miR-212/132. Los resultados muestran que la morfina aumenta la unión a 24 hpf. Sin embargo, se observa una disminución a 48 hpf, probablemente debido a la unión de MeCP2 al sitio RE1. Los datos se representan como la media $\pm$ SEM. ** $p < 0,01$; $N = 3$

Figura 12. La señalización de Notch modula los efectos de la morfina sobre la expresión miR-212/132. ChIP-qPCR para CREB fosforilada (pCREB) unida a los sitios CRE del promotor de miR-212/132 24hpf. DMSO corresponde al grupo de control ya que DAPT no se puede disolverse en agua. DAPT reduce la unión de pCREB al promotor, incluso en presencia de morfina. Los datos se representan como media $\pm$ SEM. $N = 2$

Figura 13. Los niveles de expresión de miR-29a durante el desarrollo del pez cebra siguen un patrón cíclico de expresión ascendente y descendente. **Expresión temporal de miR-29a durante el desarrollo de pez cebra a 5, 8, 16, 24 y 48 hpf.** Los resultados muestran que este miRNA está altamente expresado durante las primeras etapas de desarrollo, especialmente durante 5 y 16 hpf. Sin embargo, se observa un aumento a 48 hpf, lo que indica un supuesto papel importante para este miRNA durante el desarrollo. Estos resultados se correlacionan con el hecho de que la señalización de Notch, uno de los targets de miR-29a es necesario en la especificación temprana de destino de la célula. Los datos se expresan como cantidad absoluta de miRNA-29a por 2/µg de cDNA. Los datos se expresan como la media $\pm$ SEM, N = 3.

Figura 14. La cascada de señalización opioide y Notch fosforilan y activan NF- κ B. Western Blot contra NF- κ B fosforilada a 24 hpf en embriones de pez cebra AB y mutantes *mindbomb*. La morfina induce la activación de NF- κ B, y también lo hace la inhibición de la vía MAPK con el inhibidor U0126 en embriones de tipo AB (control). En mutantes *mindbomb* (Mb), donde la señalización de Notch se encuentra interrumpida, la administración de morfina parcialmente revertir los efectos, pero no cuando la ruta MAPK se interrumpe con U0126. Los datos de Western Blot se normalizaron contra la actina. Los datos se representan como la media $\pm$ SEM. N = 4. * P < 0,05, ** p < 0,01

Figura 15. Control de la expresión del miR-29a por las vías señalización opioide y Notch. Los niveles de miR-29a fueron medidos por qPCR en embriones de pez cebra a 24 hpf . La inhibición de la morfina y MAPK (con U0126) reduce la expresión de miR-29a. Por otra parte, la interrupción de la señalización Notch con DAPT aumenta dramáticamente la expresión de este miRNA, excepto cuando se coadministra con morfina. Los datos se representan como media $\pm$ SEM. * $P < 0,05$, * $p < 0,001$ $n = 3$. MD, Morfina 10 nM + DAPT; MU, Morfina 10 nM + U0126.**

Figura 16. La señalización opioide y Notch están reguladas recíprocamente. Se determinaron los niveles de OPRM1 y NICD mediante Westernblot después de la adición de varios fármacos a células SH-SY5Y. DAPT regula positivamente a OPRM1. La morfina también aumenta OPRM1, como se ha descrito anteriormente (Garcia-Concejo et al., 2016). La inhibición de las MAPK también mostraron un aumento de la expresión de OPRM1. La naloxona, sin embargo, no revirtió los efectos, incluso cuando se coadministró con morfina. Sin embargo, la exposición a DAPT y morfina indujo una disminución de los niveles de OPRM1, en comparación con los otros grupos (A). La morfina induce una regulación negativa de NICD. El inhibidor de la vía MAPK U0126 también redujo la activación de Notch. Los mismos resultados se observaron cuando la morfina se coadministró con DAPT (B). Estos resultados sugieren que ambas cascadas de señalización están relacionadas entre sí, probablemente mediada por la interacción de NICD con pCREB vinculante a sitios CRE. Los resultados se expresan como el cambio respecto al control. Media $\pm$ SEM, N = 3. Nx, naloxona; MN, morfina 10 nM + Naloxona; MU, morfina 10 nM + U0126; DM, DAPT + U0126. *, P <0,05, **, p <0,01, ***, p <0,001

Figura 17. La morfina induce cambios en la agregación celular y demora la diferenciación de los precursores neuronales. Fotogramas de un cultivo de neuronas primarias de antígeno de pez cebra transgénico: GFP experimento de lapso de tiempo durante 8 horas en un grupo de control (A) y expuestos a 10 nM morfina (B). Los resultados muestran una prolongada expresión de GFP después de la exposición a la morfina (Figura B). La morfina ejerció un fuerte efecto sobre la agregación de precursores neuronales. Además, la expresión de GFP se retrasó y duró durante las 8 horas del experimento. Las cabezas de flechas negras indican células GFP positivas, mientras que las cabezas de flecha blancas indican neuronas maduras.

Figura 18. DAPT y morfina induce cambios en la agregación celular y demora la diferenciación de los precursores neuronales Fotogramas de un cultivo de neuronas primarias a partir de transgénicos zebrafish nestin: Experimento de lapso de tiempo GFP durante 8 horas en un grupo DAPT (A), DAPT + Morfina 10nM (B). Los resultados muestran una prolongada nestin expresión después de DAPT exposición (Figura A], aunque el aumento de la agregación celular no se observó. La morfina coadministrada con DAPT no sólo redujo la agregación de precursores neuronales, sino que también redujo la expresión de GFP, lo que indica que se promovió la diferenciación.

Figura 19. Morfina y DAPT control de la agregación de células y la proliferación de R1 ES células cultivadas diferenciadas en neuronas dopaminérgicas. TH inmunohistoquímica de R1 células ES proliferación y agregación cambios durante la diferenciación dopaminérgica de ES R1 células. La morfina indujo un aumento de la proliferación celular en comparación con el grupo control. Las células agrupadas mostraron y aumento de células TH positivas. DAPT redujo la agregación celular en comparación con el grupo DMSO. TH: verde, DAPI: azul, Phalloidin: rojo. CM, morfina control, M, morfina, CD, DMSO control, D, DAPT. Las flechas indican la célula más representativa entre los grupos estudiados.

Figura 20. La morfina reduce el número total de células diferenciadas durante el protocolo de diferenciación en neuronas dopaminérgicas. Porcentaje de diferenciación de las células R1 ES en neuronas dopaminérgicas (células TH positivas). Para cada inmunocitoquímica se contaron 20 campos para determinar el porcentaje de diferenciación. Este porcentaje se calculó como la división de las células TH positivas entre las células totales (marcadas con DAPI). La morfina redujo la diferenciación de las células R1 ES, mientras que DAPT no tuvo efecto sobre el número total de células TH positivas. Los resultados se obtuvieron a partir de tres experimentos IHC diferentes. Los datos se expresan como Media $\pm$ SEM. **, $p < 0,01$. CM, grupo control; M, 10 nM de morfina, CD, DMSO de control; D, DAPT.

Figura 21. Western Blot de varios marcadores dopaminérgicos implicados en la diferenciación dopaminérgica en la primera etapa de desarrollo de células R1 ES. Los resultados muestran un aumento de SIRT1 en ambas etapas después de la administración de morfina (Figura A). La morfina también aumenta FoxA2 y NGN2, ambos factores de transcripción necesarios para el correcto desarrollo de precursores neuronales (Figura B y C). DAPT induce un notable aumento de FOXA2 en la primera etapa de desarrollo (Figura B). Los experimentos se realizaron por triplicado. Todos los resultados se normalizaron contra actina (control de carga). Los datos se representan como media $\pm$ SEM. **, $p < 0,01$. N = 3. CM, control; M, morfina; CD, DMSO; D, DAPT. El número indica la etapa de diferenciación.

Neural Stem Spheres (Stage 2)

Figura 22. Western Blot de varios marcadores dopaminérgicos implicados en la diferenciación dopaminérgica en la segunda etapa de desarrollo de células R1 ES. Los resultados muestran un aumento de SIRT1 en ambas etapas después de la administración de morfina (Figura A). La morfina también aumenta FoxA2 y NGN2, ambos factores de transcripción necesarios para el correcto desarrollo de precursores neuronales (Figura B y C). DAPT induce un notable aumento de FOXA2 en la primera etapa de desarrollo (Figura B). Los experimentos se realizaron por triplicado. Todos los resultados se normalizaron contra actina (control de carga). Los datos se representan como media $\pm$ SEM. **, $p < 0,01$. N = 3. CM, control; M, morfina; CD, DMSO; D, DAPT. El número indica la etapa de diferenciación.

Neural Precursors (Stage 3)

Figura 23. Western Blot de varios marcadores dopaminérgicos implicados en la diferenciación dopaminérgica en la tercera etapa de desarrollo de las células R1 ES. Los resultados muestran que SIRT1 no se expresa en la cuarta etapa del desarrollo (A), así como Pitx3 (C). DAPT reduce los niveles de expresión de varios marcadores dopaminérgicos como Nurr1 (B) y Pitx3 (C), lo que resulta en una expresión TH reducida (D). Los experimentos se realizaron por triplicado. Todos los resultados se normalizaron contra actina (control de carga). Los datos se representan como media $\pm$ SEM, N = 3. CM, control; M, morfina; CD, DMSO; D, DAPT. El número indica la etapa de diferenciación.

Dopaminergic Neurons (Stage 4)

Figura 24. Western Blot de varios marcadores dopaminérgicos implicados en la diferenciación dopaminérgica en la cuarta etapa de desarrollo de las células R1 ES. Los resultados muestran que SIRT1 no se expresa en la cuarta etapa del desarrollo (A), así como Pitx3 (C). DAPT reduce los niveles de expresión de varios marcadores dopaminérgicos como Nurr1 (B) y Pitx3 (C), lo que resulta en una expresión TH reducida (D). Los experimentos se realizaron por triplicado. Todos los resultados se normalizaron contra actina (control de carga). Los datos se representan como media $\pm$ SEM, N = 3. CM, control; M, morfina; CD, DMSO; D, DAPT. El número indica la etapa de diferenciación.

Data are represented as mean $\pm$ SEM, N=3.

Figura 25. miR-212, miR-132 y miR-29a controlan la expresión de varios factores de transcripción dopaminérgicos. La expresión de los marcadores dopaminérgicos después de la transfección con mimics del miRNA 212, 132 o miR29a coadministrada con morfina en células SH-SY5Y. Estos resultados muestran que miR-132 disminuye NGN2 (A). Sin embargo, la expresión del miR-132 induce un aumento de SIRT1 (B). Además, miR-132 sobreexpresa TH, aunque esto se invierte por la administración de morfina (C). MiR-212 sólo parece tener un efecto sobre la expresión TH, en presencia y ausencia de morfina (C). MiR-29a sólo disminuye NGN2 cuando se coadministra con morfina (A). La sobreexpresión de miR-29a induce una regulación positiva de TH, pero se invierte después de la administración de morfina (C). Además, la sobreexpresión de miR-29a indujo la expresión de PITX3, un marcador dopaminérgico no expresado en células SH-SY5Y (D). Los datos se representan como media $\pm$ SE o área relativa. N = 3. C, control, morfina, morfina 10 nM.

Figura 26. Implicaciones de señalización opioide y Notch en la diferenciación dopaminérgica. Las imágenes corresponden a imágenes ISH contra el mRNA de los tres genes de 36 hpf embriones de pez cebra. (A', B' y C'). Mindbomb, muesca que marca mutantes defectuosos (D', E' y F'). Como se observa en las imágenes, el silenciamiento de señalización opioide indujo una downregulation de *nurr1* y *pitx3*, así como la expresión *th*. Por otro lado, la disrupción de la muesca favorece la lateralidad de la diferenciación dopaminérgica, induciendo la expresión de los marcadores dopaminérgicos y de las neuronas a lo largo de la línea media.

Figura 27. Morfina y DAPT reduce la apoptosis inducida por MPTP en células SH-SY5Y. TUNEL en SH-SY5Y después de la apoptosis inducida por MPTP. A) Control B) Control de apoptosis positivo C) Control de MPTP D) DMSO E) Morfina 10 nM F) DAPT G) Resveratrol H) U0126 I) Porcentaje de células TUNEL positivas. La morfina reduce el efecto apoptótico de MPTP. DAPT y U0126 también reducen el número de células apoptóticas. El resveratrol no indujo ningún cambio significativo. Se contaron 40 campos para medir el porcentaje de apoptosis. Resv, Resveratrol. Las flechas indican células TUNEL (apoptóticas) positivas. Verde, TUNEL células

Figura 28. Morfina y MAPK señalización interrupción inducir neuroprotección a MPTP. TUNEL en SH-SY5Y después de la apoptosis inducida por MPTP. A) Control B) Control de apoptosis positivo C) Control de MPTP D) Morfina + DAPT E) Morfina + U0126 F) Morfina + Naloxona G) Morfina + Resveratrol H) DAPT + Resveratrol I) TUNEL células positivas. La morfina coadministrada con U0126 indujo un mayor efecto neuroprotector que las dos moléculas separadas. El resveratrol no indujo ningún cambio significativo, aunque su administración con DAPT invirtió los efectos de la interrupción de señalización Notch. Se contaron 40 campos para medir el porcentaje de apoptosis. Se contaron 40 campos para medir el porcentaje de apoptosis. MD, morfina + DAPT; MU, morfina + U0126; MN, morfina + naloxona; RM, morfina + resveratrol; DR, DAPT + Resveratrol. Las flechas indican células TUNEL (apoptóticas) positivas. Verde, TUNEL células positivas; Azul, DAPI.

Figura 29. Efectos neuroprotectores frente a MPTP en embriones de pez cebra de 48 hpf. MPTP inducida por la apoptosis específica de las neuronas dopaminérgicas del cerebro mediano de pez cebra. La morfina y el DAPT demostraron ser un agente neuroprotector eficaz, aunque específicamente en los grupos dopaminérgicos anteriores (A1 v A2) y no en el posterior (A4-A6). . Azul: DAPI Rojo: TH

Figura 30. Efectos neuroprotectores frente a MPTP en embriones de pez cebra de 48 hpf. MN, morfina + naloxona. MPTP inducida por la apoptosis específica de las neuronas dopaminérgicas del cerebro mediano de pez cebra. Resveratrol no mostró ningún efecto neuroprotector contra MPTP. La naloxona, sin embargo, mostró un efecto en la contrarrestación de los efectos del MPTP. U0126 interrumpió la distribución dopaminérgica, indicando que esta vía es necesaria para la diferenciación dopaminérgica correcta. Azul: DAPI Rojo: TH

Figura 31. Efectos neuroprotectores contra MPTP en embriones de pez cebra de 48 hpf. DM, DAPT + Morfina; DR, DAPT + Resveratrol; MR, Morfina + Resveratrol; MU, Morfina + U0126. MPTP inducida por la apoptosis específica de las neuronas dopaminérgicas del cerebro mediano de pez cebra. La morfina y la coadministración DAPT indujeron una separación entre los diferentes grupos dopaminérgicos, pero no protegieron a ninguno de los grupos de MPTP. Morfina + Resveratrol no mostró efectos neuroprotectores. La disrupción del patrón dopaminérgico por U0126 fue parcialmente revertida por la morfina, y los grupos resultantes fueron resistentes a MPTP. Azul: DAPI Rojo: TH

Figura 32. La morfina regula la expresión de miR-212 y oprm1 mediante la activación de Oprm1 en embriones de pez cebra a 24 hpf. La activación de Oprm1 por la morfina induce una sobre-regulación del pri-miR-212/132, que después del posterior splicing y maduración conduce a un aumento de la forma madura miR-212. Además de eso, la activación de Oprm1 induce su propia expresión en un mecanismo de feedforward. miR-212 controla la regulación positiva del receptor dirigiendo el 3'UTR del mRNA. Sin embargo, el mecanismo mediante el cual la administración de morfina 10 nM induce la sobreexpresión del miRNA primario puede estar mediado por otra vía, ya que el silenciamiento del receptor no invierte el mecanismo de regulación positiva de la morfina.

Figura 33. La morfina regula la expresión de miR-212 y *oprm1* en embriones de pez cebra a 48 hpf, aunque el mecanismo no implica la activación de Oprm1. El silenciamiento de *oprm1* induce un aumento de pri-miR-212/132 que se exagera después de la administración de morfina. Esto indica una regulación putativa de la expresión del miRNA primario por otros receptores, como los receptores opioides delta 1/2. Sin embargo, esta regulación positiva del miRNA primario no induce un aumento en los niveles de la forma madura, lo que indica que el proceso de splicing debe regularse después de la administración de morfina. Por otra parte, el aumento de los niveles de miR-212 a 24 hpf ahora está reprimiendo los niveles de *oprm1*.

Figura 34. Mecanismo propuesto para la regulación de la expresión pri-miR-212/132 mediante la activación del receptor opioide mu (Oprm1) por la morfina a 24 hpf. El mecanismo propuesto a 24 hpf implica tres vías diferentes: 1, la inhibición de la adenilato ciclasa mediada por α_{Gi} que induce una disminución de cAMP y, por tanto, reduce PKA actividad 2, la subunidad α_{Gi} cierra los canales de calcio, por lo tanto, reducir la fosforilación de MeCP2 y prevenir la represión de la expresión del pri-miR-212/132 3, la activación de PKC induce la vía de MAP quinasa, fosforilando CREB. La represión de la señalización del calcio impide la activación de CAMKII y la fosforilación de MeCP2, por lo que no se observa una represión de la transcripción. La fosforilación de CREB mediada por la vía MAPK induce la sobreexpresión de miR-212/132, como se observa en nuestros estudios ChIP-qPCR. Las líneas punteadas implican que estas vías no están activas durante el proceso.

Figura 35. Mecanismo propuesto para la regulación de la expresión pri-miR-212/132 mediante la activación del receptor opioide mu (Oprm1) por morfina a 48 hpf. El mecanismo propuesto a 48 hpf implica tres vías diferentes: 1, la hiperactivación de la PKA induce la fosforilación de CREB, controlando así la expresión de oprm1. La reapertura de los canales de calcio induce la fosforilación de MeCP2 a través de CaMKII y se une al sitio R1 promotor miR-212/132, reprimiendo la expresión de los miRNAs, 3, la liberación de calcio intracelular induce la fosforilación de la CaMKIV, potenciando los efectos represores de MeCP2 en el RE1 de los promotores oprm1 y miR-212/132. Las líneas punteadas indican caminos que no están activos durante este proceso..

Figura 36. Las cascadas de señalización opiode y Notch están relacionadas por los tres miRNAs estudiados (miR-212, miR-132 y miR-29a) ya través de la modulación de CREB y NF-κB. La morfina activa OPRM1, y a través de la vía PI3K / Akt induce la activación de IKK y la fosforilación de NF-κB y los niveles de miR-29a disminuyen, aunque la señalización de MAPK, a través de un intermediario desconocido, reduce la fosforilación de NF-κB (líneas punteadas y signo de interrogación). Esta reducción induce un incremento en la expresión de ADAM12, y la escisión de Notch aumenta y los niveles de NICD se incrementan. NICD, por otro lado, induce una disminución en la unión de pCREB al promotor de los miR-212/132, disminuye los niveles de estos miRNAs y aumentan por esa razón los niveles de OPRM1.

Figura 37. Expresión de los factores de transcripción dopaminérgicos en los embriones de pez cebra AB (A) y en los mutantes mindbomb (B). Las regiones donde estos factores se colocan inducen la expresión de *th* en el área del mesencéfalo (C). Estos resultados sugieren que la vía de Notch es necesaria para la correcta especificación de las neuronas dopaminérgicas y determinar la lateralidad y las regiones donde se generan las neuronas dopaminérgicas.

Figura 38. La interacción de señalización de Notch y opioide median la expresión de TH a través de la expresión de NURR1. La morfina induce la expresión de NURR1 y también su fosforilación y activación, promoviendo la expresión de TH. Sin embargo, NICD puede inducir la expresión de NURR1 mediante la reducción de la unión a su promotor de varios complejos represores mediados por CSL. Por otra parte, SIRT1 puede modular la actividad de NICD por su desacetilación (Guarani et al., 2011) y actuar también como un modulador de la actividad de Nurr1 (Kim et al., 2013). Estos resultados indican que NURR1 per se no es suficiente para inducir la expresión de TH que podría explicar las diferencias observadas en diferentes grupos neuronales durante la diferenciación

ANNEX II

First author Publications

RESEARCH ARTICLE

μ Opioid Receptor Expression after Morphine Administration Is Regulated by miR-212/132 Cluster

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Abstract

Since their discovery, miRNAs have emerged as a promising therapeutic approach in the treatment of several diseases, as demonstrated by miR-212 and its relation to addiction. Here we prove that the miR-212/132 cluster can be regulated by morphine, through the activation of mu opioid receptor (Oprm1). The molecular pathways triggered after morphine administration also induce changes in the levels of expression of *oprm1*. In addition, miR-212/132 cluster is actively repressing the expression of mu opioid receptor by targeting a sequence in the 3' UTR of its mRNA. These findings suggest that this cluster is closely related to opioid signaling, and function as a post-transcriptional regulator, modulating morphine response in a dose dependent manner. The regulation of miR-212/132 cluster expression is mediated by MAP kinase pathway, CaMKII-CaMKIV and PKA, through the phosphorylation of CREB. Moreover, the regulation of both *oprm1* and of the cluster promoter is mediated by MeCP2, acting as a transcriptional repressor on methylated DNA after prolonged morphine administration. This mechanism explains the molecular signaling triggered by morphine as well as the regulation of the expression of the mu opioid receptor mediated by morphine and the implication of miR-212/132 in these processes.

Introduction

The analysis of the molecular signalling cascade activated by morphine may lead to unveil new molecular targets that could be used to palliate the undesired effects of opiate drugs. The opioid receptors have been widely studied, and their regulation after being activated could explain the molecular basis of addiction, especially the mu opioid receptor (Oprm1), since it is the receptor which morphine binds to with the highest affinity [1]. Opioid receptors are G-protein coupled receptors, and the signalling cascade they activate induces the activation of cAMP Response Element Binding (CREB) protein [2, 3], a transcription factor involved in many molecular processes.

Since their discovery, microRNAs (miRNAs) have emerged as extremely promising tools in the modulation of the expression of many genes [4]. miRNAs are small non coding RNA transcripts that regulate gene expression at the post-transcriptional level. These molecules control gene expression by binding to complementary sequences in the 3' untranslated region (3' UTR) of their target mRNA transcripts to facilitate their degradation and/or inhibit their translation [5], although binding to other regions have been described [6, 7]. miRNAs are involved in many developmental processes, such as differentiation, proliferation, and their dysregulation has been linked to a wide variety of diseases [8, 9]. Several other miRNAs, such as miR-let7d and miR-133b [10, 11], have been described to be regulated by morphine and play a role in behavioural effects of this drug.

miR-212 and miR-132 form a cluster, although their functions may not be fully correlated [12, 13]. Previous studies [14, 15] reported that expression of miR-212 was increased in the dorsal striatum of rats with extended but not restricted daily access to intravenous cocaine self-administration. Moreover, CREB activation has been shown to regulate miR-212 expression after exposure to cocaine in murine models [16]. Taking these results into consideration, it is of interest to unravel whether the regulation mechanism triggered by cocaine could also be activated by morphine through the activation of Oprm1, or more importantly, if both drugs share the functional cascade that describe their activity. The results obtained in this work show that morphine is regulating the expression of miR-212 through CREB signalling, and that miR-212 can also modulate miR-132 and *oprm1* expression inhibiting its translation by binding to its 3' UTR. This regulation mechanism could explain some of the molecular components that remain unclear concerning opioid signalling.

Material and Methods

Experimental animals

Zebrafish from the AB strain were bred and raised in the Fish Facilities of our lab following standard protocols [17]. In all experiments, adequate measures were taken to minimize pain or discomfort and animals were handled according to the guidelines of the European Communities Council Directive 2010/63/UE, to the current Spanish legislation for the use and care of animals RD 53/2013 and to the Guide for the Care and Use of Laboratory animals as adopted and promulgated by the U.S. National Institutes of Health. For ISH experiments, embryos were fixed in 4% paraformaldehyde, and for RNA extraction embryos were frozen in liquid nitrogen. These experiments were approved by the University of Salamanca Ethics Committee.

Drug Treatment and inhibitors

Zebrafish embryos were exposed to 10 nM or 10 μ M morphine-sulphate in E3 embryo buffer from 5 hours post fertilization (hpf) (50% epiboly) to 24/48 hpf. Fresh morphine solution was added at 24 hpf. A control group (E3 only) was used in parallel for each experiment. Morphine was provided by the Spanish Ministry of Health. Concentrations used for each inhibitor were: U0126 (Promega) 20 μ M, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7, Sigma) 10 μ M and Forskolin (Sigma) 20 μ M.

RNA Extraction and qPCR

Total RNA, including miRNA, was extracted using Trizol (Life Technologies), following the manufacturers protocol. To study the levels of expression of the miRNAs of interest, a previous polyadenylation was performed using Poli-A polymerase (New England Biolabs) and a retro-transcription reaction using M-MuLV retrotranscriptase (New England Biolabs), as described

in the manufacturer's protocol. The retrotranscription of *oprm1* samples were carried out using the High Capacity RT Kit (Applied Biosystems). The absolute quantification of the PCR products was accomplished with a standard curve using the SYBR-Green method, and specifically designed oligonucleotides as described by Balcells et al., [18] The SYBR-Green was included in a 2× Master Mix (Life Technologies, SYBR Green PCR Master Mix). The final volume of each reaction was 20 μ l, distributed as follows: 10 μ l of Master Mix, 0.8 μ l of each oligonucleotide, 7.4 μ l of distilled water, and 1 μ l of cDNA in a concentration of 25 ng/ μ l. A standard curve was constructed for each experiment by serial dilutions of cDNA. The amplification reaction were performed in an ABI 7300 qPCR Thermal Cycler (Applied Biosystems), with the following conditions: 15 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 70°C. qPCR was performed by triplicate, and each experiment was repeated with three different samples. The sequences of the primers used were the following: *oprm1* qF, ACGAGCTGTGCAAGA TTGTG; *oprm1* qR, CCGATTGCAGATGAAAGAT; dre-miR-132 qF, AGTAAACAGTCTACA GCCATG; dre-miR-132 qR, TCCATTTTTTTTTTTTTTTTTTTGTCTG; dre-miR-212 qF, CGCAG TAACAGTCTACAGTC; dre-miR-212 qR, CCAGTTTTTTTTTTTTTTTTTTTGGCCAT; pri-miR-F, TCTGGCAACCTTCCACAG, pri-miR-R, TGGAGACAGCAGGAGGG.

Total protein extraction, immunoprecipitation and western blot

Total proteins were extracted from zebrafish embryos at 24 and 48 hpf using cold Ringer solution and 300 μ l of protein extraction buffer (10 mM tris pH 7.4, 2% triton X 100, 1 mM PMSF, 1 μ l/ml protease inhibitors [Sigma]). Embryos were aspirated using a syringe and centrifuged 10 minutes at 10000 g at 4°C. The supernatant containing proteins was frozen at -80°C. Proteins were quantified by Bradford method. 350 μ g of total protein were used for the immunoprecipitation of each group and incubated with 25 μ l of Oprm1 antibody (Spanish patent license: P201330193) conjugated with protein A agarose beads. Beads were washed with lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS, 1x protease inhibitors) and centrifuged at 14000 rpm. Pellets were resuspended in 25 μ l of 4X loading buffer (10% SDS, 600 μ l of glycerol, 150 μ l of 2-mercaptoethanol, 1M Tris-HCl pH: 6.8 and 30 μ l of Bromophenol blue). Samples were boiled for 5 minutes and centrifuged at 14000 rpm. Supernatants were loaded in 10% polyacrylamide SDS gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked with 5% milk in TBS. Oprm1 primary antibody was used at 0.25 μ g/ml to detect the immunoprecipitated protein. After incubation, membranes were washed with TBS containing 20% Tween 20 and incubated for 1 hour with the goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology) diluted 1:5000. Blots were developed with ECL (Amersham). Experiments were performed in triplicates and quantified with ImageJ software.

Morpholino microinjection

The morpholino antisense (MO) oligomers used to knock down *oprm1*, *miR-212* and *miR-132* were purchased from Gene Tools, LLC. The MO was diluted in sterilized water to a stock concentration of 1 mM. In addition to the three MO experimental groups (untreated, 10 nM morphine, and 10 μ M morphine), each experiment included a control MO group injected with morpholino that exhibits no binding target or biological activity (control morpholino). Morpholino was injected into the yolk at the one cell stage according to the published protocols [19]. The concentrations of *target* MO and control MO used were 0.33 and 1 μ M, respectively (3 nl were injected into each embryo). The sequences of the morpholinos used were the following: *oprm1* MO, AATGTTGCCAGTGTTTTCCATCATG; dre-miR-132 MO, ACAGTAAC AATCTAATGCCACGGTC; dre-miR-212 MO, AGCCATGACTGTAGACTGTTACTGT.

Whole-mount in Situ Hybridization (WISH)

Embryos at 24 hpf and 48 hpf were dechorionated, fixed with 4% paraformaldehyde (PFA) in phosphate saline buffer (PBS) overnight at 4°C, washed twice in PBS for 5 min at room temperature (RT), and stored in absolute methanol at −20°C until use. The hybridization was carried out as described in Sanchez-Simon et al. [20], and the probes used for this experiment were designed as described by He et al. [21].

Luciferase assay

For chemiluminescence detection we used the Dual-Glo Luciferase Assay (Promega). The sequences were cloned into PmirGlo (Promega) using the protocol for digestion, ligation and transformation described in GeneClip™ U1 Hairpin Cloning Systems (Promega). HEK293 cells were transfected with a wild-type and mutated construct using Lipofectamine 2000 [22]. Briefly, 300ng of reporter plasmid were diluted in 25 μl of DMEM in combination with 20 nM or 50nM of miR-212 or miR-132 miRNAs mimics (small, chemically modified double-stranded RNAs that mimic endogenous miRNAs by up-regulation of miRNA activity) or 50nM of miR-1 mimics (Ambion) when required. This solution was combined with diluted Lipofectamine (1.5 μl/well) and the transfection was carried out for 48 hours at 37°C in a 5% CO₂ incubator. Luminescence was measured during 10 seconds as recommended by the manufacturer using a luminometer (Appliskan, Thermo Scientific). The ratio between luminescence from firefly and the control (*Renilla*) was used to check if the miRNA was binding to the sequence cloned into the plasmid. The mean for each group (n = 8) was calculated and normalized with the wells transfected only with the empty plasmid. The primers cloned into the vector were the following: wtF, CTAGCTAGGTACCTAGTTAGTTTGAGGAAAGACTGTTGG; wtR, TCGACCAACAGTCTTTCCTCAAATACTAGGTACCTAG; misF, CTAGCTAGGTACCTAGTTAGTTTGAGGAAATCAGTGTGG; misR, TCGACCACACTGATTTTCCTCAAATACTAGGTACCTAG

Primary neuron culture

Brains from 42 hpf zebrafish embryos were placed in 1x E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO₄, 150 μM KH₂PO₄, 50μM Na₂HPO₄, 1 mM CaCl₂, 0.7 mM NaHCO₃, 0.5 mg/l methylene blue) with 1x tricaine as described by Chen et al. [23]. Briefly, cells were grown in Dulbecco's modified medium to 48 hpf. Morphine (1 nM) was added after plating. 48 hours later neurons were fixed in 4% paraformaldehyde (PFA) (w/v). Immunocytochemistry analysis for primary cultured cells was carried out as described by Chen et al. [23]. Neurons were fixed with 4% PFA (w/v) for 20 minutes and blocked with antibody buffer and 5% goat serum; 1:250 dilution was used for Oprm1 antibody. To-PRO (1:1000, ThermoFisher) and Alexa fluor 594 Phalloidin (1:10, ThermoFisher) were used as nuclei and soma markers, respectively. Primary cultures were analyzed with a life cell microscopy (Zeiss).

Statistical analysis

qPCR ct values were first included in REST-384 v2 software to calculate the relative expression of groups respect to control embryos. Data were analysed by one-way analysis of variance (one-way ANOVA). Tukey's post hoc test was then performed for multiple comparisons. Differences were considered significant at p < 0.05. Results are shown as means ± S.E.M. All statistical analyses were performed with Prism 5 (GraphPad Software, Inc).

Results

miR-212 and miR-132 temporal expression is closely related

miR-212 and miR-132 form a genetic cluster [12, 13], and its expression and function may be related to each other. To determine the relevance of miR-212 and miR-132 during development, we studied their temporal expression at 5, 8, 16, 24 and 48 hpf (Fig 1A and 1B). Although both are highly expressed between 5 and 16 hpf, miR-212 expression decays during development. In addition, we have observed an increase in expression of miR-212 at 8 hpf and an increase of miR-132 at 16 hpf. Besides, the number of copies for miR-132 is greatly increased at 48 hpf when compared to miR-212 (Fig 1B), suggesting the importance of this miRNA at this particular stage. To study a possible relation of the levels of miRNAs, we knocked-down miR-212 and miR-132 in order to analyze the independence of each other of their expression profiles (Fig 1C and 1D). After the knock-down of miR-212, and at several stages (5, 8 and 16 hpf), we have observed a decrease in expression of miR-132 (Fig 1C). In addition, the levels of miR-212 were downregulated in miR-132 morphants (Fig 1D). Even though miR-132 is more expressed during development, miR-212 has been linked to addiction [16] and it is probably crucial during the first stages of development.

Morphine modifies the levels of expression of miR-212, of pri-miR-212/132 and *oprm1*

The levels of expression of miR-212 in zebrafish embryos were measured by qPCR after the administration of two different doses of morphine (10nM and 10 μ M) from 5 hpf (Fig 2). We have previously used these two doses to cover a wide variety of pharmacological responses triggered by morphine. As for a biochemical rationale, we have already proven that both doses

Fig 1. miR-212 (A) and miR-132 (B) temporal expression, and miR-212 expression levels in 132 morphant embryos (C), and miR-132 expression levels in 212 morphant embryos (D). Mo: morphant. The results show differences in the temporal expression of the clustered microRNAs. Figs C and D show a downregulation of one of the miRNAs when the other is knocked-down, indicating a possible regulation between the expression of these molecules in the context of their cluster. Results are shown as the number of copies per 2 μ g of cDNA (A and B) or the fold change respect to control levels at 24 and 48 hpf. (C and D) Data are expressed as mean $\pm$ SEM. Control group is shown as the dotted line. Statistical significance; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Fig 2. miR-212 expression levels in control and *oprm1* morphant embryos after morphine (10 nM and 10 μ M) treatment (A, 24hpf; B, 48hpf). pri-miR-212/132 expression levels in control and *oprm1* morphant embryos after morphine administration (10 nM and 10 μ M) (C, 24hpf; D, 48hpf). *oprm1* expression levels in control and miR-212 morphant embryos after morphine (10 nM and 10 μ M) treatment (E, 24hpf; F, 48hpf). Oprm1 protein levels measured by IP-WB in control and miR-212 morphant embryos after morphine (10 nM and 10 μ M) treatment (G and I, 24hpf; H and J, 48hpf). C-: Negative control Mo: morphant. IgG HC: immunoglobulin gamma heavy chain. Morphine regulates both miR-212 and *oprm1* expression, inducing an upregulation of both molecules at 24 hpf, but a downregulation at 48 hpf. Results are shown as the fold change respect to control (dotted line) at 24 and 48 hpf. Data are expressed as mean $\pm$ SEM. Statistical significance; *, $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$.**

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bind to mu opioid receptor and could exert different responses [24]. However, the concentrations used can be considered neither clinical nor abusive, since we are not able to evaluate behavioral changes related to these conditions in the zebrafish embryos. Our group has also antagonized these doses of morphine with naloxone, obtaining a clear reversal of the opioid effects [20]. At 24 hpf (Fig 2A), an increase in the levels of the miRNA was observed for both concentrations of morphine, although it was higher at 10 μ M. In contrast, miR-212 was down-regulated at 48 hpf. Additionally, we analyzed the implication of mu opioid receptor in miR-212 expression. After knocking-down the mu opioid receptor, the up-regulation induced at 24 hpf by morphine administration was not observed (Fig 2A). However, the decrease in the expression was not reversed by *oprm1* morpholino at 48 hpf (Fig 2B). Also, as shown in the Figs (Fig 2A and 2B), the levels of miR-212 were not modified in the control *oprm1* morphants. In addition, we measured the levels of the pri-miR-212/132 (Fig 2C and 2D) and we observed a correlation between the expression of the pri-miR and miR-212, except for *oprm1* morphants exposed to 10 μ M morphine in which an upregulation is observed. Furthermore, we analyzed the differences in the results when zebrafish embryos were exposed to morphine from 20 to 24 hpf, instead of 5 hpf. We observed the same results in the levels of miR-212 in both cases, which possibly indicates that the changes observed at 24 hpf are an acute response to morphine exposure. As miR-212 expression is affected by morphine administration, we analyzed if mu opioid receptor was being regulated by this miRNA (Fig 2E and 2F). After morphine treatment, mu opioid receptor was strongly up-regulated at 24 hpf (Fig 2E). In addition, we have observed a decrease in the levels of *oprm1* at 48 hpf after morphine administration (Fig 2F). However, the knock-down of miR-212 induced an increase in the expression of the receptor, much higher than the observed when miR-212 was present at both 24 and 48 hpf.

Finally, to determine whether these changes observed in the mRNA levels were correlating with the protein levels, we performed an immunoprecipitation-western blot (IP-WB, Fig 2G and 2H). The results obtained with this technique correlate with our qPCR experiments (Fig 2I and 2J). However, Oprm1 was downregulated in miR-212 morphants exposed to 10 μ M morphine at both 24 hpf and 48 hpf (Fig 2I and 2J) and considering that miR-212 forms a cluster with miR-132, we decided to analyze if both miRNAs could regulate *oprm1* translation.

miR-212/132 cluster regulates *oprm1* mRNA expression binding to its 3'UTR

In order to determine whether these miRNAs were effectively regulating *oprm1* by targeting its mRNA, we performed a bioinformatic analysis of putative binding sites of mu opioid receptor [25]. A possible binding site for miR-212/132 was observed in the 3' UTR of *oprm1*, as well as an additional site in the second exon of the mRNA. By means of a luciferase assay we confirmed that the binding site in *oprm1* 3' UTR was actively repressing mu opioid receptor (Fig 3). miRNA mimics (small, chemically modified double-stranded RNAs that mimic endogenous miRNAs by up-regulation of miRNA activity) were cotransfected with the plasmids, and we observed a significant decrease in the luminescence on the unmutated 3' UTR binding site

Fig 3. Complementarity between *oprm1* mRNA 3'UTR and miR-212 (A), and between *oprm1* 3' UTR and miR-132 (C). Luciferase assay of the binding site for miR-212 in the 3'UTR of *oprm1* (B) and miR-132 (D). wt, unmutated plasmid; mis, mutated plasmid. Results are shown as the fold change respect to control. The groups transfected with the wild-type plasmid and the plasmid containing the mismatch were cotransfected with 20nM, and 50nM miR-212 or miR-132 mimics, and also with 50nM miR-1 mimics as negative control. The cluster formed between miR-212 and miR-132 regulates *oprm1* expression, as observed in the significative changes when the wild type plasmid is cotransfected with an active form of the miRNAs. Data are expressed as mean $\pm$ SEM, N = 8. Statistical significance; *, $p < 0,001$.**

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(wild type, wt). The mutation of the seed region (mismatch, mis) reversed this decrease, indicating that the predicted binding site was physiologically functional. Moreover, the increase on the concentration of miRNA mimics reduced the luciferase activity, but only when cotransfected with the wt plasmid, corroborating our findings. These results prove that miR-212 and miR-132 are binding to the 3'UTR region of *oprm1* mRNA, and physiologically repressing *oprm1* expression (Fig 3). The decrease in the luminescence on both wt and mis plasmids is probably due to the endogenous activity of the miRNAs present in HEK293 cells.

Morphine modifies miR-212 expression in the midbrain and hindbrain of zebrafish embryos

The results observed by qPCR were corroborated by using an ISH probe against the pri-miR-212/132 (Fig 4A–4F). These miRNAs are expressed in midbrain and hindbrain, and the expression on these areas correlates with the changes obtained in the qPCR experiments. In addition, we obtained a primary neuron culture and performed an IHC against Oprm1 to determine if the changes observed *in vivo* occurred at the cellular level (Fig 4G and 4H). The expression of the Oprm1 receptor was increased 24 hours after the administration of 1nM morphine (Fig 4H), corroborating the results of the qPCR. The morphology of neurons was also modified, showing a rounded shape after morphine exposure.

miR-212 expression is regulated by MAPK, calmoduline and PKA

To analyze in detail the signaling cascade triggered by the activation of Oprm1, the levels of both miR-212 and *oprm1* were studied after the coadministration of morphine and U0126, an inhibitor of MEK1/2 (Fig 5). The upregulation of miR-212 expression after morphine exposure at 24 hpf is reversed to normal levels when the MAP Kinase pathway was blocked (Fig 5A). In addition, we inhibited calmoduline (CaM) and calmoduline kinase II (CaMKII) using W7 to determine the implication of calcium signaling in miR-212 expression. As observed in the Fig 5A, calcium signaling inhibition induces an overexpression of miR-212 in control, 10nM and 10 μ M groups. Using Forskolin, an activator of PKA, we observed a strong upregulation of this miRNA in the three groups (Fig 5A). In contrast, at 48 hpf, miR-212 levels remained decreased after inhibiting MEK1/2, calcium signaling or activating PKA (Fig 5B). The inhibition of MAP kinase pathway and CaM/CaMKII after 10nM morphine administration did not induce the strong downregulation observed in the 10nM control embryos (Fig 5B). The mild downregulation observed in the 10 μ M group is potentiated by disrupting MEK and calcium signaling. PKA over-activation mimicked the decrease of 10 μ M control group, even when coadministered with morphine. *oprm1* overexpression after morphine exposure is mediated by MEK, and blocked by U0126 at 24 hpf (Fig 5C). Moreover, forskolin prevents the induction of the expression of *oprm1*. At 48 hpf, the downregulation in *oprm1* expression levels is partially reverted after MEK and calcium pathways inhibition and by the increase of cAMP (Fig 5D).

Discussion

In our analysis of the miR-212/132 cluster, the expression of miR-132 is higher than that observed for miR-212. Furthermore, the sequence of miR-132 is more conserved during evolution when compared to that of miR-212 [26], meaning that the function of this miRNA may be essential during the early stages of development. miR-212 presents the maximum expression at 8 hpf, while the highest expression observed for miR-132 is at 16 hpf. These miRNAs may be related to somite differentiation (directly related to muscle formation) which are being developed at these stages. The importance of miR-132 in muscle differentiation has been previously described by Bijkerk et al. [27]. Several authors have described a downregulation of miRNAs

Fig 4. ISH against miR-212 (A-F) and IHC against Oprm1 (G-H). miR-212 pattern of expression in control (A, D) and after morphine treatment (10 nM (B,E) and 10 μ M (C,F)) (left, 24hpf; right, 48hpf). Zebrafish primary neuron culture (G, H), control (G) and 1nM morphine (H). The arrows show the regions in the midbrain and in the hindbrain where the changes observed in miR-212 expression levels are more relevant. The IHC performed on the primary neuron culture showed changes in the levels of Oprm1 (green), which correlates with the results observed in the qPCR and ISH experiments.

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Fig 5. miR-212 expression levels after morphine administration and inhibition of MEK1/2 (U0126, M), CaM/CaMKII inhibitor (W7, W) and PKA hyperactivation (Forskolin, F) (A, 24hpf; B, 48hpf). *oprm1* expression levels after morphine administration and inhibition of MEK1/2 (U0126, M), CaM/CaMKII inhibitor (W7, W) and PKA hyperactivation (Forskolin, F) (C, 24hpf; D, 48hpf). Results are shown as the fold change respect to control (dotted line). These results show that the three pathways studied are involved in miR-212 expression, but only MAP kinases are related to *oprm1* changes after morphine administration. Data are expressed as mean $\pm$ SEM, N = 3. Statistical significance; *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$.

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that belong to the same cluster in some diseases [28, 29, 30]. Although the mechanism for this regulation has been studied, it still remains unclear [31]. The downregulation observed in miR-132 levels after knocking-down miR-212 may indicate that miR-212 is regulating the expression of the miRNAs in the cluster, and may have evolutionarily emerged as a regulator for miR-132, which could be crucial during development [32]. The upregulation observed at 24 hpf in the expression of miR-212 after morphine administration is mediated by the activation of the mu opioid receptor, since *oprm1* morphants do not show any significant change. In contrast, a decrease of this miRNA is observed at 48 hpf, but it is not reversed in the morphants. These results indicate that the up-regulation of this miRNA observed after morphine exposure in the early stages of development is mediated by the mu opioid receptor. On the other hand, the decrease of miR-212 levels induced after prolonged morphine administration is mediated by a different pathway than that triggered by the activation of mu opioid receptor, as we have observed in the primiR-212/132 levels after 10 μ M morphine administration in *oprm1* morphants. Previous results from our group have demonstrated a differential expression of miR-132 and miR-212 in 48 hpf zebrafish embryos [33]. However, the knock down of the mu opioid receptor did not modify the levels of expression of miR-212, thus the physiological expression of this miRNA is not controlled by *Oprm1*, but this receptor is responsible for the changes observed after morphine exposure. Hence, *Oprm1* activation by morphine modifies the levels of miR-212. Moreover, the changes in the levels of its expression were also observed in the primiR-212/132 levels. These results suggest that morphine is not only preventing the degradation of the miRNA but effectively inducing its expression. However, the regulation of the expression and maturation of these miRNAs may be mediated by any other factors, leading to a differential expression of these two miRNAs during the development, as observed in *oprm1* morphants exposed to 10 μ M morphine. Several authors have already observed similar findings as a result of a post-transcriptional regulation of clustered miRNAs [34]. The localization of the precursor, as seen in the ISH pictures, also correlates with the results measured by qPCR. We have also observed several changes in the shape of the neurons in the primary neuron culture, possibly due to an alteration in the differentiation, as previously described by other authors [14, 35].

An upregulation of *oprm1* levels at 24 hpf has also been obtained after the administration of morphine. This receptor has been shown to induce its own expression in a feedforward mechanism after an acute morphine dose in zebrafish embryos [20]. In contrast, a decrease in the levels of *oprm1* mRNA is observed at 48 hpf, probably due to a prolonged activation of the receptor or to a reduction in the expression exerted on its promoter by some of the known repressors, such as MeCP2 [36] or PU.1 [37]. On the other hand, we have observed an increase at the protein levels after morphine administration. These results suggest that the reduction on miR-212 levels observed after the exposition to morphine is preventing the blockage of the translation. Furthermore, the role of the endogenous opioids in *oprm1* expression has been already studied. These molecules, widely expressed in the brain, are affected by morphine and could also be interacting with the normal activation of *Oprm1* [38]. After knocking down miR-212, we observed an increase in the expression of *oprm1*. Furthermore, the decrease induced by morphine at 48 hpf was reversed and turned into a mild increase. However, the decrease observed after exposing *oprm1* morphants to 10 μ M morphine could be mediated by miR-132

overexpression. The differential expression between miR-212 and miR-132 has already been described, and this upregulation could be mediated by other receptor rather than Oprm1. These results show a novel regulation by the cluster formed between miR-212/132 exerted on *oprm1* translation, preventing the translation of *oprm1* mRNA by targeting the binding site on the 3' UTR. At the same time, this mechanism prevents further overexpression of the levels of *oprm1* after morphine exposure. In summary, these results indicate that morphine, through the activation of Oprm1, induces miR-212 overexpression. This epigenetic factor, along with the other transcriptional regulators, reduces the translation of *oprm1* mRNA, controlling the expression of the receptor at the post-transcriptional level. Furthermore, the regulation could be also mediated by miR-132, and other pathways such as neurotrophins or other opioid receptors.

To study the molecular mechanisms underlying Oprm1 activation, we selectively inhibited several pathways related to opioid activity, such as MAP Kinases, Calmoduline/Calmoduline Kinase II and Protein Kinase A (PKA) (Fig 5). We have demonstrated that the upregulation of miR-212 induced at 24 hpf is mediated by the MAP kinase pathway (through the activation of MEK1/2) using the inhibitor U0126. The overexpression of *oprm1* is also reversed after the addition of U0126. However, we have observed a decrease in the levels of miR-212 and *oprm1* at 48 hpf compared to 24 hpf. This reduction is not fully reverted by U0126, indicating that this response may not only be mediated by the MAP kinase pathway, and hence by the activation of CREB. This transcription factor has been described to induce the expression of miR-212, while the REST protein, binding to RE1 sites, has been demonstrated to reduce the activity of its promoter [39] as well as for *oprm1* [36]. Besides, methyl CpG binding protein 2 (MeCP2) is also involved in the reduction of the expression of this miRNA forming an epigenetic protein complex [15]. Hence, the repression exerted on these two genes is also probably mediated through the activation of MeCP2 by binding to methylated RE1 sites. In addition, our results show that calcium signaling is necessary to induce the upregulation of *oprm1* after morphine administration, since W7 prevented the overexpression of the mRNA.

Furthermore, the induction of the activity of MeCP2 is mediated by calmodulin (CaM) and calmodulin kinase II (CaMKII) [40]. The activation of mu opioid receptor is known to block calcium channels via the alfa subunit Gi (α Gi), modifying the levels of intracellular calcium [41]. CaM induces MAP kinase activation mediated by PKC, RAS and Raf1 activation [42], therefore triggering CREB phosphorylation. In addition, the activation of $\beta\gamma$ subunits induces the release of the intracellular deposits of calcium of the RE mediated by inositol triphosphate (IP₃) through phospholipase C (PLC) activation [43]. In addition, cAMP signaling inhibition has been demonstrated to regulate morphine response [44]. Therefore, while CaM/CaMKII inhibition reduces MeCP2 phosphorylation and activation, it also increases CREB activity and miR-212 expression, as we have shown after using the inhibitor W7. On the other hand, PKA activation mediated by forskolin may be inducing CREB phosphorylation and therefore activating miR-212 and *oprm1* transcription. In fact, the levels of miR-212 are downregulated compared to the control group. This indicates that MAP kinase signaling, activated by other pathways (such as neurotrophins, as described by Numakawa [45] and Remenyi [46] and) is necessary to maintain miR-212 physiological levels. If CaMKII activity is inhibited by W7, we observe a strong increase in the levels of the microRNA. CaMKII phosphorylation of Ser421 of MeCP2 inhibits the expression of this miRNA by blocking transcription through the binding to RE1 sites [15]. The loss of activity in this calcium pathway induces a loss of phosphorylation on MeCP2, and thus reduces its activity. On the other hand, this epigenetic inhibition does not control mu opioid receptor expression, as CaM/CaMKII inhibition does not involve any significant change. $\beta\gamma$ subunits activation of PLC and posterior IP₃ release induces the liberation of intracellular calcium stored in the endoplasmatic reticulum, and the activation of CaMKIV

Fig 6. Proposed mechanism for the relationship between the mu opioid receptor (Oprm1), activated by morphine, and the cluster miR-212/132. The responses observed at 24 hpf involve different steps: 1: inhibition of adenylyl cyclase mediated by α Gi. 2: α Gi subunit closes calcium channels 3: PKC activation induces MAP kinase pathway, phosphorylating CREB. 4: $\beta\gamma$ subunits induce calcium release of endoplasmic reticulum. The downregulation observed at 48 hpf involves two steps: 5: changes in the methylation patterns on miR-212 and *oprm1* promoters induce MeCP2 repression, blocking transcription, 6: PKA overactivation. CREB phosphorylation induces miR-212 and *oprm1* expression. In addition, miR-212 targets *oprm1* 3'UTR and prevents its translation, controlling the levels of the receptor. At 48 hpf, changes in the methylation pattern and the joining of MeCP2 to the promoters of miR-212/132 and *oprm1* prevents CREB action, downregulating both genes.

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[47]. This kinase induces the activation of MAP kinase pathway [48], and the posterior phosphorylation of CREB, resulting in an overexpression of miR-212 and *oprm1*. In addition, miR-212 targets *oprm1* mRNA blocking the translation mediated by RISC complex [49], preventing further overexpression of the receptor. This mechanism serves as a feedback control of mu opioid receptor expression: morphine induces miR-212 and *oprm1* transcription, increasing their levels. This miRNA, on the other hand, targets *oprm1* mRNA and reduces the levels of the protein.

Furthermore, the changes in the methylation pattern of miR-212 and *oprm1* have been already studied [34, 35]. MeCP2 is actively repressing their promotor binding to RE1 sites and preventing the overexpression observed after morphine administration. Since the inhibition of CaM/CaMKII induces an increase of the expression of these two genes, our results indicate that MeCP2 phosphorylation on Ser421 [40] is necessary for the activity of this epigenetic factor. PKA activation by forskolin partially rescues the normal expression of these molecules. CREB phosphorylation mediated by PKA could provoke an induction of the expression of miR-212 and *oprm1*, but as MeCP2 is still activated and bound to the promoter, the transcription is partially blocked, as has been described in the regulation of other genes of many cellular processes [50].

It is clear, taking the above into consideration, that there is a relationship between the activity of the mu opioid receptor and miR-212. We propose the following mechanism after morphine administration (Fig 6): the activation of Oprm1 (light green) induces several intracellular changes; 1: activation of α Gi subunit (dark green) inhibits adenylyl cyclase (AC, red), interrupting the production of cAMP and preventing the activity of Protein Kinase A (PKA, yellow); 2: In addition, α Gi subunit closes calcium channels (CC, light blue); 3: Oprm1 activation of Protein Kinase C (PKC, green) induces the activation of RAS and Raf1, which activate MAP Kinase pathway (MEK, ERK and MNK1/2, orange), inducing the phosphorylation of CREB (orange); 4: βγ subunits induce the release of IP₃, and the posterior release of intracellular calcium, stored at the endoplasmatic reticulum (ER). In contrast, the downregulation observed in all the experimental groups at 48 hpf seems to be mediated by several factors, but not by Oprm1 activation. Moreover, the expression of miR-212 and *oprm1* is clearly reduced, indicating that the mechanism undergoing after morphine administration must be different. The regulation mechanism involved in the responses observed is mediated by two processes; 5: the changes on the methylation pattern in miR-212 and *oprm1*, probably in RE1 sites, inducing the repression of the transcription mediated by MeCP2 and 6: cAMP overproduction after prolonged morphine administration induces PKA activation [39].

These mechanisms present novel insights into the signaling cascade triggered by the activation of Oprm1. These results, together with receptor endocytosis studies, may explain the first steps of morphine activity in the CNS.

Author Contributions

Conceived and designed the experiments: RER. Performed the experiments: AG AJ. Analyzed the data: AG AJ. Contributed reagents/materials/analysis tools: RER. Wrote the paper: AG RER.

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Review Article

miRNAs Mediated Regulation of Neuronal Proliferation and Differentiation during Zebrafish Development

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Abstract

Morphine is one of the first-line therapies used in pain treatment, although its side effects and abuse as recreational drug make its use controversial. This opioid is involved in several biological processes and has remarkable effects in Central Nervous System (CNS) development. In this sense, miRNAs have been postulated as possible targets to control the molecular pathways triggered by exposure to morphine. Previous research has shown that in zebrafish embryos morphine alters the dopaminergic pathway-related genes *th*, *dat* and *pitx3*, as occurs with other drugs of abuse such as cocaine. These changes observed lead to a failure in the correct differentiation of the dopaminergic neurons. The modification of those genes is similar and it is related to the changes induced in the expression of other genes such as *wnt1*. Besides, morphine is also able to alter proliferation by changing the pattern of the proliferative cells around the periventricular area. *mir133b*, *mir212* and *mir132* are involved in these changes induced by morphine during zebrafish CNS development. On the one hand, *mir133b* is strongly related to the dopaminergic system changing the expression of *th* and *dat* through *pitx3* regulation after morphine exposure. On the other hand, *mir212* and *mir132* are altered by morphine administration but they are also involved in *oprm1* and *mecp2* expression by targeting their mRNA sequences. This negative regulation of *Mecp2* induces the over expression of *Bdnf*. This review highlights the importance of assessing morphine effects on development and the key role of miRNAs in this process.

ABBREVIATIONS

CNS: Central Nervous System; hpf: hours post fertilization; miRNA: microRNA; Mo: morphants; NMDA: N-methyl-D-aspartate; SCI: Spinal Cord Injury; VTA: Ventral Tegmental Area

INTRODUCTION

The opioid morphine, an alkaloid and the main active compound of *opium*, can be obtained from the seed of the poppy plant, *Papaver Somniferum*. This drug has been used for centuries as a medical and recreational agent [1] although its use is known to cause undesirable side effects on peripheral tissues and on the central nervous system (CNS). Moreover, morphine has been related to several alterations on the normal development of the CNS. These findings have special relevance in relation to pregnant women, in which the administration of this drug could induce the modification in the pattern of expression of several genes,

which at the same point could cause alterations in the brain structure of the fetus or possibly later neurobehavioral problems [2]. In addition, morphine administration has been related to alterations in cell proliferation, which is of great relevance in oncology patients, in which morphine could induce division of tumor cells [3,4].

Morphine, as other opioid drugs, mainly exert its action by the activation of the μ opioid receptor (*Oprm1*) [5-8], inducing a molecular cascade that involves several processes, such as cell proliferation, apoptosis, alteration in gene expression, epigenetic regulation and neuronal differentiation [9,10].

The zebrafish (*Danio rerio*) is used as an experimental model, to study genetics and development and also to study disease-related pathways, given its easy *in vivo* manipulation [11,12]. In contrast to mammalian embryos, which develop in the uterus

and are influenced by the maternal biochemical processes, zebrafish embryos develop externally and are protected by a transparent chorion, avoiding the maternal effect on these embryos and allowing observation of possible morphological alterations. This is essential when dealing with drug exposure, as the effects observed in mammalian embryos might be due to the susceptibility of the mother and not the embryo *per se*. In this sense, the study of the effects of the drugs using zebrafish is rapidly growing [13,14]. In relation to opioids, the endogenous opioid system in the zebrafish has been characterized including a mu opioid receptor (*Oprm1*), two delta duplicates (*Oprd1* and *Oprd2*), a kappa opioid receptor (*Oprk*) and an opioid receptor like (*Oprdl*) gene [15-18]. Hence, the extensive characterization of opioid receptors in zebrafish and the characteristics of this model allow us to extrapolate key components of the opioid system in the zebrafish to other biological organisms. Besides, zebrafish is an excellent model to study early differentiation, since the transparent embryos and the fast development allow the observation of proliferative and apoptotic cells. Moreover, this model is extremely efficient for the study of the response to chemicals during the early developmental stages [19,20].

Morphine effects on these processes are mainly exerted by the action of CREB, a transcription factor closely related to mitotic regulation and the expression of genes involved in differentiation, such as *nurr1*. Morphine administration and withdrawal have been related to the alteration of other pathways, such as modifications in serotonin levels after the exposure to this drug [21].

miRNAs are a group of 19-25 nucleotides non coding RNAs, which post-transcriptionally regulate gene expression [22,23]. miRNAs are evolutionary well conserved and affect 60% of mammalian genes, becoming a central topic of research [24-27]. In mammals, the binding between miRNAs and their targets is inducing the blockage of mRNA translation [28]. In addition, the perfect complementarity between both sequences produces mRNA degradation [29]. Besides this type of binding, a seed region, which represents 2-8 nucleotides from the 5' end of the miRNA, needs perfect complementarity with the target to, at least, block translation. Several miRNAs have seed regions which bind to several mRNAs [30]. This fact means that a single miRNA can control the expression of hundreds of genes.

Current studies concerning miRNAs involve several biological functions such as cellular differentiation, development, metabolism pathways and disease biogenesis [31-33]. It has been described in zebrafish that miRNAs exert a key regulation through several developmental stages [34-36].

Here, we summarize some of the alterations that morphine, as many other addictive drugs, causes in the development and differentiation of the zebrafish CNS. These changes involve several pathways, but many of these effects are mediated by the alteration of the levels of several miRNAs, which have important transcription factors as targets.

Morphine effects in zebrafish development and differentiation

Morphine alters dopaminergic differentiation by modifying the levels of expression of several transcription

factors: Drugs of abuse have been related to alterations in the levels of biogenic amines [37]. In particular, morphine increases dopaminergic neurotransmission in several brain regions, such as the ventral tegmental area (VTA) and in the *nucleus accumbens*. These alterations in the dopaminergic pathway are directly linked to the addictive properties of morphine [38], and similar systems have been described in zebrafish [39]. A specific subset of dopaminergic neurons (A11), the far projecting neurons in this teleost, is located in the ventral diencephalic and posterior tuberculum and expresses specifically the transcription factor *Otp* [40]. The alterations observed in this area after morphine administrations are similar to those observed with other social used drugs as cocaine. Cocaine has been described to alter the expression of several genes involved in dopaminergic differentiation, thus modifying the normal development of this system [41]. This process is mediated by the alteration of the expression of several transcription factors involved in early development, such as *Ndr2*, *Otpa/Otpb*, and *lmx1b1/lmx1b2*. *Ndr2* is known to be a positive regulator of *Otp* duplicates, and its absence induces a complete lack of dopaminergic neurons from the preteectum to the posterior tuberculum [42]. Moreover, *Otpa* and *Otpb* are required for the correct development of dopaminergic neurons [43].

The exposure of zebrafish embryos to cocaine [41] from 5 to 24 hours post fertilization (hpf) increased the expression of both *lmx1b1* and *nurr1*, upregulating the expression of tyrosine hydroxylase (*th*) which results in an increased dopaminergic differentiation at this stage. However, if the embryos were exposed to morphine until 48 hpf, a downregulation of *th* was observed, probably due to a decrease of *ndr2* and *optb*. Cocaine exposure also modified the expression of the dopamine receptors of the zebrafish [44]. This alteration of dopaminergic differentiation was described to be mediated by the transcription factor *pitx3*. This factor is a positive regulator of *th*, the dopamine transporter (*dat*) and the dopamine receptors (mainly *drd2*) [45].

This alteration in dopaminergic differentiation observed after cocaine administration was also present after morphine exposure [46]. Morphine, via activation of mu opioid receptor (*Oprm1*) regulates several signaling pathways. In particular, the mitogen-activated protein kinases (MAPK) are directly involved in dopaminergic differentiation, as observed in 24 hpf zebrafish embryos. These results showed that after morphine administration, the expression levels of *th* were downregulated, and the levels of *dat* and *pitx3* upregulated. These results were reversed after the inhibition of the pathway, showing that the activation of MAPK was necessary to the correct differentiation of these cells. These observations correlate with previous findings that relate MAPK and *Oprm1* activity with TH activation [47].

Morphine alters other differentiation pathways through several genes: In addition to altering dopaminergic differentiation in the early stages of the development, morphine is known to modify the levels of expression of other transcription factors, such as *wnt1* [48]. This factor plays an important role in the development of the central nervous system, regulating neurogenesis and neuronal differentiation. Besides, it is important in adult neuronal plasticity [49]. Moreover, *Wnt1* has been proven to be involved in the differentiation of the dopaminergic neurons

[50], thus it may be related with the other alterations previously described [48]. Morphine administration downregulates *wnt1* expression in the early stages of development (8 hpf) but induces an upregulation at the later stages (16, 24, 48 and 72 hpf). However, those changes are not induced by other important mitogenic factors such as Shh [48].

In addition to these changes, a microarray study [51] showed a differential regulation of several genes in other pathways in zebrafish embryos treated with morphine up to 24 hpf. *Copb2*, a gene involved in dopaminergic receptor D1 transport [52], was shown to be dysregulated. Moreover, several other genes as *dao.1*, involved in glutamatergic activity and addiction [53] was also altered. Another gene down-regulated by exposure to morphine and identified as a gene related to *oprm1* expression is *wls*, a putative orphan G-protein coupled receptor conserved from worms to human [54]. Wls is closely related to development, as it can inhibit the secretion of Wnt, indicating that this factor could be critical in neuronal development and morphogenesis [55].

Morphine alters cell proliferation and apoptosis: BNIP3, a protein related to cell death/survival [56] has been described to be altered in zebrafish embryos exposed to morphine [51]. The alteration of the normal expression of this gene indicates that morphine in zebrafish embryos may produce an unfavorable pro-apoptotic state of the neuronal cells. In zebrafish, morphine upregulates Bdnf and TrkB at 48 hpf [10]. Also, the proliferation

pattern of the cells around the periventricular area is altered in morphine exposed embryos (Figure 1). This mechanism shows that morphine is closely related to proliferation, as described by many other authors [57,58].

In addition to the changes observed after morphine administration, *Oprm1* has an important activity in cell proliferation [59]. The knock-down of the receptor altered the normal proliferation pattern around the periventricular area, in a similar fashion than that observed after morphine administration [10]. These results indicate that the endogenous opioid system is related to normal proliferation. In addition, morphine administration enhances cell proliferation at several cell populations at 24 and 48 hpf, and act as a neuro-protector against glutamatergic excitotoxicity [60]. However, other authors have described a differential regulation of cell proliferation depending on the dose used [61]. Low doses of morphine promote cell proliferation in undifferentiated SH-SY5Y cells, while higher doses inhibited proliferation [62].

miRNAS roles in zebrafish development after morphine treatment

mir133b role during development: The characterization of *mir133* was first done in mice [63], after which its homolog's were discovered in several other species. Three different miR-133 sequences are known: *mir133a-1*, *mir133a-2*, and *mir133b*.

Figure 1 Morphine effects on cell division and apoptosis after morphine exposure and knocking down *mir212* and *mir132*. The mitotic marker phospho-histone 3 (H3-P) (red) and TUNEL technique (green) were used to determine whether morphine treatment (10 nM) alters proliferation and apoptosis in the CNS. Changes in the pattern of proliferation (Arrows) were observed between control (A: 1-3) and treated embryos (A: 4-6) at 48 hpf and *mir212* and *mir132* morphants (Enlarged in 1'-6'). Mitotic marker and TUNEL positive cells (Enlarged in 1''-6'') were quantified around the peri ventricular area and the midbrain-hindbrain boundary (B-C) (n=3). Cells nuclei were stained with DAPI (blue). Embryos are oriented anterior toward the left and posterior toward the right (A'). Mb: Midbrain. Hb: Hindbrain. Mo: Morphant. Unt: Untreated embryos. Treated: 10 nM morphine treated embryos. Taken from Jimenez-Gonzalez et al., (BBA General Subjects, 2016).

Table 1: *mir133b* involvement in several diseases and biological processes.

Disease	Mechanism	Role	Model	Reference
Parkinson disease	Axonal degeneration	Regulating RhoA/Decreasing α -synuclein mRNA levels	Rat dopaminergic neuron primary culture/ PC12 cells	[62]
Renal carcinoma	Apoptosis/Cell viability	Targeting JAK2/STAT3	Human renal carcinoma cell lines	[63]
Gastric cancer	Warburg effect	Targeting PTBP1	Human cell lines	[64]
Glioblastoma	Migration	Targeting matrix metalloproteinase 14	Human glioma cell lines	[65]
Diabetic nephropathy	---	Biomarker	Human	[66]
Glioma	Proliferation	Targeting Sirt1	Human Glioma cells	[67]
Acute Myocardial Infarction	---	Biomarker	Human	[68]
HIV-associated dementia	Apoptosis	Targeting Hsp70	Rats	[69]
Tumor proliferation	Mitosis	Targeting NUP124	Cell lines	[70]

In particular, *mir133b* plays an important role in several diseases and biological processes, summarized in table (1). This miRNA has an effect on zebrafish spinal cord regeneration [73] since one of its multiple targets is RhoA [74]. This protein increases after spinal cord injury (SCI) [64] therefore the inhibition of RhoA exerted by *mir133b* enhances the re-growth of the corticospinal tract after SCI. In cancer, *mir133b* can participate in promoting or suppressing tumors. When overexpressed, *mir133b* can behave as an oncogene, inducing tumor cell proliferation [69], or when under-expressed, it functions as a tumor suppressor, negatively regulating oncogenes [65].

Additionally, *mir133b* regulates the differentiation, maturation, and function of dopaminergic neurons by downregulating the homeobox gene *pitx3* [75] which is related to CNS development. In particular, at 24 h post fertilization, the dopaminergic system begins its differentiation and the first TH-positive neurons are detected [76].

Morphine modulates the expression of *mir133b* and dopaminergic markers through *Oprm1* during zebrafish CNS development: The analysis of a microarray carried out in zebrafish embryos after morphine administration revealed a decrease in the expression of several miRNAs at three developmental stages: 16,24,and 48hpf. *mir133b* was chosen due to its reported effect on dopaminergic neurons, an essential component in drug addiction processes and CNS development [75]. The qPCR validation of *mir133b* showed its levels were decreased in 24hpf embryos exposed to 10nM and 1 nM morphine. The opioid antagonist naloxone did not significantly change the expression of this miRNA, but it blocked the effect of morphine, proving that the changes in *mir133b* were mediated by morphine binding to the opioid receptors [46]. The transcription factor *Pitx3* is a well-known *mir133b* target. *Pitx3* regulates the transcription of the dopaminergic markers *Th* and *Dat*. Since miRNAs normally regulate the stability or the translation of their targets, morphine exposure should increase the expression levels of these transcripts by reducing *mir133b*.

qPCR studies proved that the RNA levels of *pitx3*, *th* and *dat* increased after morphine exposure in 24hpf zebrafish embryos while the treatment with naloxone effectively abolished the morphine induced changes in the expression levels of *pitx3*, *th*, and *dat*, suggesting that morphine regulates the level of the

dopaminergic genes via the control of *mir133b*.

In order to establish the role of *Oprm1* in regulating *mir133b*, *oprm1* was knocked-down by morpho lino oligonucleotide injection. The amount of *mir133b* increases with in embryos injected with *oprm1* morpholino (morphants). Furthermore, 1or10 nM morphine exposure did not alter the *mir133b* level in *oprm1* morphants while the same concentrations of morphine treatment resulted in a decrease of *mir133b* levels in embryos injected with control morpholino. The increased expression in *mir133b* detected in the *oprm1* knock-down embryos also led to a decrease of the subsequent *mir133b* targets, i.e., *pitx3*, *th*, and *dat*. These results clearly indicate that *Oprm1* is the mediator for the morphine-induced regulation of *mir133b* and its targets [46].

mir212/132 cluster role in the molecular cascades triggered after morphine administration: *mir212* and *mir132*, both codified in the same locus, located on chromosome 10 in rat and 11 of mouse [78,79]. In humans they also share a primary transcript encoded by a locus on chromosome 17 [80]. In addition, *mir212* and *mir132* share the seed region although they have specific targets and are regulated by CREB and REST transcription factors, so most of their functions are strongly related to the neural compartment. In fibroblast culture it has been proved that *mir132* increases in the presence of negative form of REST [81]. Similarly, CREB was firstly identified as regulator of this locus in cortical neurons of rats stimulated with neurotrophins [79].

Besides, *mir132* induces neurite out growth and modulates the dendritic morphology of immature neurons in the hippocampus and cortex after the inhibition of one of its targets, P250GAP. This target (a brain enriched GTPase-activating protein) is important in neuronal development as it controls N-methyl-D-aspartate (NMDA) receptor signaling [82]. It also modifies the dendritic plasticity by controlling *MeCP2* expression [83] and this protein is fundamental for the correct neural maturation [84]. These results show the relevance of this locus in neural morphogenesis.

To determine the relevance of *mir212* and *mir132* during zebrafish development, their temporal expression was studied at 5, 8, 16, 24 and 48hpf. Although both are highly expressed, *mir212* expression decreases during development while *mir132* increases from 5 to16 hpf. Besides, the number of copies for *mir132* is greatly increased at 48hpf when compared to *mir212*,

suggesting the importance of this miRNA at this particular stage [7]. The levels of expression of *mir212* in zebrafish embryos were measured by qPCR after the administration of two different doses of morphine (10nM and 10μM). These morphine doses have been also antagonized with naloxone, obtaining a clear reversal of the opioid effects [60]. At 24 hpf, an increase in the levels of the miRNA was observed for both concentrations of morphine, although it was higher at 10μM. In contrast, *mir212* was down-regulated at 48 hpf. Additionally, the implication of mu opioid receptor in *mir212* expression was analyzed. After knocking-down the mu opioid receptor, the levels of *mir212* were back to normal after morphine administration at 24 hpf. However, *oprm1* morpholino did not revert the effects of morphine at 48 hpf. Also, the levels of *mir212* were not modified in the control conditions in *oprm1* morphants pointing to the relevance of *oprm1* for morphine effects on *mir212* expression but not in its physiological levels. As *mir212* expression is affected by morphine administration, it was analyzed if this miRNA was regulating mu opioid receptor. After morphine treatment, mu opioid receptor was strongly up-regulated at 24 hpf. In contrast, morphine administration induced a decrease in the levels of *oprm1* at 48 hpf. However, the knock-down of *mir212* induced an increase in the expression of the receptor, much higher than the observed when *mir212* was present at both 24 and 48 hpf [7]. 10nM morphine exposure increased the expression of *mir132* at 48 hpf. The incubation with opioid antagonist, naloxone, induced the opposite effect exerted by morphine exposure, whereas both, morphine and naloxone treatment, did not change the levels of 132 [10]. These results show that morphine is changing both *mir212* and *mir132* by the specific activation of *Oprr1*.

miR-212/132 cluster regulates *oprm1* mRNA expression binding to its 3'UTR: In order to determine whether *mir212/132* cluster was effectively regulating *oprm1* by targeting its mRNA, a bioinformatic analysis of putative binding sites of μ opioid receptor was performed [85]. A possible binding site form iR-212/132 was observed in the 3'UTR of *oprm1*, as well as an additional site in the second exon of the mRNA. By means of aluciferase assay it was confirmed that the binding site in *oprm1* 3'UTR was actively repressing μ opioid receptor. miRNA mimics (small, chemically modified double-stranded RNAs that mimic endogenous miRNAs by the up-regulation of miRNA activity) were co-transfected with the plasmids inducing a significant decrease in the luminescence on the non-mutated group. Moreover, the increase in the concentration of miRNA mimics reduced the luciferase activity, but only when co-transfected with the wild type plasmid. These results prove that *mir212* and *mir132* are binding to the 3'UTR region of *oprm1* mRNA, and physiologically repressing *oprm1* expression [7].

It has been described that *mir212* levels are modified after cocaine administration in the hippocampal region of adult rat brains [86]. These results suggest that the addictive properties of cocaine and morphine, and probably other drugs, could be controlled by this miRNA. In addition, cocaine has also been related to the alteration of the levels of expression of *mir-let7d* in zebrafish embryos [87] and with the regulation of *oprm1* mRNA expression in mice [88].

***mir212* expression is regulated by MAPK, calmoduline and PKA:** To analyze in detail the signaling cascade triggered by the activation of *Oprr1*, the levels of both *mir212* and *oprm1* were studied after the co-administration of morphine and inhibitors of MEK1/2 and calmoduline or an activator of PKA pathway [7]. *mir212* quantification revealed the relevance of MEK1/2 on its expression after morphine treatment at 24 hpf. CaM/CaMKII and PKA also showed an effect on *mir212* expression at 24 hpf but not 48 hpf. *oprm1* did not change at 24 hpf while its expression decreased in all the experimental groups at 48 hpf. These results point to the great relevance of the developmental stage analyzed and the importance of MEK1/2 and the balanced effect between CaMKII and PKA on the expression of *mir212* at the earlier stages.

Bdnf and TrkB expression analysis after morphine exposure and in miRNAs morphant embryos: The modification induced by morphine in the localization of mitotic cells at 48 hpf points to a possible role of neurotrophins in morphine effects (Figure 1) [10]. It has also been observed that miR-132 and miR-212 are regulating the pattern of expression of proliferating cells around the periventricular area in 48 hpf zebrafish embryos. As miR-212/132 cluster has been previously related to Bdnf pathway [80], Bdnf and TrkB expressions were studied in *mir212* and *mir132* morphants. In addition, *oprm1* morphants were also analyzed to assess if *Oprr1* is one of the possible effectors in the changes induced by morphine in the expression of Bdnf. In all groups, a significant decrease of Bdnf expression was found. When TrkB was analyzed, the levels in control embryos were higher after knocking down *mir212*, where as morphine treatment induced a decrease in the levels of this protein in those morphants. No significant changes could be observed in the expression levels of TrkB in *mir132* or *oprm1* morphants.

Bdnf expression is inhibited by *MeCP2*, which was firstly identified as an epigenetic factor which binds to methylated DNA, avoiding its transcription [86] and bioinformatic predictions showed a target region for both miRNAs in the third exon of the *mecp2* sequence. In this sense, a luciferase assay confirmed the regulation of *mir212* and *mir132* on *mecp2* gene expression.

DISCUSSION AND CONCLUSION

The use of morphine is known to cause undesirable effects, which includes changes in cell proliferation (modification in the number of dividing and apoptotic cells) and in the differentiation of several neuronal populations. In the last years, zebrafish has been proven to be an excellent model to study these processes triggered after morphine administration.

Morphine, as well as other drugs of abuse, is known to induce alterations in the dopaminergic and serotonergic positive cells. These changes are mediated by several transcription factors such as CREB, *nurr1* or *pitx3*, which are also involved in the responses after cocaine intake. These modifications in the normal development of those specific neuronal groups observed in zebrafish embryos are likely related to the appearance of the addictive symptoms.

Moreover, miRNA alterations observed after drug exposure also interfere with the normal physiological development, through the modification of the levels of expression of their targets. *mir133b* has been related to alterations in zebrafish

development through the transcription factors *pitx3* and *wnt1*. Besides, *mir212* and *mi132* are involved on the expression of neurotrophins such as Bdnf and TrkB through *Mecp2* expression, and can also modify the number of proliferative cells around the periventricular area of the zebrafish hindbrain. Hence *mir133b*, *mir212* and *mir132* are novel regulators of morphine effects during two relevant stages of zebrafish development (24 and 48 hpf). These findings have also established a relationship between these miRNAs and several systems with key roles for CNS formation such as opioid, dopaminergic, neurotrophins and several other regulatory pathways.

Here we have discussed the most relevant contributions highlighting the importance of miRNAs in cell proliferation and differentiation using zebrafish as a research model. These processes are involved in morphine response and may be related to tolerance and the appearance of addiction. Thus, the better understanding of these mechanisms could lead to the design of new drugs lacking the drawbacks that the chronic use of morphine induces.

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ANNEX III

Other Publications of the candidate

Chiarlone A, Börner C, Martín-Gómez L, Jiménez-González A, García-Concejo A, García-Bermejo ML, Lorente M, Blázquez C, García-Taboada E, de Haro A, Martella E, Höllt V, Rodríguez R, Galve-Roperh I, Kraus J, Guzmán M. MicroRNA let-7d is a target of cannabinoid CB1 receptor and controls cannabinoid signaling. *Neuropharmacology*. 2016 Sep;108:345-52. doi: 10.1016/j.neuropharm.2016.05.007. Epub 2016 May 11.

Jimenez-Gonzalez A, García-Concejo A, López-Benito S, Gonzalez-Nunez V, Arévalo JC, Rodriguez RE. Role of morphine, miR-212/132 and mu opioid receptor in the regulation of Bdnf in zebrafish embryos. *Biochim Biophys Acta*. 2016 Jun;1860(6):1308-16. doi: 10.1016/j.bbagen.2016.03.001. Epub 2016 Mar 4.