INSTITUTO DE NEUROCIENCIAS CASTILLA Y LEÓN

Laboratorio de Trastornos Audiomotores

VNiVERSiDAD D SALAMANCA

Efectos de la administración de un inhibidor de la recaptación de serotonina, Sertralina, sobre los cambios inducidos por el estrés prenatal

Tesis Doctoral

INÊS PEREIRA de FIGUEIREDO

Salamanca, 2016

INSTITUTO DE NEUROCIENCIAS CASTILLA Y LEÓN

Laboratorio de Trastornos Audiomotores

Los abajo firmantes, Drs. Dña. Mª Dolores E. López García, Dña. Consuelo Sancho Sánchez y D. Orlando Castellano Benítez, miembros del Instituto de Neurociencias de Castilla y León

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Que el presente trabajo titulado **"Efectos de la administración de un inhibidor de la recaptación de serotonina, Sertralina, sobre los cambios inducidos por el estrés prenatal"**, ha sido realizado bajo su dirección por Dña. Inês Figueiredo y reúne las condiciones necesarias de calidad y rigor científico para su exposición pública y defensa con el fin de optar al título de Doctor por la Universidad de Salamanca.

En Salamanca, a 19 de Febrero de 2016

Fdo:
Dra. Mª Dolores E. López

Fdo:
Dra. Consuelo Sancho Sánchez

Fdo:
Dr. Orlando Castellano Benítez

Para la planificación, realización y redacción de este trabajo de investigación hemos obtenido financiación de diversas instituciones, sin cuya ayuda no hubiera sido posible: MCINN (BFU2007-65210 y BFU2010-17754) y Junta de Castilla y León (GR221).

AGRADECIMIENTOS

¿Qué se puede decir para agradecer el trabajo y el apoyo de tantas y tantas personas que han hecho posible la culminación de una tesis de doctorado, sin las cuales esta empresa no hubiera sido posible? Quiero empezar dedicando unas palabras de gratitud para mis directores, la Dra. Mª Dolores E. López García, Lita para los amigos, Dra. Consuelo Sancho y al Dr. Orlando Castellano. Todos han sido el principal artífice y motor de mi carrera profesional; y no sólo eso, también me ha brindado un apoyo personal impagable. Me siento profundamente en deuda con todos y simplemente decir que algún día me gustaría corresponderles. A Lita, quiero darle un agradecimiento especial, ya que sin su apoyo e incentivo esta tesis no hubiera sido posible. No sólo por la concepción del proyecto, sino por la paciencia, orientación, amistad y por estar siempre presente durante estos largos años de concretización. Quisiera agradecerle su constante preocupación humana conmigo y mi familia.

Esta tesis ha coexistido con los momentos más especiales que alguien puede querer en su vida, el nacimiento de sus hijos, en mi caso, mis cuatro hijos, Joana, Francisco, Rodrigo y María. También ellos (aunque sin saberlo) me han motivado a seguir con este proyecto, a buscar e investigar más y mejor.

En segundo lugar, también me gustaría agradecer al Dr. Luis Muñoz las metodologías que he aprendido a lo largo de estos años, y al Dr. Juan Carro, mi "psicólogo de cabecera".

El laboratorio ha sido una fuente de motivación que sin duda me ha ayudado a crecer profesionalmente y a madurar. Quiero hacer una mención especial hacia Richard y Adela, cuyos consejos y soporte han sido fundamentales estos años. No me quiero olvidar de Sebastián, David y tantos amigos, por haber estado presentes durante la realización de los experimentos, compartiendo experiencias.

Agradezco el apoyo técnico y personal que he recibido del INCYL y de sus integrantes, especialmente del Dr. Miguel Merchán y de Nacho Plaza. No me quiero olvidar de las Dras. Luisa Mateus, Maria João Fradinho y Graça Ferreira Dias, de la

Facultad de Medicina Veterinaria de Lisboa, por haberme ayudado a poner a punto mis experimentos de bioquímica.

Por último y no menos importantes quiero también agradecer a mi familia, de forma particular a mi abuela “Avó Zé”, por su sabiduría y palabras de ánimo; a José, por todo su apoyo y por cuidar de los niños cuando estaba haciendo experimentos y, de una forma especial, a mi Madre, por su paciencia infinita.

“No existe un camino para la felicidad.

La felicidad es el camino.”

Mahatma Ghandi

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ABREVIATURAS

5-HT- 5-hidroxi triptamina (Serotonina)
AAE- actividad locomotora antes del inicio de cada ensayo
ACTH- hormona adrenocorticotropa
AVP- arginina vasopresina
BLA- núcleo basolateral de la amígdala
BNST- núcleo del lecho de la estría terminal
CA- campo abierto
CER- respuesta emocional condicionada
CNS- sistema nervioso central
CRH - hormona liberadora de corticotropina
DEF- postura defensiva o de defensa
EA- evitación activa
EC- estímulo condicionado
eHHA- eje hipotálamo-hipófisis
EI- estímulo incondicionado
eSA- eje simpato-adrenomedular
GC(s)- glucocorticoide o glucocorticoides
Glu- glutamato
GR (s)- receptor de glucocorticoides o receptores de glucocorticoides
GOT- glutamato-oxalacetato transaminasa
GPT- glutamato-piruvato transaminasa
IA - exploración interna (*inner crossing activity*)
IA/OA- cociente de la exploración interna/exploración externa
IEE – actividad locomotora entre ensayos
IMO- inmovilización
i.p.- intraperitoneal
ISRS(s)- inhibidor o inhibidores selectivos de la recaptación de 5-HT
IS- choques inescapables (*inescapable footshocks*)
LDH- lactato deshidrogenasa
LC- *locus coeruleus*
LH- desamparo aprendido *(Learned helplessness)*
LTC- alteración a largo plazo (*long term change*)
MC(s)- mineralocorticoide o mineralocorticoides
MCH - hemoglobina corpuscular media
mPFC- área medial de la corteza prefrontal
MPV - volumen plaquetario medio
MR (s)- receptor de mineralocorticoides o receptores de nineralocorticoides
N- número de animales
N. accumbens- núcleo accumbens
NDR- núcleo dorsal del rafe
No-IMO- no sometidos a estrés por inmovilización
No-IS- no sometidos a choques inescapables
NR- núcleos del rafe
n.s.- no significativo
N. tracto Solitario- núcleo del tracto solitario
OA- exploración externa *(outer crossing activity*)
PFC- corteza prefrontal
PPI- inhibición del RAS por un estímulo previo (*pre-pulse inhibition)*
P - día postnatal
PS - estrés prenatal

PVN- neuronas hipotalámicas periventriculares
RAS- reflejo auditivo de sobresalto
RS- estrés por restricción
SERT- Sertralina
SEM - error estándar de la media
SGP- sustancia gris periacueductal
SNA- Sistema Nervioso Autónomo
TEPT- trastorno por estrés postraumático
Veh - vehículo
vPFC - área ventral de la corteza prefrontal
VTA- área tegmental ventral.
W- Vatio

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PREÁMBULO

En las postrimerías del siglo pasado, se constató una vez más que las alteraciones causadas por estrés y ansiedad en la sociedad moderna, se encuentran entre los problemas de salud más comunes. Según la Organización Mundial de Salud (OMS), al menos el 25% de los adultos sufrirá alguna forma de trastornos por ansiedad en algún momento de su vida (Gordon y Hen, 2004; Anacker y cols., 2010), siendo las mujeres más susceptibles a este tipo de trastornos (Olff y cols., 2007).

Dada la importancia y el elevado coste económico y social de dichos trastornos, muchos investigadores han dirigido sus esfuerzos a estudiar los mecanismos desencadenantes de la ansiedad y la depresión que provocan la aparición del estrés, desarrollando modelos animales de esta patología, ya que tanto el hombre como muchos animales responden de forma similar ante situaciones estresantes.

Teniendo en cuenta lo anterior, y en esa línea de pensamiento, nuestro trabajo se orienta a profundizar en los efectos que provoca el estrés, fundamentalmente si aparece en las primeras etapas de la vida, cuando puede modular el impacto psicológico inducido por experiencias aversivas posteriores (Cordero y cols., 2003), jugando un papel muy importante en la etiopatogenia de algunos trastornos comportamentales (Edwards y Burnham, 2001; Maccari y cols., 2003; Van den Hove y cols., 2005; Pittenger y Duman 2008).

En humanos se ha descrito la existencia de una alta incidencia de anomalías comportamentales, y una mayor vulnerabilidad a sufrir afecciones psiquiátricas, en niños nacidos de madres que, durante el embarazo estuvieron sometidas a eventos estresantes, tales como conflictos familiares, conflictos armados o la muerte del esposo (Chen y cols., 2010).

En el momento actual, se conocen bien los efectos a largo plazo de las experiencias aversivas precoces en la repuesta al estrés y a los procesos alostáticos en los humanos. El estrés prenatal afecta al aprendizaje, a la memoria y a la toma de decisiones, siendo responsable directo de la etiología de variadas psicopatologías y un factor de riesgo importante para el desarrollo de trastornos mentales (Pittenger y Duman, 2008; Alleva y Francia, 2009). Sin embargo, se desconoce por qué algunos individuos son más susceptibles a desarrollar enfermedades en respuesta al estrés, mientras que otros parecen ser más resistentes y se recuperan rápidamente. La posibilidad de identificar precozmente los factores de riesgo y cómo revertir los efectos del estrés abriría una variedad de estrategias de intervención que, en última instancia, podría evitar consecuencias patológicas a largo plazo.

La hipótesis de nuestro trabajo se basa en que, tratamientos que actúan sobre los niveles de serotonina, 5-hidroxi triptamina (5-HT), consiguen revertir los efectos del estrés precoz. La 5-HT es un neurotransmisor central que juega un importante papel como estabilizador de la

actividad nerviosa, mediando los efectos del ambiente y específicamente del estrés (Jeffrey Newport y cols., 2001).

Tendremos en cuenta un factor fundamental, el género. Se sabe que los hombres y mujeres presentan diferente respuesta al estrés y a los fármacos, aunque se desconoce la razón de este hecho. Una forma plausible de estudiarlo, evitando los obvios dilemas éticos de la experimentación en humanos, es utilizando un modelo animal como la rata Wistar, en el que tras someter a ratas gestantes al estrés en la última semana de gestación, estudiamos sus crías, machos y hembras, tratadas con Sertralina (SERT).

Por todo lo anterior, pensamos que los resultados del presente trabajo podrán servir para comprender mejor los fundamentos de los tratamientos que actúan sobre los niveles de 5-HT, que modifican las alteraciones comportamentales inducidas por el estrés precoz, evaluando las diferencias entre sexos.

I. INTRODUCCIÓN

1.1. Generalidades sobre estrés

"Estrés" es un término que deriva del latín ***stringere,*** y que significa apretar, atar o provocar tensión. El concepto de estrés es relativamente antiguo, habiendo sido planteado ya desde el siglo XVII, y se utilizó frecuentemente para expresar la privación o las experiencias adversas que padecían las personas (González y Escobar, 2002). Ya en el siglo XVIII, el concepto deja de referirse a las consecuencias emocionales del individuo y pasa a entenderse como el factor desencadenante de tales reacciones. Años más tarde, el fisiólogo Walter Cannon (1935) introdujo por primera vez el término estrés en Medicina. Él propone la respuesta de estrés como la "adaptación del organismo para hacer frente a las emergencias". Ante éstas, el organismo sufriría una reacción inespecífica y generalizada, un estado de alarma, que le permitiría "pelear o huir". Cannon demostró que, con tal activación, el organismo podría hacer frente a la emergencia y recuperar el estado de equilibrio fundamental (Cannon, 1935). Por otro lado, el endocrinólogo Hans Selye (1936) describió las características generales de la respuesta al estrés, definiendo el estrés como un "síndrome general de adaptación", como una respuesta no específica del organismo ante una demanda impuesta, un desequilibrio al que se debe hacer frente (Pacak y cols., 1998).

Actualmente, el término estrés se utiliza para aludir a cualquier condición interna o externa que perturbe el equilibrio dinámico del organismo u homeostasis (Leonard, 2005; Esch y Stefano, 2010). Estas perturbaciones, los estresores, pueden ser cualquier estímulo, externo o interno (físico, químico, acústico, somático, social, etc.), pero puede ser también el resultado de la anticipación mental acerca de lo que puede ocurrir o de algo que ya ocurrió.

1.2. Sustrato neural de la respuesta al estrés

El cerebro es el órgano donde comienza el estrés y el más afectado por este evento (McEwen, 2008); determina qué es estresante y las respuestas fisiológicas y comportamentales a desarrollar frente a cada estresor presentado.

1.2.1. Sistemas de respuesta al estrés

Los aspectos centrales del sistema de respuesta al estrés implican **percepción** (depende de la atención), **procesamiento** (respuestas innatas o dependientes de sucesos previos) y **transducción** de esa información en respuestas neurohormonales, neurobiológicas y comportamentales (que pueden ser o no las adecuadas) (Vermetten y Bremner, 2002).

Para Selye (1986) (in Pacak y cols., 1998), el estrés sería "un estado del organismo manifestado por un síndrome inespecífico", haciendo énfasis en que se produce la misma respuesta tras la exposición a cualquier estresor. Numerosos estudios posteriores han demostrado lo contrario, la existencia de diferentes respuestas neuroendocrinas por exposición a diferentes estresores. Pacak y su equipo presentan una propuesta alternativa de "especificidad primitiva" en la respuesta de estrés - es decir, cada tipo de estrés tendría una "firma" neuroquímica - con mecanismos centrales y periféricos cuantitativos y, quizá, cualitativos distintos (Pacak y cols., 1998). Estas alteraciones ocurrirían, no de forma aislada, sino en concordancia con los cambios fisiológicos, comportamentales y con las experiencias vividas por cada individuo.

Hoy en día, se conoce la existencia de una íntima comunicación entre los sistemas fisiológicos, nervioso, endocrino e inmune (Furay y cols., 2008; McEwen, 2008), con lo cual, la respuesta al estrés origina una serie de modificaciones coordinadas a nivel cardiovascular, metabólico, inmunológico, neuroendocrino, sensorial, motor y cognitivo (Sapolsky y cols., 2000; Cohen y Zohar, 2004; Gordon y Hen, 2004; Samuelsson y cols., 2004; Furay y cols., 2008). Tales modificaciones permiten que el organismo se adapte a condiciones físicas, psicológicas o sociales de naturaleza múltiple. De forma colateral, durante la respuesta del estrés, se inhiben procesos fisiológicos que no son esenciales para la supervivencia a corto plazo, como la digestión, la reproducción y el crecimiento (Sapolsky y cols., 2000; McEwen, 2008), por lo que cuando la respuesta de estrés excede ciertos límites en intensidad y/o duración, las alteraciones inicialmente benéficas, pueden originar, por sí mismas, estados patológicos o exacerbar estados mórbidos latentes o preexistentes (Fumagalli y cols., 2007).

1.2.2 Circuitos implicados

Todas las modalidades sensoriales implicadas en la respuesta al estrés se procesan en el Sistema Nervioso Central a través de dos vías:

- Una vía directa y rápida, cuyas respuestas reflejas y estereotipadas se llevan a cabo en la médula espinal y en el tallo cerebral (respuestas neurovegetativas al estrés) o en el hipotálamo (respuestas neurohumorales).
- Una segunda vía, en la que la información sensorial es dirigida mediante múltiples proyecciones hacia áreas específicas de la corteza cerebral (visual primaria, auditiva, somatosensorial, etc.), que se encargan, por medio de sus conexiones con el sistema límbico de modular la información de la activación del cuerpo y de la planificación conductual. Es precisamente este proceso evaluativo el que le da el carácter adaptativo y plástico al estrés (Sapolsky, 2003).

1.2.3. Estructuras Cerebrales implicadas en la respuesta al estrés

1.2.3.1. En el Sistema Límbico

La actividad del eje hipotálamo-hipófisis (eHHA) es regulada por aferencias del sistema límbico cortical, como la corteza prefrontal (PFC), el hipocampo, la amígdala y los núcleos septales (Sapolsky y cols., 2000; Furay y cols., 2008; McEwen, 2008). Esas regiones del sistema nervioso son las encargadas de llevar a cabo los procesos intermediarios de evaluación cognitiva y emocional entre el estresor y la respuesta del organismo, por lo que todas esas estructuras reciben aferencias sensoriales directas o indirectas, y activan, a su vez, varios de los mecanismos efectores de la respuesta al estrés, como los relacionados con el sistema nervioso autónomo, el sistema motor somático y el eHHA (Sotres-Bayon y cols., 2006; Phelps y LeDoux, 2005).

La PFC corresponde al área anterior no motora del lóbulo frontal. Su área medial (mPFC) está críticamente involucrada en funciones integradoras superiores, tales como el trabajo de memoria, la atención selectiva, el control visceromotor y la toma de decisiones (Vertes, 2006; Gonçalves y cols., 2009). Este área mantiene conexiones con el sistema límbico, con el núcleo dorsomedial del tálamo y con otras zonas corticales, y recibe una densa entrada serotoninérgica, derivada principalmente del núcleo dorsal del rafe (NDR) (Amat y cols., 2005;

Amat y cols., 2008). Sin embargo, la PFC, además de recibir, tiene como característica única (entre las áreas corticales) el hecho de enviar también proyecciones directas a grupos de células colinérgicas y monoaminérgicas en el tronco encefálico, incluyendo el NDR (Vertes, 2006). En su área medial se puede ejercer un control sobre los sistemas monoaminérgicos, mediante proyecciones descendentes de la PFC hacia el NDR (Robbins, 2005; Gonçalves y cols., 2009). Con esto, la mPFC tiene la capacidad de integrar las informaciones sensitivas, externas o internas, y elegir las opciones o las estrategias comportamentales, dando al animal la posibilidad de elegir el comportamiento adaptativo adecuado (Robbins, 2005; Holmes y Wellman, 2009; Maier y Watkins, 2010).

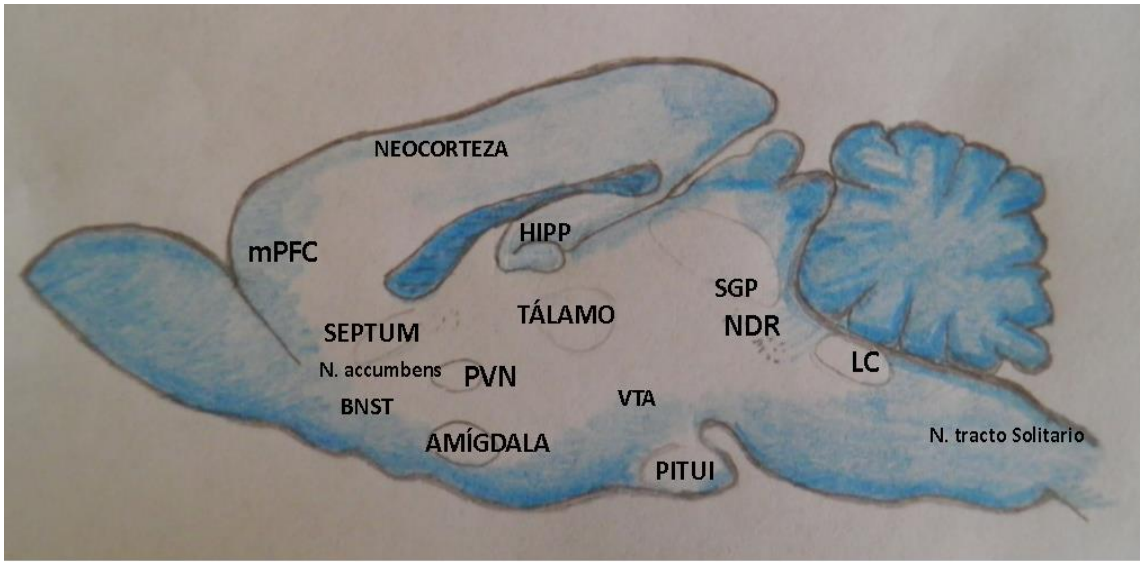


Figura 1: Corte sagital del cerebro de la rata. Se muestran algunas estructuras neurales implicadas en la respuesta de estrés**.** Abreviaturas: BNST: núcleo del lecho de la estría terminal; Hipp: hipocampo; LC: locus coeruleus; mPFC: área medial de la corteza prefrontal; NDR: núcleo dorsal de rafe; N. accumbens: núcleo accumbens; N. tracto Solitario: núcleo del tracto solitario; Pitui: glándula Pituitária; PVN: neuronas hipotalámicas periventriculares; Septum: núcleos septales; SGP: sustancia gris periacueductal; VTA: área tegmental ventral.

También el área septal lateral (**Figura 1**), recibe proyecciones inhibitorias que utilizan la hormona liberadora de corticotropina (CRH) como neurotransmisor, lo cual permite, bajo situaciones de estrés, respuestas conductuales de enfrentamiento al estresor (González y Escobar, 2002).

A su vez, el hipocampo es una estructura llave reguladora de la función de la PFC, funcionando cooperativamente, como sucede en la regulación de la memoria (Swaab y cols., 2005; Pittenger y Duman, 2008). El hipocampo constituye una importante fuente de modulación negativa de la hormona CRH, en el que la región CA3 del giro dentado tiene un papel fundamental en la memoria secuencial (McEwen, 2008) e, interviniendo en algunos aspectos del aprendizaje (Sanders y cols., 2003), juega un papel muy importante en el condicionamiento del miedo.

Sin embargo, la amígdala, otra estructura límbica muy rica en receptores de glucocorticoides (GRs), es la principal estructura en la generación de memorias emocionales (Sapolsky, 2003), estando fuertemente implicada en la persistencia y recurrencia de los recuerdos relacionados con eventos traumáticos (Swaab y cols., 2005; Castro y cols., 2010). La activación de los GRs en la amígdala, potencia la respuesta al estrés (Goldstein y cols., 1996; LeDoux, 1998), lo que sugiere la importancia de esta estructura en la asociación entre el estímulo aversivo y las respuestas (comportamentales, neuroendocrinas y corticales) inherentes al estrés psicológico (González y Escobar, 2002; Sapolsky, 2003; Phelps y LeDoux, 2005).

Otra estructura importante que interviene mediando los efectos del estrés en el estriado ventral es el núcleo accumbens (N. accumbens). El N. accumbens tiene un papel central en los mecanismos de recompensa natural, estando relacionado con los síntomas de anhedonia presentes en pacientes depresivos (Pittenger y Duman, 2008). El estrés activa las proyecciones dopaminérgicas hacia el N. accumbens desde el área tegmental ventral (VTA); esta activación puede contribuir en la mediación de la respuesta homeostática al estrés o en el aprendizaje adaptativo relacionado con el estrés (Nestler y Carlezon, 2006).

1.2.3.2. Estructuras extra-límbicas

En el tronco cerebral, hay estructuras extra-límbicas también importantes en la modulación de la respuesta al estrés, como el locus coeruleus (LC), los núcleos del rafe (NR), la

sustancia gris periacueductal (SGP) y el núcleo del tracto solitario (**Figura 1**), donde se establece la red de conexiones con las otras estructuras cerebrales (Ropper y Brown, 2005; Girotti y cols., 2006).

La SGP es una estructura situada alrededor del cuarto ventrículo, considerada como una región clave del circuito cerebral involucrado en la coordinación de la respuesta defensiva y aversiva ante el miedo y el estrés. Su importancia se debe a su elevada densidad de receptores de aminoácidos excitatorios (glutamato, aspartato) y neurotransmisores como la 5-HT, cuya actividad e interacción es muy importante en la modulación de los comportamientos defensivos (Moraes y cols., 2008).

Ante varios estímulos potencialmente amenazantes, se activan también las neuronas del LC (Valentino y Van Bockstaele, 2008), iniciándose un sistema de retroalimentación positivo entre este núcleo y las regiones prosencefálicas que modulan la síntesis y secreción de CRH, lo que conlleva al incremento en las respuestas conductuales al estresor (González y Escobar, 2002; Swaab y cols., 2005; Maier y Watkins, 2010), reconociéndose su importancia en el mantenimiento del estado de alerta y en los procesos de atención.

A su vez, localizados en la línea media del mesencéfalo, los NR**,** deben su importancia al hecho de que es una región del cerebro que proyecta a todas, o casi todas las regiones mediadoras del comportamiento, liberando 5-HT en alteraciones comportamentales y ante una situación de estrés (Robbins, 2005; Holmes y Wellman, 2009; Maier y Watkins, 2010). Así, por ejemplo, la neuronas serotoninérgicas ubicadas en los NR envían fibras nerviosas a estructuras del sistema límbico y a la SGP, llevando al animal a alejarse de la fuente aversiva (Graeff, 2004). Aunque no se ha descrito todavía la relación de las variadas conexiones con sus diferentes funciones, el mecanismo de retroalimentación desde la PFC hacia el NDR, sugiere, además, un papel fundamental de esta región en el control cognitivo del estrés (Amat y cols., 2008).

1.2.4. Neuroquímica del estrés

Siempre que la homeostasis sea amenazada, el mantenimiento del estado de equilibrio del organismo se realiza mediante el Sistema Nervioso Autónomo (SNA), que actúa a través del

eje eHHA y el eje Simpatoadrenomedular (eSA) (Samuelsson y cols., 2004).

Así, una vez que el organismo se pone en contacto con el estresor, se desencadena la respuesta aguda al estrés (**Figura 2**) (Walker y cols., 2001; de Kloet, 2003; Swaab y cols., 2005; Aisa y cols., 2008; Lai y Huang, 2011), proporcionando al organismo una reacción que permite minimizar sus efectos (*coping strategies*), activando las respuestas periféricas, tales como aumento de la respiración, frecuencia cardiaca y presión arterial, y asignando los recursos fisiológicos para promover el comportamiento defensivo más adecuado (Beck y Luine, 2010; Maier y Watkins, 2010; Raio y Phelps, 2015).

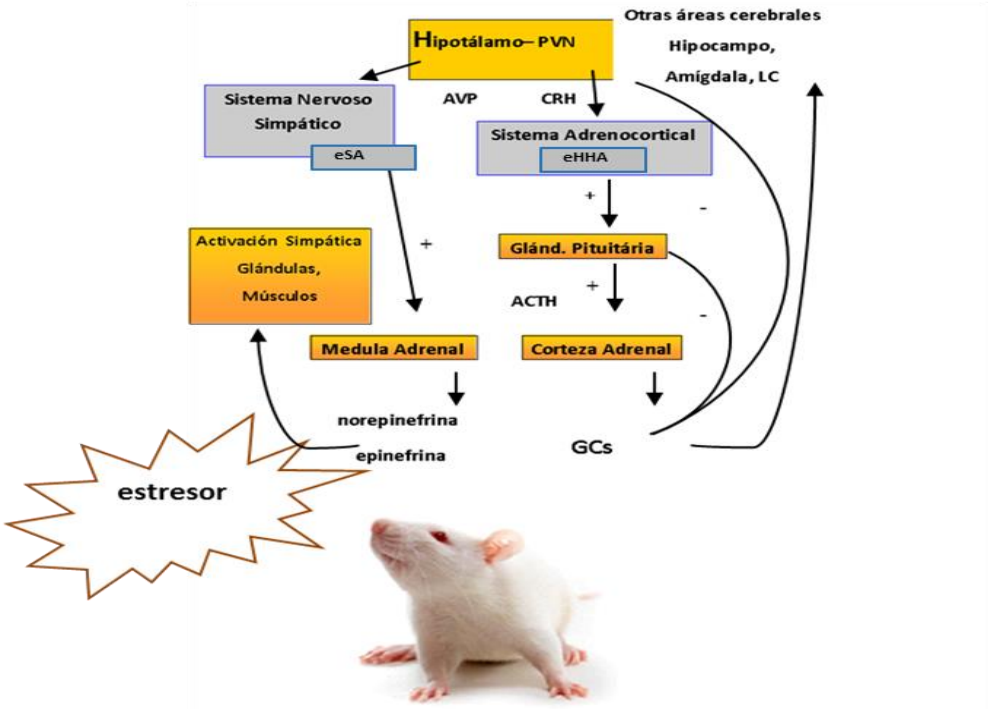


Figura 2: Esquema de la respuesta aguda al estrés. La respuesta por estrés agudo se caracteriza por la excitación de las neuronas hipotalámicas periventriculares (PVN), que estimulan la secreción de la hormona liberadora de corticotropina (**CRH)** y de arginina vasopresina (**AVP**), que, a su vez, estimulan la secreción de ACTH por el lóbulo anterior de la hipófisis. También la **ACTH** estimula la liberación de **corticoesteroides** -glucocorticoides (GCs) y algunos mineralocorticoides (MCs)- por la corteza suprarrenal. También, el eSA se activa y mediante la secreción de catecolaminas (epinefrina y norepinefrina) por la médula adrenal, se induce una respuesta simpática. Finalmente, los GCs actúan directamente sobre la hipófisis y sobre neuronas del hipotálamo, ejerciendo una retroalimentación negativa sobre su secreción, volviéndose al nivel inicial de corticoides al frenarse la secreción de CRH y de ACTH.

1.2.4.1. Glucocorticoides y sus Receptores

Con la respuesta aguda al estrés, aumenta mucho la secreción de GCs por la glándula suprarrenal, lo que proporciona un beneficio inmediato para el animal (Meaney y cols., 1991; McEwen, 2008; Swaab y cols., 2005). Los GCs actúan sobre muchos órganos y en varias áreas cerebrales, uniéndose a dos tipos de receptores nucleares, los receptores de mineralocorticoides (MRs) y los GRs, que actúan como reguladores transcripcionales (Swaab y cols., 2005). Los GRs y MRs no solamente son diana de los GCs, sino que también ejercen un efecto inhibitorio sobre el eHHA, constituyendo el principal sistema de retroalimentación negativa (Liberzon y cols., 1997; de Kloet, 2003; Pittenger y Duman, 2008).

En el cerebro, las regiones límbicas poseen la mayor cantidad de GRs y MRs (Furay y cols., 2008), en el hipocampo (Swaab y cols., 2005; McEwen, 2008), la amígdala (LeDoux, 1998; Furay y cols., 2008) y la PFC (Sotres-Bayon y cols., 2006). Los MRs están concentrados más intensamente en el hipocampo y en el área septal, mientras que los GRs están extensamente distribuidos en todo el cerebro, aunque predominan en el hipotálamo y en la hipófisis (Edwards y Burnham, 2001).

Los MRs tienen alta afinidad por los GCs (Anacker y cols., 2010), e incluso en condiciones basales, más del 70% de los MRs de las zonas límbicas están ocupados por la hormona endógena (de Kloet, 2003), mientras que los GRs sólo están significativamente ocupados en situaciones de estrés o durante el pico circadiano de secreción de esteroides (Sapolsky, 2003; Furay y cols., 2008). Por su parte, los GRs son los responsables de los efectos adversos e incontrolados del estrés crónico sobre el metabolismo, sobre la supervivencia neuronal (Anacker y cols., 2010) y sobre funciones cognitivas, estando involucrados principalmente en la consolidación de la memoria (González y Escobar, 2002; ter Horst y cols., 2012). No obstante, desde hace poco tiempo se ha asignado a ambos tipos receptores un importante papel en la regulación de las emociones (Zhou y cols., 2010).

Con lo anterior, se puede afirmar que la respuesta al estrés depende de la funcionalidad de los GRs y MRs en el cerebro. Pero las acciones de los GCs no dependen sólo de eso, ya que en sus efectos están implicadas interacciones con otros sistemas neuroquímicos, como el neuropeptídico y el monoaminérgico (Edwards y Burnham, 2001).

1.2.4.2. Neuropéptidos

En el control de la respuesta al estrés, los corticoides actúan coordinadamente con las catecolaminas y con otros importantes neurotransmisores que participan en la supervivencia neuronal y en la modulación de la conducta (Vermetten y Bremner, 2002; McEwen, 2008; Beck y Luine, 2010). Entre ellos, la colecistoquinina (Giardino y cols., 1999), las urocortinas (actuando a través de los receptores de CRH-$_2$) (de Kloet, 2003), los factores neurotróficos (BDNF), las endorfinas y las encefalinas, las citocinas (McEwen, 2008), el neuropeptido Y (Vermetten y Bremner, 2002), la angiotensina y la neurotensina (facilitadores de la liberación de ACTH), los péptidos opioides endógenos, la vasopresina (regulando el eHHA, o junto con la oxitocina, potenciando el efecto del CRH y regulando la respuesta al estrés) (Alonso y cols., 2004), y la hormona liberadora de tirotropina (Chamas y cols., 2004). Se incluyen muchas otras hormonas que no intervienen a nivel cerebral, pero son fundamentales en la eficacia de la respuesta autónoma (Ej: la somatostatina, prolactina o el glucagón). Además, muchos neuropéptidos son hormonas hipotalámicas, por lo que pueden controlar directamente la secreción de las hormonas hipofisarias y funcionar tanto como hormonas, en el sistema porta hipotalámo-hipofisario, o como neurotrasmisores en el Sistema Nervioso Central (Vermetten y Bremner, 2002).

1.2.4.3. Monoaminas

Dentro del cerebro, también se activan en respuesta al estrés, varios sistemas de monoaminas que inervan la corteza y, en particular el área medial de la corteza prefrontal (mPFC) (Goldstein y cols., 1996; Mangiavacchi y cols., 2001; Beck y Luine, 2010). Entre las monoaminas más importantes en esta respuesta están el Glutamato (representante de los aminoácidos excitatorios) y el ácido gama-aminobutírico (GABA) (prototipo de los aminoácidos inhibitorios) (Vermetten y Bremner, 2002; Maier y Watkins, 2010).

También, la 5-HT y la dopamina desempeñan un papel crucial en la actuación del organismo frente a una situación de estrés (Goldstein y cols., 1996; Mangiavacchi y cols., 2001); el estrés induce la activación de los sistemas dopaminérgico y serotoninérgico ascendentes (Berendsen y cols., 1996) y la consecuente liberación de 5-HT y dopamina en estructuras corticales y subcorticales límbicas (Beck y Luine, 2010), como la mPFC (Amat y cols., 2005).

De forma particular, la 5-HT es un neurotransmisor central que juega un papel muy importante como estabilizador de la actividad nerviosa. Influye en varias funciones cerebrales, entre las que se destaca la regulación del estatus emocional y la función cognitiva (Jeffrey Newport y cols., 2001). Además, parece estar implicada en las variaciones ontogénicas que determinan la capacidad de fijación de los GRs en el hipocampo y en la PFC, mediando los efectos del ambiente. Dicho efecto es específico para esas regiones cerebrales y para los GRs, ya que otras regiones no son afectadas por el ambiente y los MRs por su parte, no son afectados por la 5-HT (Smythe y cols., 1994).

Por otro lado, la 5-HT juega un papel crítico en la regulación de las funciones autónomas, muy dependientes del sistema serotoninérgico (Siepmann y cols., 2003), e incrementa directamente la secreción de GCs en las glándulas suprarrenales (González y Escobar, 2002), potenciando los efectos de CRH y ACTH.

1.3. Consecuencias de la exposición al estrés

1.3.1. Alteraciones fisiológicas y comportamentales

El estrés podría entenderse como una amenaza a la homeostasis, producida por estímulos potencialmente dañinos que, al ser detectados por el individuo, activan respuestas específicas compensatorias en el organismo (Pacak y cols., 1998). Estas respuestas se denominan alostáticas (Darnaudéry y Maccari, 2008) y, según su significado, deben permitir al organismo mantener la homeostasis, al "conseguir estabilidad a través de la alteración" (McEwen, 2008). Sin embargo, situaciones de estrés prolongadas o incontrolables, inducen una pérdida de resistencia y una fase de extinción de los procesos destinados a mantener la homeostasis (de Kloet, 2003; Gadek-Michalska y Bugajski, 2003; Leonard, 2005). Este hecho se explica por un fallo en el fenómeno de adaptación (Armario y cols., 2008), produciéndose un fenómeno que se denomina "carga alostática" (McEwen, 2008).

El mantenimiento de una condición estresante puede conducir a enfermedades, por incapacidad para poder superarla (Leonard, 2005), por no finalizar las respuestas al estrés cuando estas ya no son necesarias (McEwen, 2008), o porque el organismo no se habitúa al estresor. Ejemplos de las consecuencias de la carga alostática lo constituyen síntomas de tipo

físico (entre otros, la enfermedad coronaria, hipertensión, diabetes tipo 2, arteriosclerosis, obesidad, etc.) (Mueller, y Bale, 2006; Vanbesien-Mailliot y cols., 2007; von Kanel y cols., 2009; Cox y cols., 2011), o síntomas psicológicos (trastornos por ansiedad, depresión, o el trastorno por estrés postraumático (TEPT), etc.)(Swaab y cols., 2005).

Lo que parece claro es que una historia previa de estrés, sea agudo (Belda y Armario, 2004) o crónico (Armario y cols. 2008), provoca importantes alteraciones fisiológicas que originan una respuesta aumentada de los ejes HHA y SA, causando importantes alteraciones comportamentales y fisiológicas en respuesta a posteriores desafíos (Van Dijken y cols., 1993; Jaferi y Bhatnagar, 2006; Ostrander y cols., 2006).

1.3.2. Alteraciones cerebrales

El cerebro es también el órgano diana del estrés, que se altera estructural y químicamente en respuesta a éste. Una de las formas por las cuales las hormonas modulan la función cerebral es alterando la reestructuración y la reorganización neuronal, provocando una "reprogramación" del cerebro por exposición al estrés, que puede ser transitoria o permanente (McEwen, 2000; Rocher y cols., 2004; Czeh y cols., 2005; McEwen, 2008).

Los efectos del estrés en el cerebro pueden tener formas muy diferentes, dependiendo de la región y de las vías implicadas. El hipocampo es una de las estructuras más sensibles y plásticas del cerebro, que experimenta un sin número de alteraciones adaptativas en respuesta al estrés agudo y crónico (Conrad, 2010). Como el hipocampo está implicado en los procesos de memoria y aprendizaje, si se mantiene la condición de estrés, suelen producirse desajustes en las funciones cognitivas (Conrad y cols., 1999; Moita y cols., 2003; Sapolski, 2003; Conrad, 2010). A pesar de que estudios anteriores hayan atribuido los déficits encontrados en la conducta a una muerte neuronal en el hipocampo, lo cierto es que el número de células hipocampales se preserva en animales expuestos de forma prolongada a corticosteroides (Malberg, 2004; Rocher y cols., 2004), aunque sí se producen cambios en la neuroplasticidad (Sapolsky y cols., 2000; Czeh y cols., 2005), y ciertos tipos de estrés agudo y muchos tipos de estrés crónico, suprimen la neurogénesis hipocampal (Pittenger y Duman, 2008). Asimismo, las alteraciones en la formación hipocampal pueden ser reversibles. De esta manera, con el cese del estrés o la exposición a GCs, el proceso puede ser lentamente reversible, con formación de

nuevas neuronas (Conrad y cols., 1999; Sapolsky, 2003). Eso es fundamental en la protección del hipocampo contra el daño permanente, y permite que el animal readquiera sus competencias.

Las alteraciones en el hipocampo inducidas por estrés, no reflejan las alteraciones adaptativas que ocurren simultáneamente en otras regiones del sistema límbico, donde también se observan desajustes de plasticidad estructural, como en la mPFC (Banasr y cols., 2007; Holmes y Wellman, 2009) y la amígdala (Drevets, 2000). Recientemente, en estudios realizados con modelos animales, se sugiere que las neuronas de la PFC son muy sensibles al estrés (Holmes y Wellman, 2009). En ratas, se ha demostrado que la exposición a breves períodos de estrés es suficiente para causar una remodelación estructural significativa en las neuronas de la mPFC (Alonso y cols., 2004). El estrés crónico disminuye la complejidad dendrítica en la mPFC (Baran y cols., 2009), y hace que se reduzca la proliferación glial y de las células endoteliales (Alonso y cols., 2004; Banasr y cols., 2007). Como la glía proporciona soporte metabólico a las neuronas, una reducción en su número puede tener impacto en la función y morfología de las células piramidales de la mPFC (Pittenger y Duman, 2008).

Por otro lado, el estrés crónico puede aumentar la plasticidad sináptica en la amígdala**,** mejorar su función y el aprendizaje amígdala-dependiente. El estrés prolongado no sólo aumenta la cantidad y complejidad de las espinas dendríticas en la amígdala (Vyas y cols., 2006), sino que aumenta su conectividad sináptica (Conrad y cols., 1999; Drevets, 2000). Estas alteraciones no revierten después de cesar el estrés crónico (Vyas y cols., 2004), un efecto bastante distinto de la atrofia que se induce en el hipocampo y en la mPFC. Ambos procesos son el resultado de una sobreactivación de los circuitos neurales que controlan el miedo, la ansiedad y la emoción. Los efectos del estrés en la amígdala pueden ser de larga duración, habiéndose descrito un incremento del tamaño de la amígdala en la edad adulta, en ratas sometidas a estrés prenatal (Vyas y cols., 2006).

1.4. Susceptibilidad y sensibilidad al estrés

Se ha mencionado antes cómo el estrés altera su función inicial, de protectora a dañina y es causante de trastornos y enfermedades. La duración, intensidad y naturaleza del estresor son elementos importantes, pero queda por dilucidar por qué algunos individuos reaccionan y

siguen manteniendo una salud perfecta, mientras que otros sufren y se colapsan bajo las mismas condiciones adversas.

1.4.1. Factores genéticos y ambientales

La diversidad genética entre individuos origina su variabilidad en relación a la resistencia o vulnerabilidad ante los acontecimientos ambientales, en diferentes momentos de la vida. El ser más o menos susceptible al estrés, viene determinado por la convergencia entre la influencia genética y la influencia ambiental en un sustrato neuronal concreto.

El genoma codifica la base inicial del desarrollo del sistema nervioso, pero la experiencia y la interacción con el ambiente, determinan el acabado final, existiendo una "plasticidad" que lo moldea y modifica (Meaney y cols., 1991; Weinstock, 1997; Maccari y cols., 2003; Amiel-Tison y cols., 2004). Las experiencias precoces son muy determinantes en este contexto, modulando el sistema nervioso y dejando sus marcas a lo largo de la vida (Sapolsky, 2003; Alleva y Francia, 2009). El resultado final delinea la sensibilidad y la respuesta del organismo al ambiente (Vermetten y Bremner, 2002).

Así, eventos similares pueden originar diferentes resultados, explicados por un fondo genético distinto o por una interacción con el ambiente distinta, y es esta interacción la que tiene un papel fundamental en cómo el individuo responde a las experiencias (Fumagalli y cols., 2007). En este contexto, se puede concluir que el ambiente no causa necesariamente la enfermedad, sino que es la respuesta que adopte el individuo ante el estresor lo que determina el resultado final (Caspi y cols. 2003).

1.4.2. Experiencias precoces

Según se ha expresado anteriormente, las respuestas adaptativas al estrés ocurren por la activación de circuitos neuronales que están programados genéticamente, pero por su sensibilidad, pueden ser modificados por la experiencia y por el ambiente. Si las experiencias estresantes son precoces, las consecuencias parecen ser más duraderas y profundas (Amiel-Tison y cols., 2004; Swaab y cols., 2005; Ostrander y cols., 2006).

En este sentido, es bien conocida la hipótesis sobre la etiología de la esquizofrenia, según la cual, deben existir alteraciones previas acontecidas durante el desarrollo (hipótesis del

desarrollo), como malnutrición, infecciones virales o déficits genéticos para que se manifieste este trastorno. Esta hipótesis ha sido recientemente revisada, sugiriéndose que las agresiones durante el neurodesarrollo no serían suficientes para que se manifieste la enfermedad, sino, que sería necesaria una combinación de dichas agresiones, con una exposición del sujeto al estrés cuando es joven (Choy y cols., 2009). Rosen y Schulkin (1998) habían verificado que la exposición precoz a estímulos aversivos establecía y sensibilizaba específicamente los circuitos neurales del miedo, que serían reactivados en el adulto por situaciones inductoras de miedo. De hecho, las experiencias adversas precoces provocan modificaciones en el sistema nervioso que persisten a lo largo de la vida (Aisa y cols., 2008), y constituyen las experiencias con mayor peso en lo que respecta a la predisposición futura para la reactividad (manera de como el individuo reaccionará a nuevas situaciones) (McEwen, 2008), en la vulnerabilidad para la psicopatología (Aisa y cols., 2008; Choy y cols., 2009), en la susceptibilidad a sufrir depresión, ansiedad, anhedonia (Leventopoulos y cols., 2009) o déficits cognitivos en la edad adulta (Fumagalli y cols., 2007; Alleva y Francia, 2009).

En ese sentido, es interesante apuntar que si un factor de estrés precoz vuelve durante la vida adulta, puede que se exacerben y precipiten episodios de depresión, esquizofrenia y del trastorno por estrés postraumático (Barros y cols., 2006; Fumagalli y cols., 2007). Todo ello sugiere que el estrés precoz puede alterar el umbral de vulnerabilidad de los sistemas cerebrales, determinando una especie de sensibilización a estresores subsiguientes, y que esta sensibilización es necesaria para que se manifiesten algunas enfermedades (Nadal y Armario, 2010).

Todos los sistemas neuronales son susceptibles de sufrir modificaciones por entradas aferentes durante el desarrollo. De acuerdo con el momento del desarrollo, el evento adverso puede interferir en la maduración de un fenotipo celular específico o en un circuito cerebral concreto. Por ejemplo: el perfil de neuronas y astrocitos en el cerebro adulto de un animal que durante su desarrollo haya sufrido privaciones, será el resultado del efecto de las sustancias asociadas al estrés recibidas a través de la placenta, del efecto del cuidado maternal recibido durante el período perinatal y de todas las circunstancias que ocurren durante la infancia, pubertad y juventud. Con este supuesto, se han diseñado varios modelos animales para

predecir la susceptibilidad al estrés inducidas por el estrés precoz: Modelo de "estrés prenatal" (Day y cols., 1998; Fumagalli y cols., 2007; Clinton y cols., 2008), de "privación de la madre" (Aisa y cols., 2008), de "manipulación precoz del animal" (Pryce y cols., 2005), o "del ambiente enriquecido precoz" (Chen y cols., 2010).

1.4.3. Estrés prenatal

El estrés prenatal (**PS**- siglas del inglés *Prenatal stress*) es un síndrome comportamental que padecen las crías de madres expuestas a estrés durante la gestación. El feto en desarrollo es particularmente vulnerable a las agresiones o condiciones adversas experimentadas por la madre gestante y por sus reacciones frente a ellas. En respuesta al estrés, el feto puede sufrir adaptaciones o modificaciones, cuyos efectos pueden persistir hasta que se convierta en adulto, o incluso hasta las generaciones siguientes (Meaney y cols., 1991; Weinstock, 1997; Maccari y cols., 2003; Amiel-Tison y cols., 2004; Alleva y Francia, 2009). La plasticidad de los sistemas fisiológicos fetales puede alterar su funcionalidad, influyendo en la maduración de componentes del sistema nervioso (Barros y cols., 2006; Aisa y cols., 2008; Fumagalli y cols., 2007; Popa y cols., 2008; Choy y cols., 2009), con efectos profundos y persistentes en las funciones cerebrales.

El modelo de PS en la rata fue desarrollado por Thompson (Thompson, 1957), y pretendía dilucidar los mecanismos subyacentes a las alteraciones físicas y comportamentales profundas. Posteriormente, han surgido otros modelos de PS parecidos al suyo, usando diferentes períodos y tipos de estresores durante la gestación (Peters, 1986; Hayashi y cols., 1998; Samuelsson y cols., 2004; Koenig y cols., 2005; Louvart y cols., 2009; Wilson y cols., 2013).

El PS es un modelo animal extensamente usado hoy en día, y muy relevante para todos los trabajos que intentan dilucidar los efectos de la exposición temprana a ambientes adversos, sobre la vulnerabilidad de los individuos a desarrollar desórdenes neurofisiológicos. En humanos, se describen las experiencias traumáticas precoces, entre ellas el PS, como un factor que potencia el riesgo de enfermedades (Vanbesien-Mailliot y cols., 2007), alteraciones del desarrollo (Mueller y Bale, 2006), trastornos comportamentales o el desarrollo de diversas psicopatologías (Fagundes y cols., 2013). En los animales, el PS influye profundamente sobre el neurodesarrollo y puede predisponer a presentar dificultades de adaptación en la vida posnatal

(Barbazanges y cols., 1996; Darnaudéry y Maccari, 2008). Este tipo de experiencia aversiva aplicada a ratas, induce anormalidades en las crías desde la vida temprana, hasta la edad madura (Maccari y cols., 2003; Bhatnagar y cols., 2005; Son y cols., 2006; Wilson y cols., 2013).

En el cerebro, el PS puede afectar el proceso de pérdida de arborización dendrítica y sináptica, que ocurre normalmente desde la pubertad hasta el adulto (Barros y cols., 2006), induce una disminución en los niveles de 5-HT en el cerebro de las crías jóvenes (Hayashi y cols., 1998) y afecta negativamente la proliferación celular en el hipocampo (Malberg, 2004; Samuelsson y cols., 2004).

1.5. Factores que permiten revertir los efectos del estrés

1.5.1 Factores ambientales

Experiencias positivas tempranas en la vida tendrán efectos opuestos a los observados en animales estresados (Meaney y cols., 1991; Leonard, 2005; Alleva y Francia, 2009). Las experiencias positivas pueden reflejar respuestas adaptativas a situaciones de estrés posteriores. En este sentido, se ha descrito que incluso breves períodos de manipulación en las primeras semanas de vida, se asocian con marcadas reducciones en las respuestas frente a estímulos aversivos en la edad adulta (Meaney y cols., 1991). También el aprendizaje, un ambiente enriquecido e incluso el ejercicio físico (Sapolsky, 2003), colaboran en mantener la salud de las neuronas hipocampales y permiten restablecer comportamientos basales.

1.5.2. Tratamiento farmacológico

También se pueden revertir los efectos nocivos del estrés, manipulando en la vida temprana los receptores de 5-HT o mediante tratamiento farmacológico (Maeng y cols., 2008; McEwen, 2008; Ulloa y cols., 2010). Aunque en el período precoz se recuperen los receptores de 5-HT, en el adulto ya no se produce dicha mejoría. Sin embargo, se ha descrito que, en el adulto el tratamiento crónico con fármacos serotoninérgicos, que activan los receptores 5-HT, tiene eficacia terapéutica en el tratamiento de varios trastornos psicopatológicos (Maier y Watkins, 2005).

1.5.2.1. Inhibidores selectivos de la recaptación de la Serotonina

Se han asociado diversas situaciones patológicas con niveles bajos de 5-HT, como patologías depresivas, trastornos alimentarios, ansiedad, esquizofrenia, epilepsia o drogodependencias (Yamada y cols., 1999; Kim y cols., 2002; Zazpe y cols., 2007; Bilge y cols., 2008). Los inhibidores selectivos de la recaptación de 5-HT (ISRSs) son actualmente los fármacos de elección para el tratamiento de la mayoría de trastornos depresivos (Jones y Blackburn, 2002), de ansiedad (Matar y cols., 2006), de personalidad y trastornos por pánico (Zanoveli y cols., 2007).

Los ISRSs ejercen su efecto antidepresivos inhibiendo la recaptación de la 5-HT en el espacio intersináptico (Manfridi y cols., 1992; Bilge y cols., 2008). Son muy efectivos y, comparados con los antidepresivos tricíclicos, tienen la misma eficacia clínica, aunque son más selectivos (Siepmann y cols., 2003) y presentan menos efectos colaterales, dada su poca afinidad por los transportadores de noradrenalina, muscarínicos, de histamina y de dopamina (Jones y Blackburn, 2002).

1.5.2.2. Sertralina

La SERT pertenece a la clase de antidepresivos -ISRSs- arriba descrita. Este derivado naftalenamino [1S, 4S-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthylamine] (Koe y cols., 1983), es un potente inhibidor de recaptación de 5-HT (Manfridi y cols., 1992).

La SERT es un fármaco altamente efectivo en el alivio de la depresión, de la ansiedad (Giardino y cols., 1999; Siepman y cols., 2003), del TEPT (Matar y cols., 2006), del trastorno obsesivo compulsivo, en los ataques de pánico (Zanoveli y cols., 2007) y en la fobia social. Este fármaco no presenta afinidad por la mayoría de los receptores de los neurotransmisores, no afecta la función psicomotriz y es bien tolerado (Kim y cols., 2002).

1.5.2.3. Efectos de los ISRSs bloqueando los efectos del estrés

Las neuronas serotoninérgicas se localizan fundamentalmente en los NR, siendo estas neuronas los blancos de las drogas antidepresivas, como las ISRSs (Leventopoulos y cols., 2009).

El sistema serotoninérgico es muy complejo, como lo evidencia la gran diversidad de subtipos de receptores sobre los cuales actúa este neurotransmisor (al menos 14 subtipos

distintos) y la variedad de funciones reguladas por cada uno de ellos (Hensler, 2003). Mientras los receptores presinápticos de 5-HT (autorreceptores somatodendríticos) se encuentran en los NR, los receptores de 5-HT postsinápticos abundan en el sistema Límbico (Leventopoulos y cols., 2009).

Los ISRSs actúan atenuando las secuelas de la exposición al estrés. Aunque se conoce que los ISRSs actúan principalmente al unirse a los transportadores de 5-HT, algunos ISRSs actúan sobre otras monoaminas neuroactivas, inhibiendo parcialmente la recaptación de norepinefrina, de dopamina, o disminuyendo la actividad de las neuronas liberadoras de CRH (Matar ycols., 2006).

Junto a las alteraciones celulares y moleculares inducidas por el tratamiento crónico con ISRSs, están los efectos beneficiosos, como los efectos neuroprotectores y neurotróficos de la 5-HT (Manjiy cols., 2001; Hajsan y cols., 2005; Pittenger y Duman, 2008), bloqueando los efectos dañinos del estrés sobre las neuronas. La 5-HT incrementa los niveles de factores neurotróficos, particularmente el BDNF, que ha sido propuesto como elemento crítico en la acción de estos antidepresivos en el hipocampo.

Los efectos de los antidepresivos en la neurogénesis hipocampal parecen ser, en parte, modulados por otras áreas cerebrales como la amígdala (Castro y cols., 2010), que a su vez es una de las dianas de los ISRSs.

1.6. Determinación de los efectos del estrés

1.6.1. Pruebas comportamentales

1.6.1.1. El reflejo auditivo de sobresalto y sus modulaciones como modelo experimental

Cuando se produce un estímulo auditivo intenso e inesperado, se desencadena una reacción de alarma generalizada en el organismo: el Reflejo Auditivo de Sobresalto (RAS). En los mamíferos, esta reacción se manifiesta como una respuesta refleja de amplios grupos musculares estriados, fundamentalmente de los segmentos proximales de las extremidades superiores y se acompaña de una reacción del sistema nervioso autónomo (Koch, 1999). El reflejo de sobresalto es la respuesta más rápida a un estímulo amenazante (Li y cols., 2009).

La respuesta refleja de sobresalto puede ser modificada cuantitativa o cualitativamente por diversas condiciones naturales o experimentales, lo que refleja la capacidad del organismo para ajustar su respuesta a determinadas circunstancias internas o externas (Davis, 1980; Koch, 1999; Lehmann, 2000). La respuesta del RAS es variable entre especies, por sus diferencias genéticas y de sexo (Koch, 1999). Dentro de cada especie, las características del RAS son muy similares en todos los individuos sanos y, por ello, la medición de su latencia e intensidad se ha convertido en una prueba muy usada para evaluar el estado del sistema auditivo, pero también, para evaluar el estado de normofuncionalidad del sistema nervioso, tornándose como una herramienta válida para la investigación de mecanismos cerebrales relacionados con la plasticidad comportamental y la gestión de estados emocionales en varias especies (Grillon, 2002; Geyer, 2006, Geyer y cols., 2001; Hoenig y cols. 2005).

Con la habituación**,** hay un descenso de la amplitud de respuesta debida a la repetición del estímulo, igual que sucede en la inhibición prepulso, PPI (del término anglosajón "pre-pulse inhibition"), paradigma en el que la respuesta de sobresalto ante un estímulo queda reducida o casi abolida, cuando ese sonido es precedido por otro estímulo sensorial de baja intensidad (Brauer y cols., 2009). Por otro lado, se puede intensificar la magnitud del RAS y de sus modulaciones, por sensibilización, asociando al estímulo sensorial un estímulo aversivo (Li y cols., 2009).

El éxito de la evaluación del RAS en la rata como herramienta para realizar estudios comportamentales se debe a su facilidad de medición, por su simplicidad, al basarse en la simple detección de un movimiento en respuesta a un estímulo sonoro, por su plasticidad, por el número de modificaciones o modulaciones que se pueden inducir: la habituación, la sensibilización, la inhibición prepulso y la potenciación por miedo (Braff y Geyer, 1990; Geyer, 2006; Ishii y cols., 2010). Pero una de las grandes características del RAS, es que la plasticidad ocurre de igual forma entre las diferentes especies, desde roedores hasta humanos (Braff y cols., 2001; Swerdlow y cols., 2001), convirtiendo el paradigma de evaluación del RAS en un candidato ideal para la investigación traslacional en Neurociencias.

1.6.1.2. Prueba del campo abierto

Desde hace algunos años, se usa la prueba del campo abierto (CA) como una prueba básica de medición de la ansiedad, que consiste en colocar al animal en un ambiente desconocido, del cual no puede escapar y observar su conducta (Walsh y Cummins, 1976; Prut y Belzung, 2003). Este procedimiento implica la confrontación forzada de la rata a una situación que es naturalmente adversa para los roedores (la rata es un animal nocturno que vive y se protege en madrigueras), como estar en un espacio abierto desconocido y expuesto a luz intensa (Palanza, 2001; Prut y Belzung, 2003). La simplicidad y economía de la prueba, combinada con la apreciación etológica de su validez y su estandarización entre especies y condiciones de trabajo, hacen que resulte un procedimiento de laboratorio muy válido.

1.6.1.3. Prueba de condicionamiento de evitación activa

También la prueba de condicionamiento de evitación activa (EA), por choques escapables, ha demostrado ser una herramienta muy útil para la investigación de los procesos comportamentales y mecanismos neurobiológicos del aprendizaje emotivo (Sanders y cols., 2003; Cain y cols., 2010). Recientemente, se ha comenzado a utilizar la prueba de evitación bidireccional en estudios que analizan los efectos del estrés en modelos animales de depresión (conocido como *learned helplessness*), al someter a los sujetos a choques inescapables (IS) previos a los choques escapables (Santos y cols., 2006). Según lo descrito en la bibliografía, la exposición previa a choques sin posibilidad de escape, los IS, interfiere con las subsecuentes tareas de aprendizaje operantes (Leuner y cols., 2004; Zazpe y cols., 2007), por lo que las respuestas adquiridas de evitación, pueden tornarse inadaptadas y desarrollarse déficits en la conducta de escape y el desamparo (Mangiavacchi y cols., 2001; Santos y cols., 2006).

1.6.1.4. Prueba de respuesta emocional condicionada

La prueba de Condicionamiento Emocional (CER) es una prueba también ampliamente usada para estudiar los efectos del estrés.

En el paradigma del CER, se induce una situación de estrés de la cual el animal no puede escapar y los estímulos se asocian con dolor (como choques no controlables u otros eventos

desagradables), que adquieren propiedades emocionales aversivas. Así, la rata exhibe una respuesta emocional condicionada, al someterla a un ambiente hostil (el EC) (Cordero y cols., 2003; Sanders y cols., 2003; Ishii y cols., 2010). La prueba CER mide la respuesta emocional condicionada, si se ha desarrollado, inhibido, o retardado, y, lo que es más importante, mide la capacidad de cada animal para suprimirla o extinguirla. La extinción del miedo ocurre con la repetición de exposición al estímulo condicionado en ausencia del estímulo traumático, y así la capacidad de inducir miedo se reduce gradualmente hasta que está ausente cualquier manifestación emotiva (Gordon y Hen, 2004; Pittenger y Duman, 2008). Se emplea esta prueba para caracterizar los comportamientos depresivos (Vermetten y Bremner, 2002; El Yacoubi y cols., 2003), el trastorno por estrés postraumático (Blundell y cols., 2011) y trastornos de ansiedad (Ishii y cols., 2010) inducidos por el estrés.

1.6.1.5. Pruebas fisiológicas

Además de cambios conductuales, la respuesta de estrés induce una serie de modificaciones fisiológicas a nivel neuroendocrino, pero también hematológico, cardiovascular, metabólico e inmunológico (Meaney y cols., 1991; Sapolsky y cols., 2000; Gordon y Hen, 2004; Cohen y Zohar, 2004; Mueller y Bale, 2006; Furay y cols., 2008; McEwen, 2008). Así, los parámetros utilizados para identificar los efectos del estrés, se relacionan con sus diferentes fases de respuesta, que debe ser mayor en la fase aguda, pero que deberán volver a sus valores normales finalizada la condición que la provocó (von Kanel y cols., 2009; Bierhaus y cols., 2006; Dhabhar y cols., 2007).

II. HIPÓTESIS Y OBJETIVOS

2.1. Hipótesis experimental

Las experiencias por las que pasa un organismo en los primeros momentos de su vida, tienen efectos profundos y persistentes en sus funciones cerebrales (Cordero y cols., 2003; Fumagalli y cols., 2007; Alleva y Francia, 2009). En esa línea, el estrés prenatal provoca alteraciones en el comportamiento emocional (Van den Hove y cols., 2005), en los procesos de atención, e induce cambios fisiológicos importantes, tanto en animales como en los seres humanos (Barbazanges y cols., 1996; Hayashi y cols., 1998; Son y cols., 2006; Mueller y Bale, 2006), lo que puede representar un factor de riesgo en el desarrollo posterior de determinadas psicopatologías. Los hijos de madres que han sufrido estrés durante la gestación, presentan alteraciones sensoriomotoras y trastornos comportamentales que responden, de forma general, positivamente a tratamientos farmacológicos.

Entre la amplia variedad de fármacos utilizados para paliar estos trastornos, se encuentran los tratamientos que directa o indirectamente intervienen en el metabolismo de la 5-HT (Manfridi y cols., 1992). En este grupo, la SERT es uno de los fármacos más seguros y eficaces para el tratamiento de la ansiedad y de los trastornos causados por el estrés, presentando pocos efectos secundarios (Jones y Blackburn, 2002; Siepman y cols., 2003; Matar y cols., 2006; Skaer y cols., 2009).

En el presente trabajo nos hemos propuesto determinar si la administración de SERT revierte los efectos del estrés prenatal sobre los procesos conductuales y fisiológicos en los animales experimentales.

La hipótesis del trabajo se sustenta en que, analizando las respuestas conductuales, fisiológicas y atencionales de las ratas frente a ambientes ansiogénicos, podremos evaluar objetivamente los efectos del tratamiento crónico con SERT sobre las afectaciones que induce el estrés prenatal.

2.2. Objetivos

El objetivo general del trabajo es evaluar el papel del estrés prenatal en la rata como elemento determinante de la susceptibilidad a padecer alteraciones neuropsicológicas en la edad adulta, y valorar los efectos del tratamiento crónico con Sertralina sobre los cambios producidos por el estrés prenatal en machos y hembras.

2.2.1. Objetivos específicos

1. Evaluar si el procedimiento de estrés por restricción es un modelo eficaz en la inducción de estrés en ratas adultas.

2. Determinar los efectos de la administración de Sertralina intraperitoneal en ratas adultas sometidas a estrés por restricción.

3. Caracterizar los efectos inducidos por estrés prenatal en la conducta emocional y en la atención en ratas adultas.

4. Evaluar los efectos de la administración crónica de Sertralina en ratas sometidas y no sometidas a estrés prenatal.

5. Analizar los efectos metabólicos y fisiológicos del estrés prenatal y de la Sertralina en ratas adultas.

2.3. Diseño Experimental

Para alcanzar el ***objetivo 1****, "Evaluar si el procedimiento de estrés por restricción es un modelo eficaz en la inducción de estrés en ratas adultas"*, sometemos a ratas jóvenes de ambos sexos a un protocolo de estrés postnatal por restricción, valorando los posibles cambios en el reflejo auditivo de sobresalto y en su inhibición por un estímulo previo antes y después del proceso de estrés, teniendo como control un grupo de animales similares en edad, no sometidos a estrés.

Para alcanzar el ***objetivo 2****, "Determinar los efectos de la administración de Sertralina intraperitoneal en ratas adultas sometidas a estrés por restricción*" administramos SERT durante 8 días consecutivos, a los animales sometidos a estrés por restricción. Posteriormente, evaluamos posibles cambios en el RAS/PPI y los cambios fisiológicos inducidos.

Para alcanzar el ***objetivo 3****, "Caracterizar los efectos inducidos por estrés prenatal en la conducta emocional y en la atención en ratas adultas*", comparamos animales de ambos sexos que hayan sido sometidos a estrés prenatal con sus controles. Nos planteamos:

a. caracterizar las alteraciones en el comportamiento emocional (evaluado mediante el RAS; prueba de campo abierto y la prueba del CER).
b. caracterizar deficiencias en la atención (evaluado mediante la PPI) y en el aprendizaje en un contexto aversivo (mediante la prueba de EA)

Para alcanzar el ***objetivo 4****, "Evaluar los efectos de la administración crónica de Sertralina en ratas sometidas y no sometidas a estrés prenatal*", nos planteamos evaluar si la administración crónica de un fármaco antidepresivo (SERT) en la rata joven, va a ejercer efecto sobre los cambios inducidos por estrés prenatal durante el desarrollo hasta la fase adulta. Pretendemos determinar si el tratamiento modifica, previene o revierte los efectos negativos del estrés prenatal sobre la conducta

Para alcanzar el ***objetivo 5****, "Analizar los efectos metabólicos y fisiológicos del estrés prenatal y de la Sertralina en ratas adultas*", al finalizar el estudio *in vivo*, nos planteamos:

a. Medir los niveles de ACTH en respuesta al estrés.
b. Estudiar las alteraciones sanguíneas (hematológicas, bioquímicas e inmunológicas) en respuesta al estrés.

Para la consecución de los *objetivos 1* y 2 realizaremos un estudio preliminar, usando ratas jóvenes de ambos sexos, al que llamaremos **grupo piloto (A)**. Tras el análisis de los resultados, aplicaremos los protocolos validados en ratas gestantes, y estudiaremos sus crías, creando para ello un grupo al que llamaremos **grupo estrés prenatal (B)**.

Para alcanzar los *objetivos 3, 4* y 5, dividimos el estudio del grupo estrés prenatal (B) en tres abordajes experimentales distintos, donde serán explorados los efectos del estrés prenatal y de la SERT, mediante pruebas de evaluación de la conducta emocional y de determinación de parámetros fisiológicos. Como control para determinar los efectos del estrés prenatal, se usan ratas descendientes de madres no sometidas a estrés durante la gestación, que serán mantenidas en condiciones estándar de alojamiento. Las **Figuras 3 y 4** muestran el esquema del diseño experimental empleado en nuestro estudio. Se indican los días de duración de cada prueba y la toma de muestras para la realización del resto de los estudios llevados a cabo.

En el esquema, se representan las publicaciones resultantes de los diferentes abordajes experimentales, denominadas ANEXOS I, II, III y IV respectivamente.

A. Grupo piloto

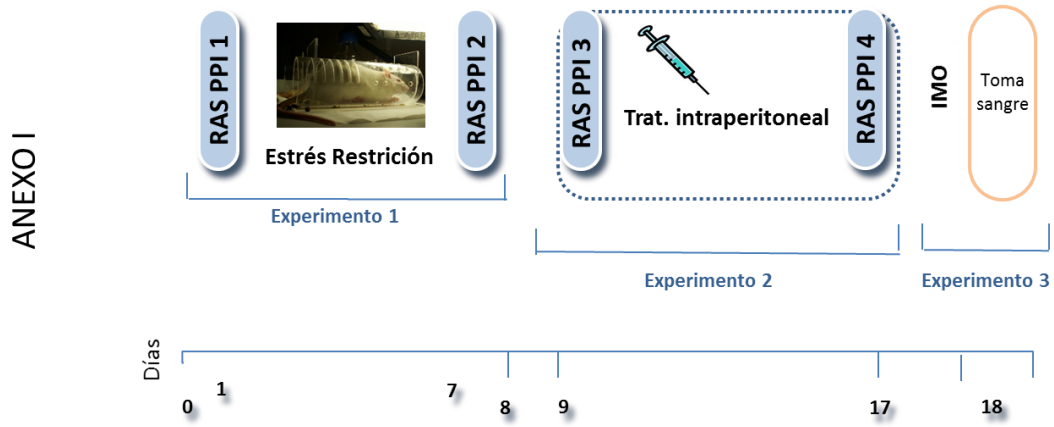


Figura 3. Diseño experimental para el grupo piloto. Esquema resumen de los pasos seguidos en este estudio para alcanzar los objetivos planteados para el grupo piloto.

Experimento 1: el día antes de iniciar el protocolo de inducción de **estrés por restricción** (RS, día 0), todos los animales son sometidos a la primera prueba comportamental (**RAS/PPI 1**), en la cual se obtienen los valores basales; el día después del final de RS, se realiza una segunda prueba conductual a todos los animales (**RAS/PPI 2**). **Experimento 2:** los animales, tanto los controles como los previamente estresados de ambos sexos, se subdividen en 6 subgrupos según el tratamiento aplicado: los animales sometidos a 8 días de inyección intraperitoneal (i.p.) (**Trat. intraperitoneal)** con vehículo (Control + Veh, RS + Veh) o SERT (SERT: 5 mg/kg/día) (Control + SERT; RS + SERT); y los animales no inyectados (Control, RS). En el primer día del experimento 2, una hora después del protocolo i.p. todos los animales se someten a la tercera prueba comportamental (**RAS/PPI 3**). El último día, una hora después del procedimiento de inyección i.p., todos los animales se someten a la cuarta prueba comportamental (**RAS/PPI 4**). **Experimento 3:** todos los animales, excepto los sometidos a restricción + inyección i.p., se someten a una sesión de estrés por inmovilización (**IMO**) y, tras 10 minutos de reposo, se anestesian, y se extrae sangre por punción cardíaca para la comparación con un grupo de animales controles no sometidos a ninguna prueba, únicamente se exponen a una extracción sanguínea (**No-IMO**). Para la determinación de las diferentes fases del ciclo estral en las hembras, realizamos una citología vaginal siempre que se mide el RAS/PPI.

B. Grupo estrés prenatal

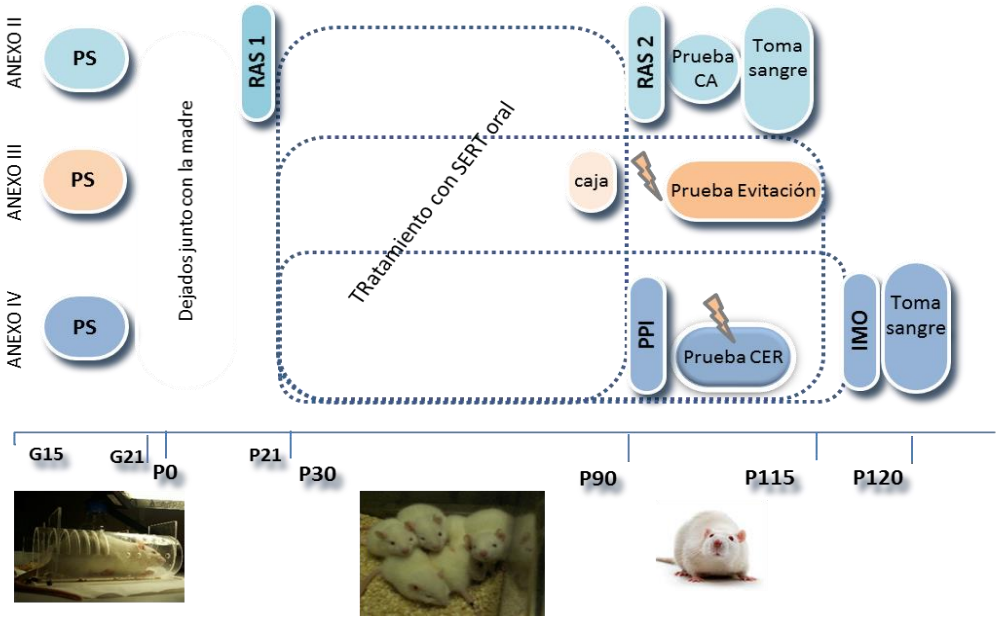


Figura 4: Diseño experimental empleado para el grupo de estrés prenatal. Esquema resumen de los pasos seguidos en este estudio para alcanzar los objetivos planteados para el grupo de estrés prenatal. El protocolo de estrés prenatal **(PS),** con estrés por restricción de la madre, comienza el día 15 de gestación hasta el parto (G15-G21). Se dejan las crías con sus madres, sin sufrir ninguna perturbación hasta el día posnatal P21. A partir de esta edad, todos los animales se pesan semanalmente hasta el final del experimento. En el primer estudio, se realiza una primera evaluación del RAS a las crías de ambos sexos de 30 días de edad sometidas a estrés prenatal y a sus controles **(RAS 1).** Desde el día posnatal P30 hasta el día posnatal P90, las ratas jóvenes reciben SERT (5mg/Kg/día) o no en el agua de bebida (**Tratamiento con SERT-oral**), tras lo cual se repite la evaluación del RAS **(RAS 2).** Se someten las ratas a la **Prueba de campo abierto (CA)** durante 3 días. Pasados 7 días, se eutanizan las ratas y se realiza un **análisis sanguíneo**. En un segundo protocolo experimental, tras el estrés prenatal, las ratas jóvenes reciben SERT desde el día posnatal P30 hasta el final del experimento (día posnatal P115). A los 88 días (P88), los animales se someten a una **Prueba de choques inescapables (IS)** con una duración de 3 días, la cual es seguida por la **Prueba de choques escapables (EA),** con una duración de 10 días. El tercer protocolo experimental, finalmente, tras el estrés prenatal, las ratas jóvenes reciben SERT desde el día

posnatal P30 hasta el final del experimento (día posnatal P120). Tras 2 meses de tratamiento farmacológico, se evalúa la PPI (P90) y se someten los animales a la **prueba del CER,** con una duración de 7 días, en la cual, los animales son expuestos a choques inescapables **(IS)**; la administración del fármaco se mantiene durante las pruebas conductuales, lo que se asume como una administración crónica del fármaco durante 3 meses. Todos los animales son sometidos a una **sesión de estrés por inmovilización** (**IMO**) y, tras 10 minutos de reposo, se anestesian y se extrae sangre por punción cardiaca para comparar los valores fisiológicos y celulares con los obtenidos en un grupo de controles **no sometidos a choques inescapables** (**No-IS).**

II. HIPÓTESIS Y OBJETIVOS

3.1. Animales de experimentación

En este estudio, se emplean ratas albinas, *Rattus norvegicus,* de la cepa Wistar, obtenidas en el Servicio de Experimentación Animal (SEA) de la Universidad de Salamanca. Las ratas fueron tratadas de acuerdo con las normas nacionales e internacionales vigentes (RD 53/2013) y en todo momento se aplicaron las directrices Europeas (2010/63/EU) para la protección de animales de experimentación y otros fines científicos.

Las ratas controles y experimentales fueron sometidas a las mismas condiciones durante todo el estudio, evitando así posibles fuentes de variación externas. Durante el desarrollo del estudio, todos los animales se mantuvieron en un ambiente de temperatura constante (21º C), con ciclo luz/oscuridad de 12 c/u y con acceso libre a la comida y al agua.

3.1.1. Grupo Piloto (A)

Como se describe en el ANEXO I, se usaron ratas jóvenes de ambos sexos, con 9-10 semanas de edad y unos 200-300 g de peso (hembras y machos respectivamente), a las que se ha estudiado durante 1 mes. Además, se emplea un grupo de animales que se mantienen sin interferencias y sirven como controles del procedimiento (**No-IMO**).

3.1.1.1. Grupos Experimentales:

Los animales se han dividido en 6 grupos de ambos sexos.

- Animales control (grupo **Control)**
- Animales control tratados con Sertralina i.p. durante 8 días (grupo **Control+ SERT**)
- Animales control tratados con solución salina i.p. durante 8 días (grupo **Control+ Veh**)
- Animales sometidos a sesiones de estrés por restricción durante una semana (grupo **RS**)
- Animales sometidos a sesiones de estrés por restricción durante una semana y tratados con Sertralina i.p. durante 8 días (grupo **RS+ SERT**)
- Animales sometidos a sesiones de estrés por restricción durante una semana y tratados con solución salina i.p. durante 8 días (grupo **RS+ Veh**)

3.1.2. Grupo estrés prenatal (B)

Como se describe en los ANEXOS II, III y IV, se usaron ratas adultas: hembras nulíparas (A), machos reproductores (B) y sus crías. Una vez fecundadas y gestantes, las hembras se separan de los machos y se mantienen cada una en su caja, asignándose aleatoriamente cada hembra a una de las dos condiciones experimentales, estrés prenatal o no estrés.

3.1.2.1. Grupos Experimentales:

Se estudiaron un total de 85 crías, 40 hembras y 45 machos distribuidos en 4 Grupos Experimentales.

- Animales no sometidos a estrés prenatal y que no reciben Sertralina (**Control**).
- Animales no sometidos a estrés prenatal y que reciben Sertralina (**Control-SERT**).
- Animales sometidos a estrés prenatal que no reciben Sertralina (**PS**).
- Animales sometidos a estrés prenatal que reciben Sertralina (**PS-SERT**).

3.2. Inducción de estrés

3.2.1. Protocolo de Estrés por restricción

La movilidad de las ratas se restringe colocándolas en cilindros de metacrilato transparente (7-8 cm de diámetro y de 13 a 14 cm de longitud) durante 45 minutos, exponiéndolas simultáneamente a una luz (lámpara con una bombilla halógena de 80 W de intensidad). La longitud del cilindro se ajusta al tamaño corporal de la rata, teniendo cuidado de no dañar al animal.

Las sesiones se hacen en una habitación silenciosa, y el protocolo se repite 3 veces al día durante 7 días, exclusivamente durante la fase de iluminación del ciclo luz-oscuridad, separadas por un período de recuperación de tres horas.

3.2.2. Protocolo de Estrés Prenatal

En el último tercio de la gestación (G15-G21), se repite el protocolo de restricción con hembras gestantes. La movilidad de las ratas se restringe colocándolas en los mismos cilindros

de metacrilato descritos anteriormente durante 45 minutos, exponiéndolas simultáneamente a la misma luz intensa, 3 veces al día (ANEXO II, ANEXO III y ANEXO IV). En estas condiciones, la rata se encuentra totalmente inmovilizada; sólo se permite un ligero movimiento de la cabeza y de las extremidades anteriores (**Figura 5**).

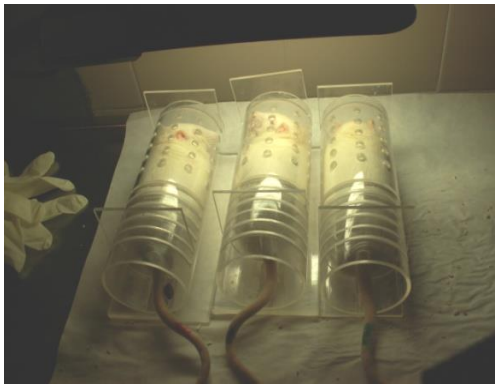


Figura 5: Método de restricción de movimientos e iluminación. Se muestran tres ratas hembras gestantes durante el proceso de inducción de estrés.

Las madres Control permanecen durante todo el período de gestación en su caja, sin someterlas a manipulaciones perturbadoras. Después del parto, las madres permanecen con sus crías durante el período de lactancia hasta el destete, sin sufrir ninguna alteración. A los 21 días de edad, se separan las crías de la madre, se pesan, se clasifican según el sexo y la camada, y se distribuyen aleatoriamente de acuerdo a la condición prenatal a que fueron sometidas y al tratamiento postnatal que van a recibir posteriormente (con o sin tratamiento farmacológico).

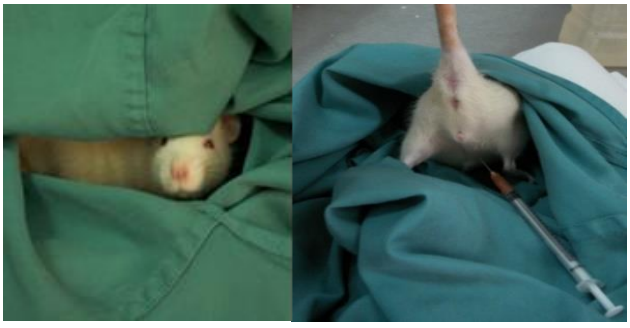
3.2.3. Protocolo de Estrés por Inmovilización (IMO).

Los animales del estudio se someten a una sesión única de estrés justo antes de ser sacrificados, y se comparan con un grupo de animales no sometidos a esta manipulación (**No-IMO**). Se usan cilindros de metacrilato y lámparas similares a las usadas en los protocolos anteriores, pero la restricción de movimientos es casi total (ANEXO I), manteniendo los animales inmovilizados durante 30 minutos (adaptado de Koenig y cols., 2005).

3.3. Tratamiento Farmacológico

3.3.1 Administración de Sertralina al grupo Piloto

El fármaco, Sertralina (Besitran© Pfizer S.A. Madrid, España), o la solución salina (cloruro sódico al 0,9%) se administra intraperitonealmente (i.p.), durante 8 días, a dosis de 5 mg/kg/día, diluido en un volumen de 1 ml de solución salina.



Figuras 6 y 7: Manipulación de las ratas para la inyección intraperitoneal. La rata para ser inyectada, se envuelve a en un paño con olor familiar para su fijación, intentando no provocarle ningún estrés adicional.

3.3.2. Administración de Sertralina al grupo estrés prenatal

Durante dos (ANEXO II) o tres meses (ANEXO III y IV), las ratas son mantenidas en grupos de 4 a 6 en cajas de policarbonato (45cm x 30cm x 20cm) administrándoles SERT por vía oral, diluida en el agua de la bebida. Cada 3 días, se cambia el agua de los biberones y se prepara nuevamente a la concentración requerida, según el peso de los animales. Usando biberones graduados, calculamos el consumo diario de líquido y, de acuerdo al peso del animal, ajustamos la cantidad del fármaco que añadir al agua para administrar una dosis de 5 mg/kg/día (de Magalhães-Nunes, 2007). A los grupos controles, se les administra agua filtrada.

3.4. Pruebas Comportamentales

3.4.1. Medición del reflejo auditivo de sobresalto y de la PPI

3.4.1.1. Equipo

Como se describe en el ANEXO II y el ANEXO IV, la medición del reflejo auditivo de sobresalto (RAS) y de su Inhibición por Estímulo Previo (PPI), se realizó empleando el sistema SR-LAB (San Diego Instruments, San Diego, CA, USA) (**Figura 8**).

Figura 8. Equipo de medición del reflejo auditivo de sobresalto. El equipo consta de varias cámaras para realizar múltiples mediciones a la vez.

El sistema está constituido por un cilindro acrílico transparente (8,2 cm de diámetro) donde se coloca el animal a evaluar. Este cilindro reposa sobre una plataforma de metacrilato, acoplada a un acelerómetro piezoeléctrico que se encarga de detectar cualquier movimiento que se produzca en el cilindro. El estímulo sonoro se produce en un altavoz, colocado a 24 cm sobre el cilindro. Todo ello se encuentra colocado dentro de una cámara de aislamiento acústico (38 x 40,5 x 58,5 cm) que posee ventilación e iluminación propia. El sistema se encuentra acoplado a un ordenador personal, que posee una tarjeta de adquisición de datos para transformar la información analógica a digital; también, mediante el ordenador y empleando el sistema informático SR-LAB, se definen las características de los estímulos acústicos. El sistema informático almacena las respuestas del animal y además, permite su visualización durante toda la ejecución de la sesión de estudio.

3.4.1.2. Procedimiento

La rata se coloca en la cámara de aislamiento y, tras 5 minutos de aclimatación, se suceden los pulsos de estimulación correspondientes a la prueba de medición del reflejo, alternando estímulos de baja intensidad (ruido blanco de 65, 70 y 80 dB SPL, respectivamente, de 20 ms de duración), que actúan como prepulsos, seguidos de un ruido intenso de 115 dB de 20 ms que desencadena el **RAS**. El tiempo entre los estímulos sucesivos es de 30 segundos, con un total de 64 estímulos. Durante toda la sesión, se mantiene un ruido de fondo de 40 dB SPL, con el fin de evitar interferencias de ruidos externos y asegurarnos una igualdad de condiciones experimentales.

La magnitud porcentual de la **PPI** se calcula para cada intensidad del prepulso según la siguiente fórmula:

$$\textbf{PPI} = \frac{\text{(amplitud del RAS en ensayos sin estímulo previo)-(amplitud del RAS en ensayos con estímulo previo)}}{\text{(amplitud del RAS en ensayos sin estímulo previo)}} \times 100$$

3.4.2. Prueba del Campo Abierto

3.4.2.1. Equipo

El campo utilizado es un área circular con 1,2 m de diámetro, una pared acrílica de 50 cm de alto, descubierto y centralmente iluminado con una lámpara de 80 W a una altura de 60 cm (**Figura 9**). El suelo del campo abierto está dividido en 12 áreas externas (pegadas a la pared) y 7 áreas internas (hacia el centro).

3.4.2.2. Procedimiento

La prueba está descrita en el ANEXO II. Se realiza repetidamente tres días consecutivos, a la misma hora para cada animal (entre las 12:00 h y las 14:00 h). El hecho de que esta prueba se realice en una superficie abierta, ancha e iluminada, hace que la rata desencadene una respuesta exploratoria y exprese conductas específicas. El animal percibe el nuevo ambiente como potencialmente peligroso, y el análisis de su respuesta sirve como el primer indicador de

la emocionalidad incondicionada (Roth y Katz, 1979). Es posible conocer la actividad exploratoria de cada animal contando el número de veces que las diferentes áreas son atravesadas, obteniendo la actividad locomotora en cada zona (Roth y Katz, 1979; Pivina y cols., 2007), o sea, la actividad exploratoria total; la exploración externa (*outer crossing activity*- **OA**); la exploración interna (*inner crossing activity*- **IA**); y el cociente de la exploración interna/exploración externa (**IA/OA**). Se mide también la actividad exploratoria vertical (***Rearings***) en cualquiera de las zonas.

Figura 9: Vista panorámica del equipamiento usado para realizar la prueba de campo abierto (CA). La habitación donde se realiza la prueba está semi oscura, siendo el centro del CA la zona más iluminada. Las divisiones están dispuestas en círculos concéntricos que el animal puede explorar libremente. Las áreas más pegadas a la pared del cilindro constituyen la zona de exploración externa (**OA**). Las áreas internas constituyen la zona de exploración interna (**IA**).

3.4.3. Prueba de evitación activa tras choques inescapables

La prueba bidireccional es un paradigma basado en el aprendizaje asociativo por un proceso de condicionamiento de Pavlov (Pinheiro y cols., 2007). La evitación o el escape activo son paradigmas de aprendizaje instrumental en el que los animales pueden controlar la exposición a los estímulos aversivos al realizar respuestas de escape, que constituyen normalmente respuestas activas que permiten a los individuos mantenerse a salvo.

3.4.3.1 Equipo

Se utiliza la caja de Mower-Millar (**Figura 10**). Es una caja metálica (50 cm de largo por 25cm de fondo y 25cm de alto), con la pared anterior transparente, para poder observar la actuación del animal. El piso está formado por barras electrizables, que permiten aplicar

choques eléctricos y posee una barrera con una puerta (10x 10 cm), que divide la caja en dos compartimientos iguales, donde normalmente se permite el paso libre del animal.

Figura 10: Caja de Mower-Miller. Sobre ella está la unidad que controla la intensidad, cantidad y duración de choques y el intervalo entre choques.

3.4.3.2. Procedimiento.

3.4.3.2.1 Choques Inescapables.

Como se describe en el ANEXO III, la prueba se inicia con 2 sesiones de habituación de 3 minutos de duración durante 2 días consecutivos, en los cuales, la rata se acostumbra a la caja experimental. En esta prueba, se introduce la rata en el lado izquierdo del aparato y no se permite el paso libre del animal hacia el lado derecho. Al tercer día, se hace una sesión con la misma duración, pero tras un minuto de aclimatación, se someten los animales a una secuencia de 3 choques eléctricos inescapables (**IS**) de 0,35 mA, con una duración de 5 segundos y con un intervalo de 20 segundos entre choques. La rata se deja reposar otro minuto, se observa el comportamiento y se retira devolviéndola a su caja.

En el día de los choques, se contabiliza la respuesta motora a los choques: número e intensidad de **vocalizaciones** y **saltos** (en la presencia de cada uno de los tres choques se evalúa la rata con: 0, indica que no responde; 0,5, si la respuesta es poco intensa; 1, responde intensamente; así la puntuación de cada animal en la prueba varía del 0 al 3). Se mide, además, el porcentaje de tiempo que el animal permanece en estado de inmovilización o ***freezing***.

3.4.3.2.2 Choques escapables.

La prueba de choques escapables comienza 4 días tras los **IS**. Esta prueba se realiza en sesiones diarias de 10 ensayos cada una durante 10 días consecutivos, y se permite el paso libre del animal, ya que la barrera está abierta. En esta prueba las barras electrizables sirven de estímulo incondicionado (**EI**). El aparato para realizar la prueba está dotado de un generador de luz (de 3 W de intensidad) y es el encendido de la luz (durante 5 segundos) el que actúa de estímulo condicionado (**EC**), que sirve como señal de aviso y antecede a la presentación del **EI** (3 segundos antes del choque). El animal debe asociar que, tras el encendido de la luz, se produce la descarga eléctrica, la cual se puede evitar cruzando hacia el compartimento que no tiene luz.

Los parámetros evaluados están descritos en el ANEXO III. Resumidamente, en cada sesión se contabiliza el número de veces que los animales adoptan cada uno de los siguientes tres tipos de respuestas:

a) **Respuesta de evitación:** es la respuesta adecuada para evitar sufrir el choque eléctrico. Cambio de compartimento en presencia del estímulo condicionado (**EC**) (la luz) y antes de la aparición del estímulo incondicionado (**EI**) (el choque eléctrico);
b) **Respuesta de huida:** es la respuesta adecuada para acortar la duración del **EI**. Se considera respuesta de huida el cambio de compartimento en presencia del choque eléctrico;
c) **Respuesta nula:** se da cuando el animal no es capaz en ningún momento de interrumpir el **EC**, ni tampoco de interrumpir el **EI** asociado con el **EC**. Cuando el animal no pasa a través de la barrera y se queda en el compartimiento donde recibió el choque eléctrico.

3.4.3.2.3. Valoración de cada tipo de respuesta.

El porcentaje de respuestas de evitación permite evaluar la capacidad de aprendizaje y retención de memoria. Por condicionamiento pavloviano, la rata aprende a evitar y no sufrir el choque eléctrico al asociar el EI con el EC. Se considera que los animales están condicionados cuando son capaces de evitar el choque eléctrico por lo menos el 70% de las veces y durante dos sesiones consecutivas.

El porcentaje de respuestas nulas permite evaluar el déficit de escape inducido por los **IS**. Consideramos como actitud o conducta de desamparo, cuando los animales no logran

escapar o huir, y responden de forma persistente con respuestas nulas a los choques. Se considera que los animales presentan una respuesta conductual extrema si presentan el 100% de respuestas nulas en 3 sesiones consecutivas de estudio.

3.4.3.2.4. Actividad locomotora y manifestaciones de emocionalidad

Se evalúa la actividad motora y exploratoria del animal bajo la influencia de la situación experimental, midiendo la actividad locomotora antes del inicio de cada ensayo (**AAE**) y la actividad entre ensayos (**IEE**), o sea, se contabiliza el número de veces que la rata cruza la puerta sin que hubiera sido estimulada a hacerlo, antes y durante cada ensayo.

Como indicadores de integridad sensoriomotora y emotiva se contabilizan las respuestas a los estímulos: **EI** (choques), midiendo las **vocalizaciones** (suma del número e intensidad de chillidos audibles durante las 10 sesiones) y **saltos** (nº e intensidad durante las 10 sesiones); y las **respuestas a los estímulos EC**: respuestas de orientación a la luz (el animal orienta la mirada a la luz sin moverse). Se contabiliza además el número de bolos fecales (Archer, 1973; Katz y cols., 1981).

3.4.4. Prueba de respuesta emocional condicionada (CER)

3.4.4.1. Equipo

Para esta prueba se usa la misma caja de Mower-Miller, utilizada para el estudio de condicionamiento de evitación. En esta prueba, la puerta se mantiene cerrada durante todo el experimento, y el animal apenas puede explorar el lado izquierdo de la caja.

3.4.4.2. Procedimiento

El procedimiento esta descrito en el ANEXO IV. Muy brevemente, la prueba se inicia con 2 sesiones de habituación de 3 minutos de duración durante 2 días consecutivos, en los cuales la rata se acostumbra a la caja experimental. Al tercer día, se hace una sesión con la misma duración, sólo que, una vez colocada la rata en la cámara experimental y, tras un minuto de aclimatación, se somete a una secuencia de 3 choques eléctricos de 0,35 mA (*footshock*), con una duración de 5 segundos y con un intervalo de 20 segundos entre choques. Se deja reposar a

la rata otro minuto, se observa el comportamiento y se la devuelve a su caja. Pasadas 24 horas, los animales se re-exponen a la prueba una vez por día y con una secuencia de 4 sesiones. Estas sesiones ocurren sin choques y sin dar todavía acceso al lado derecho de la caja, y sirven para introducir un recuerdo situacional del choque (Armario y cols., 2008).

3.4.4.3. Valoración de la prueba.

Una vez expuestas las ratas a choques a los que no pueden escapar, se permite evaluar el condicionamiento del miedo y su extinción. El condicionamiento del miedo se forma al crearse un nexo entre el estímulo neutro (**EC**), que en este caso es el contexto (la caja), y el estímulo intenso, los choques eléctricos (**EI**) (Savonenko y cols., 1999). El "miedo" es cuantificado como el porcentaje de tiempo invertido por la rata haciendo *crouching* o *freezing* durante la duración de la prueba y se define en el ANEXO IV, como postura de defensa (**DEF**). La extinción del miedo ocurre cuando, en la 4ª sesión tras los choques, la rata pasa menos del 10% del tiempo total de prueba en una postura de defensa.

Durante los 7 días de la prueba, se miden además el número de ***rearings*** (exploración vertical) y de ***groomings*** (movimientos de acicalamiento utilizando los miembros superiores o la lengua).

3.5. Estudios bioquímicos

3.5.1. Obtención del material biológico para estudios bioquímicos

Una vez terminado el estrés por **IMO**, dejamos la rata reposar 10 minutos en una caja limpia (Maccari y cols., 2003), pasados los cuales se anestesia con una sobredosis de pentobarbital sódico (60 mg/kg de peso por vía i.p.). Se realiza una punción cardiaca (en el ventrículo izquierdo) y se extrae sangre. En cada extracción, se divide el volumen de sangre obtenido en dos alícuotas, una de ellas se recoge en un tubo al vacío de 2 ml (venoject™) con EDTA (K3) para la obtención de sangre total y la otra en un vial *eppendorf*™ de 1,5 ml, sin anticoagulante para obtener suero.

Las muestras se dejan una hora a temperatura ambiente, siendo después refrigeradas y centrifugadas a 1000 *x g* a 4º C durante 20 minutos. El suero obtenido es decantado en viales *eppendorf*™ y usado inmediatamente para análisis bioquímicos. Parte del plasma obtenido sirve

para hacer los hemogramas y la otra parte se conservó a -80ºC para la posterior determinación de ACTH.

3.5.2. Bioquímica del suero y plasma sanguíneo

Como se describe en los ANEXOS I y IV, para los análisis bioquímicos usamos el equipo SPOTCHEM™ EZ *device* (QBC europe), con tiras de tm II (Liver-1, ARKRAY®) para determinar las proteínas totales, Albumina, Bilirrubina Total; y las enzimas citosólicas, Glutamato-oxalacetato transaminasa (GOT), glutamato-piruvato transaminase (GPT) y Lactato deshidrogenasa (LDH).

El hemograma se obtuvo mediante el equipo ADVIA 120 (*Hematology System*). Los parámetros hematológicos obtenidos fueron: número de eritrocitos, concentración de hemoglobina, hemoglobina corpuscular media (MCH), hematocrito, número de plaquetas, volumen plaquetario medio (MPV) y número y tipo de leucocitos.

3.5.3. Valoración de los niveles de ACTH

El estudio se realizó en el laboratorio de Fisiología de la Facultad de Medicina Veterinaria de la Universidad de Lisboa. El procedimiento de radioinmunoensayo se llevó a cabo según las especificaciones del fabricante (kit *ImmuChemm Double Antibody hACTH I RIA kit MP Biomedicals)*.

En primer lugar, se reconstituyen los reactivos congelados Anti-ACTH en el agua suministrada en el kit, se mezclan ligeramente y se dejan reposar durante 15 minutos a 4°C. Se adicionan las soluciones en el orden indicado en el protocolo, pipeteando los reactivos directamente de los viales. Se añade el agua diluyente, el hACTH estándar (para construir la curva de calibración por dilución seriada), las muestras, el antisuero, los controles para ACTH y finalmente el hACTH -^{125}I. Tras 30 segundos, se agita cada tubo en un agitador vórtex hasta obtener un color homogéneo, incubándose posteriormente a 4ºC durante 16 horas. Pasado el tiempo de incubación y tras una centrifugación (1000 *x g* durante 15 minutos a 6º C), se decanta el sobrenadante y se evalúa en el contador de radiación gamma del laboratorio.

3.6. Estudios estadísticos

Todos los análisis estadísticos se llevaron a cabo con el programa SPSS (versión 15.0 para Windows; SPSS Inc, Chicago, Illinois, USA). La descripción y evaluación de los datos se efectuó mediante el valor de la media ± error estándar de la media (SEM), tanto para variables continuas, como para frecuencias absolutas y relativas de variables categóricas. Los resultados se compararon entre los diferentes grupos haciendo uso del módulo ANOVA, y en los casos apropiados, se realizaron comparaciones múltiples *post-hoc* y empleando el test *t* de Student para variables independientes. En los estudios longitudinales, los resultados se compararon entre los diferentes grupos haciendo uso del test de ANOVA mixto split-plot, con comparaciones por pares Sheffé (análisis inter-sujetos) y Bonferoni (análisis intra-sujetos). Cuando fue adecuado, se empleó el test del "*chi*" cuadrado. El nivel de significación estadística aceptado fue $p< 0,05$.

IV. RESULTADOS

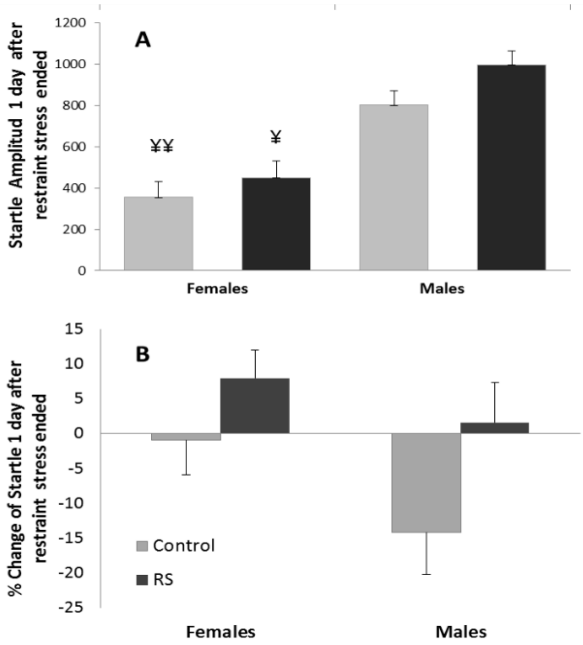
4.1. Grupo piloto: animales jóvenes sometidos a estrés por restricción y tratamiento posterior con Sertralina

4.1.1. Efecto del estrés por restricción en animales jóvenes tras completar el protocolo de estrés.

Según el diseño experimental descrito en la Figura 1 del ANEXO I, en el experimento 1 evaluamos el efecto del estrés por restricción (**RS**) en animales adultos, estudiando las modificaciones que se producen sobre el RAS y sus modulaciones. Se comparan los valores del RAS y PPI un día después de someter los animales al **RS** (RAS/PPI2) con los valores de RAS y PPI un día antes de iniciado el protocolo de estrés, i.e. con sus valores basales (RAS/PPI1).

4.1.1.1. Amplitud del RAS.

Cuando se analiza la amplitud del RAS un día después de someter las ratas al protocolo de estrés por restricción (ANEXO I, Figura 2A), se observa una tendencia a un ligero incremento en sus valores inducido por el **RS**, aunque las diferencias entre controles y animales estresados no fueron significativas ($F_{1,74}$= 2.58, n.s.). El sexo del animal es un factor determinante en la respuesta al RAS, existiendo diferencias significativas entre machos y hembras en ambos grupos, $F_{1,74}$= 4,47, p< 0,001, donde los machos presentan mayores valores de amplitud del RAS.



ANEXO I - Figura 2. Resultados de la medición del reflejo auditivo de sobresalto. A) Amplitud del RAS (expresado en unidades arbitrarias) en animales de ambos sexos después de una semana de estrés por restricción (RS) y animales que no sufren estrés (Control). B) Porcentaje de cambio en las amplitudes del RAS inducida por el estrés tanto en hembras como en machos, cuando se mide un día después de finalizado el protocolo de estrés por restricción (cada animal es su propio control). N = 17 animales por grupo y sexo. Cada columna representa las medias ± error estándar de la media (SEM). ¥¥, $p < 0,01$, y ¥, $p < 0,05$, indican las diferencias entre machos y hembras.

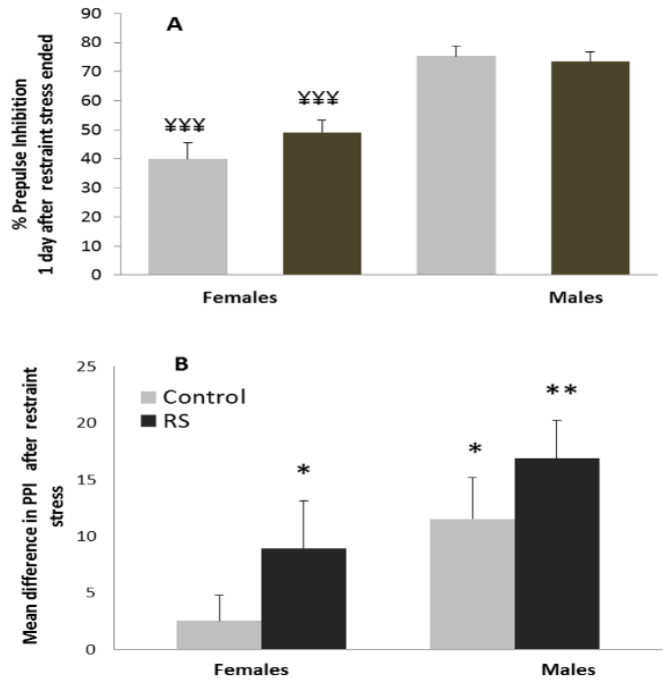
Cuando se evaluó el cambio en porcentaje (%) de las amplitudes de la respuesta de sobresalto auditivo en cada individuo (RAS2/ RAS1) × 100) − 100), el análisis confirmó que la modulación del RAS no resulta alterada significativamente por el estrés ($F_{1,74}$ = 1,29, n.s.), a pesar de que los machos control exhiban una tendencia de habituación (Figura 2 B).

4.1.1.2. Análisis de la PPI.

Para evaluar la PPI, utilizamos diversos paradigmas experimentales, con estímulos prepulso de 65, 70 y 80 dB SPL. La mayor inhibición se consigue al emplear el prepulso de mayor intensidad (datos no mostrados), por lo cual, para estudiar la PPI entre diferentes grupos, elegimos el prepulso de 80 dB SPL.

Con ese paradigma experimental, no se encontraron diferencias en los valores de la PPI como efecto del estrés (ANEXO I, Figura 3A) en ambos sexos. En general, los machos presentan valores de PPI significativamente mayores que las hembras ($F_{1,74}$= 70,60, p <0,001).

Por otra parte, el análisis de varianza teniendo en cuenta los factores *grupo × sexo × día*, indica que los valores de PPI se incrementaron significativamente en relación a sus valores basales (o sea, PPI 2 *vs*. PPI 1), $F_{1,74}$= 24,06 p<0,001, sin que el estrés ($F_{1,74}$ = 1,89) o el sexo ($F_{1,74}$ =3,89) influyeran. El análisis *post-hoc* permite apreciar que la diferencia en los machos fue significativa, independientemente del tratamiento de estrés y, en las hembras, la PPI aumenta sólo en el grupo sometido a estrés por restricción (ANEXO I, Figura 3B).



ANEXO I – Figura 3. Porcentaje de inhibición prepulso. (A) Porcentaje de inhibición prepulso en animales de ambo sexos después de una semana de estrés por restricción (RS) y en animales sin estrés (Control). (B) Medias (±SEM) de las diferencias en los valores de la PPI inducida por el estrés en animales de ambos sexos, un día después de finalizado (en relación a los niveles basales). ¥¥¥, p < 0,01, indican las diferencias entre machos y hembras; **, p < 0,01 y *, p < 0,05, indican un aumento significativo en la PPI.

4.1.1.3. Evaluación de las latencias del RAS y de su PPI.

Tanto la latencia de la respuesta de sobresalto auditivo como de su PPI no se alteran significativamente con el estrés ($F_{1,75}$= 2,17, y $F_{1,75}$= 0,021, n.s.). No se aprecian diferencias por efecto del **RS**, pero sí se pudo demostrar una interacción del grupo con el sexo ($F_{1,75}$= 6,5, p=0,013), y las hembras muestran un valor de latencia de la PPI menor que los machos (ANEXO I, Tabla 1).

Startle Latency

groups	Females		Males	
	Mean value	Difference vs. basal	Mean value	Difference vs. basal
Control	31.1 ± 2.1	+ 0.27 ± 2.6	35.3 ± 1.8	- 3.52 ± 2.3
RS	30.5 ± 2.3	- 1.82 ± 3.3	36.7 ± 1.8	+ 0.61 ± 1.7

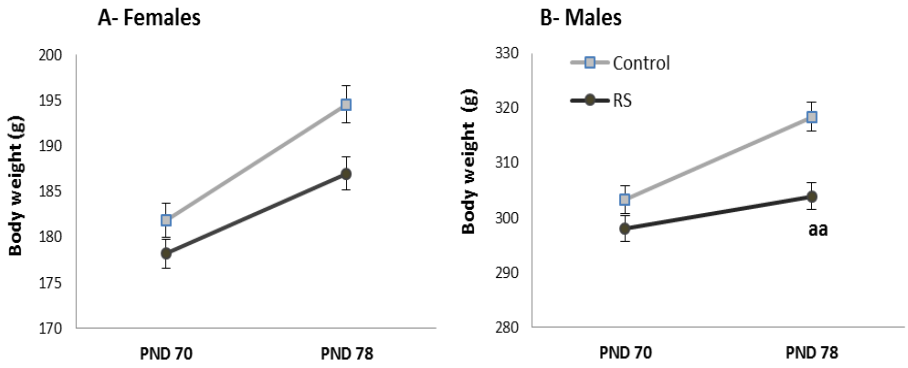
PPI Latency

groups	Females		Males	
	Mean value	Difference vs. basal	Mean value	Difference vs. basal
Control	26.9 ± 1.3 ¥	- 0.25 ± 1.6	28.7 ± 1.3	- 2.37 ± 2.1
RS	28.6 ± 2.1	+ 1.11 ± 1.4	28.9 ± 1.1	- 1.95 ± 2.2

ANEXO I – Tabla 1. Valores medios de latencia del RAS y su PPI. Se muestran los valores de las medias ± SEM tanto del RAS como de la PPI en animales de ambos sexos de los diferentes grupos experimentales (N = 17-21 animales por grupo y sexo). ¥, p < 0,05, indica diferencias significativas entre machos y hembras después del procedimiento experimental en los animales Control.

4.1.1.4. Estudio de la variación del peso corporal pasados 7 días.

El análisis entre machos y hembras se realizó por separado. Todos los animales incrementaron su peso a lo largo de la semana que duró la prueba (p <0,001, en ambos sexos). El estrés por restricción afecta a la ganancia de peso en ambos sexos, siendo la diferencia sólo significativa en los machos (p<0,01); los machos estresados pesan menos (14,2±3,7 g) que sus controles (ANEXO I, Figura 4).



ANEXO I – Figura 4. Efecto del estrés por restricción sobre del peso corporal. Valores de peso corporal en animales hembras (A) y machos (B) sometidos o no a restricción (RS y Control). Abreviaturas: PND 70 y PND 78 días postnatales 70 y 78, respectivamente; N = 17-21 animales por grupo y sexo. El peso se expresa como el valor medio ± SEM. aa, p < 0,01, indica el efecto del estrés por restricción en los machos.

4.1.2. Efecto de la administración de Sertralina intraperitoneal en ratas previamente sometidas a estrés por restricción.

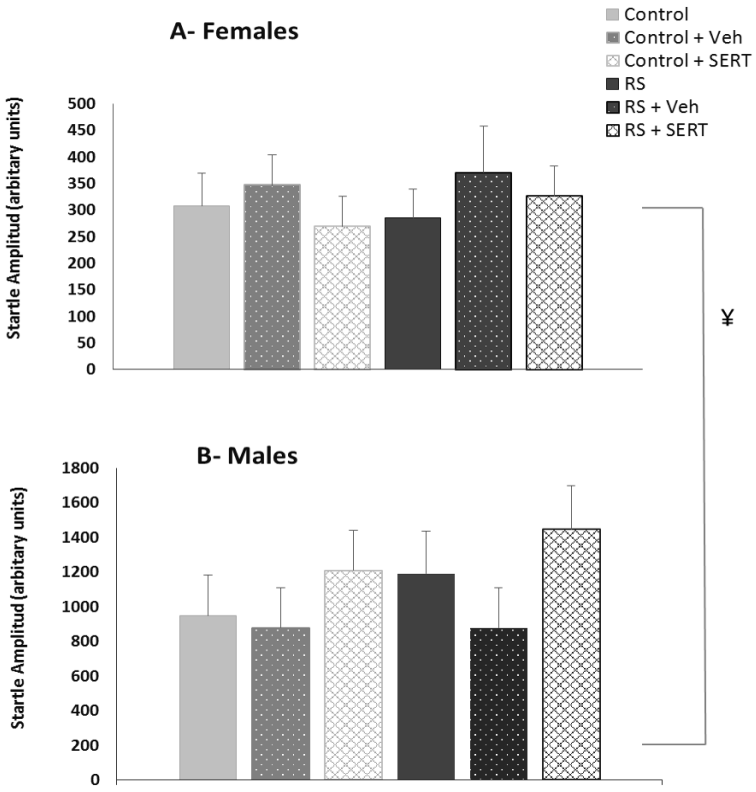
Tal y como se describe en el diseño experimental, en el segundo experimento del ANEXO I se administró SERT a los animales previamente sometidos a estrés por restricción del experimento anterior. El presente experimento permite evaluar los efectos de someter a sesiones repetidas del RAS/PPI animales Control; animales sometidos a estrés por restricción (**RS**) y animales sometidos al RS/o no y sometidos a tratamiento intraperitoneal con SERT (5 mg/kg/día) o Salino (vehículo), al comparar en cada grupo experimental los valores del RAS al final de experimento (RAS/PPI4) con sus valores basales (RAS/PPI1).

4.1.2.1. Amplitud del RAS

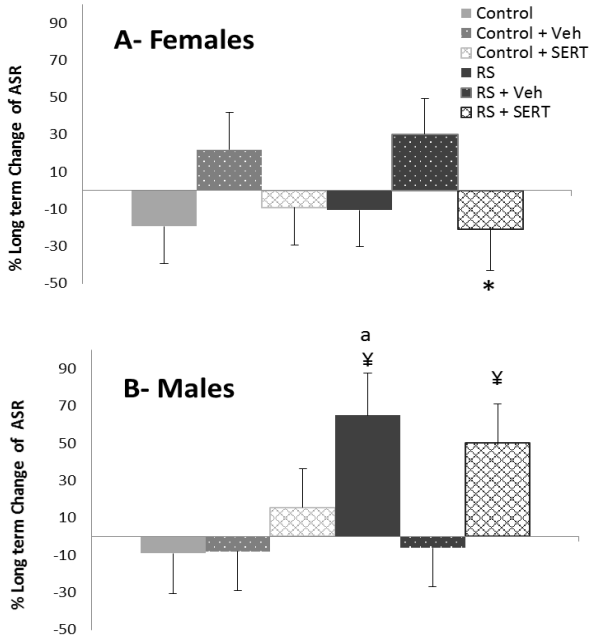
Una vez finalizado el procedimiento experimental, la amplitud del RAS no muestra valores significativamente diferentes entre los grupos de estudio (ANEXO I, Figura 5).

Dadas las grandes diferencias en los valores del RAS entre individuos, dentro de cada grupo experimental, el análisis se complementó evaluando el porcentaje (%) de alteración a largo plazo (**LTC**) de la amplitud del RAS en cada individuo (RAS4/ RAS1) × 100) −100). Con este análisis, se confirma que ni el grupo ($F_{5,73}$ = 1,22) ni el sexo ($F_{1,73}$ = 3,43) influyen en la amplitud del RAS, pero sí hay interacción del sexo con el grupo ($F_{5,73}$ = 2,93, p=0,02), afectando la modulación del RAS entre-sesiones. Cuando se separa el análisis por sexo, se aprecia un efecto

del estrés por restricción en los machos (ANEXO I, Figura 6B), efecto que se manifiesta en un incremento porcentual en la amplitud del RAS de un 74,8 ± 30,7 % en relación a los controles ($p<0,05$) y la SERT no lo revierte por completo. En las hembras, el procedimiento de inyección del fármaco ha sido el único factor que modifica ligeramente la amplitud del RAS, efecto que es revertido por la SERT (ANEXO I, Figura 6A).



ANEXO I - Figura 5. Amplitud del reflejo auditivo de sobresalto en los diferentes grupos experimentales. Valores medios de la amplitud del RAS (en unidades arbitrarias) en hembras (A) y machos (B) previamente estresados por restricción y no estresados, que fueron sometidos a 8 días de inyecciones intraperitoneales con vehículo (RS + Veh; Control + Veh), SERT (5 mg/kg/día) (RS + SERT Control + SERT), o no inyectados (Control, RS). El peso corporal se usó como covariable. N= 8 animales por grupo y sexo (valores medios ± SEM). ¥ $p < 0,05$, indica diferencias significativas entre machos y hembras en todos los grupos experimentales.

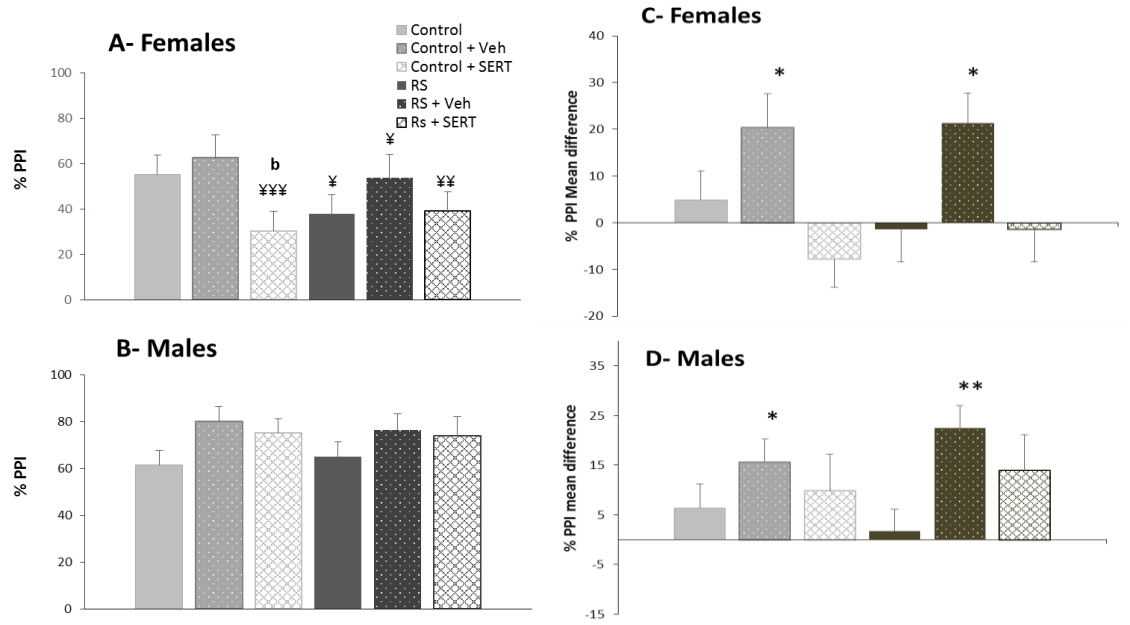


ANEXO I - Figura 6. Estudio longitudinal de la amplitud del reflejo auditivo de sobresalto. Porcentaje de los valores del cambio a largo plazo en la amplitud del RAS inducido por los paradigmas experimentales en (A) hembras y (B) machos**.** Los valores de peso corporal se toman como covariable. N = 8 animales por grupo y sexo. Medias ± SEM.*; p < 0,05, indica una disminución significativa del reflejo de sobresalto; a, p < 0,05, indica el efecto del estrés por restricción en machos (RS vs. Control). ¥, p < 0,05, indica diferencias significativas entre machos y hembras.

4.1.2.2. Análisis de la PPI

Una única inyección i.p. de fármaco o de solución salina no modifica los valores de la PPI, pero las inyecciones i.p. repetitivas sí modifican significativamente dichos valores en relación a los valores basales (grupo*sexo*días) $F_{1,58}$ = 19,92, p< 0,001. Aunque no sea significativo el efecto grupo ($F_{5,58}$ = 0,71, p=0,62), la PPI sí se modifica en relación a las dos variables combinadas, grupo*sexo ($F_{5,58}$ = 7,2, p< 0,001).

En los grupos de animales que no reciben tratamiento i.p. (**Control** o **RS**), los valores de PPI prácticamente no se modifican en ambos sexos, mientras que en los animales inyectados con suero salino (**Control+ Veh** y **RS+ Veh**) la PPI aumenta, independientemente de que los animales hayan sido previamente estresados o no (efecto pinchazo) (ANEXO I, Figura 7). El tratamiento con SERT revierte ligeramente este "efecto pinchazo" en los machos, y más notoriamente en las hembras.



ANEXO I - Figura 7. Efecto de la inyección intraperitoneal sobre el porcentaje de inhibición prepulso. Porcentaje de inhibición prepulso en (A) hembras y (B) machos. Diferencia en los valores medios de la PPI (± SEM) inducidos por los paradigmas experimentales en las hembras (C) y machos (D) para los 6 grupos experimentales: previamente estresados o no y no inyectados (RS; Control); y previamente estresados o no inyectados intraperitonealmente (i.p.) con SERT (5 mg/kg/día) (RS + SERT Control + SERT), o vehículo (RS + Veh; Control + Veh) durante 8 días**.** ¥¥¥, $p < 0,001$, ¥¥, $p < 0,01$, y ¥, $p < 0,05$, indican las diferencias significativas entre machos y hembras; b, $p < 0,05$, indica el efecto de SERT en las hembras. **, $p < 0,01$, y *, $p < 0,05$, indican el aumento significativo de la PPI como efecto de la inyección i.p. con vehículo en ambos sexos.

4.1.2.3. Evaluación de las latencias del RAS y de la PPI.

Los valores de las latencias de respuesta del RAS y de la PPI no varían con los diferentes tratamientos, pero sí se evidencia interacción entre los valores de latencia, grupo y sexo, probablemente por la diferente respuesta de cada sexo a cada protocolo experimental y por las diferencias entre sexos. En general, se observa que el procedimiento de inyección acorta la latencia de respuesta. En los machos, este efecto alcanza significación en los resultados de la medición del RAS, acortándose significativamente la latencia de respuesta independientemente del estrés anterior ($p<0,05$) (ANEXO I, Tabla 2).

Startle Latency

Experimental groups	Females	Males
	Mean values ± s.e.	Mean values ± s.e.
Control	30.1 ± 3.3	37.2 ± 3.1
Control + Veh	26.8 ± 3.2 ¥	35.7 ± 3.0*
Control + SERT	32.6 ± 3.3	37.9 ± 3.1
RS	29.5 ± 3.1 ¥	40.7 ± 3.3
RS + Veh	31.0 ± 4.5	34.6 ± 3.0*
RS + SERT	33.7 ± 3.3	40.2 ± 3.1

PPI Latency

Experimental groups	Females	Males
	Mean values ± s.e.	Mean values ± s.e.
Control	31.1 ± 2.5	34.1 ± 2.1
Control + Veh	26.7 ± 2.1	28.4 ± 2.1
Control + SERT	30.7 ± 2.3	25.6 ± 2.3 b
RS	27.2 ± 2.1 ¥¥	35.2 ± 1.8
RS + Veh	24.7 ± 3.2 ¥	32.6 ± 3.2
RS + SERT	28.6 ± 2.3	24.9 ± 2.1 b

ANEXO I - Tabla 2. Efectos de la administración i.p. de SERT sobre la latencia del RAS y de la PPI en animales que sufren estrés por restricción.
¥¥, p < 0,01 y ¥, p < 0,05, indican diferencias significativas entre machos y hembras después del procedimiento experimental; b, p < 0,05, indica el efecto de la SERT en los machos vs. Control; *, p < 0,05, indica la disminución significativa de la latencia del RAS por efecto de las inyecciones intraperitoneales con vehículo en los machos, en comparación con los valores basales. Valores medios ± SEM en los diferentes grupos experimentales (N = 8 por grupo y sexo).

4.1.2.4. Evaluación del peso corporal

Todos los animales de ambos sexos incrementan su peso de forma significativa durante los 16 días de duración del experimento (p <0,001). El tratamiento i.p. con SERT o solución salina durante 8 días no induce cambios significativos en el incremento del peso de los animales de ambos sexos, aunque en los machos (estresados o no), el tratamiento con SERT induce una ligera pérdida de peso (los machos CC+SERT y SS+SERT pesan respectivamente -8,4±3,2 y -8,3 ±3,1, en relación a sus controles).

4.1.2.5. Análisis de parámetros fisiológicos.

Un día después de la última prueba comportamental, los animales de ambos sexos de los diferentes grupos experimentales estudiados fueron expuestos a un nuevo factor

estresante, el estrés por inmovilización (**IMO**). Se analizan diferentes parámetros hematológicos que se comparan entre los diferentes grupos de animales (**No-IMO**) (ANEXO I, experimento 3).

4.1.2.5.1. Análisis hematológico.

Los animales sometidos a estrés por **IMO** (sean Controles o RS) y no inyectados, presentan un aumento en el hematocrito (%), en la concentración de hemoglobina corpuscular media (MCH) (pg) y en los factores de coagulación (número de plaquetas y volumen plaquetario), en comparación con los animales **No-IMO**, sin que el número de eritrocitos se haya cambiado (ANEXO I, Tabla 3). Sin embargo, los animales previamente sometidos a inyecciones diarias (Control+ Vehículo) no experimentaron alteraciones relativamente a los animales **No-IMO**, diferenciándose de sus controles (Tabla 3), efecto que no fue revertido completamente por el tratamiento con SERT.

Experimental groups		Erythrocytes (10^6 /µl)	Hemoglobin (g/dl)	Hematocrit (%)	MCH (pg)	Platelet (10^3 /µl)	MPV fL(µm^3)
Females	No-IMO	7.8 ± 0.1	14.9 ± 0.3	40.4 ± 0.7	19.6 ± 0.2	914.1.5 ± 60.4	7.9 ± 0.2
	Control	7.8 ± 0.3	15.7 ± 0.3 ¥	45.4 ± 1.6** ¥	19.9 ± 0.7	1036.3 ± 62.8	10.9 ± 0.3**
	Restraint stress	7.9 ± 0.3	16.6 ± 0.4	44.0 ± 1.6	20.7 ± 0.4	1411.2 ± 71.1**a	11.0 ± 0.5*
	Control + Vehicle	7.4 ± 0.2 ¥	13.8 ± 0.6 iii	37.9 ± 0.9 ii ¥	17.8 ± 0.6 **iii	771.6 ± 88.2	10.1 ± 0.5*
	Control + SERT	7.0 ± 0.5	15.2 ± 0.6	39.6 ± 1.7	20.6 ± 0.5 bb	866.2 ± 128.3	10.5 ± 0.7*
		F=1.3, ns	F=11.3, p<0.001	F=64.0, p<0.001	F=12.9, p<0.001	F=10.4, p<0.001	F=5.5, p<0.001
Males	No-IMO	8.2 ± 0.2	15.4 ± 0.2	40.7 ± 0.5	18.7 ± 0.1	908.5 ± 61.1	7.7 ± 0.1
	Control	8.4 ± 0.2	17.2 ± 0.2	47.6 ± 1.0***	19.7 ± 0.3	927.3 ± 62.8	10.8 ± 0.3**
	Restraint stress	7.7 ± 0.3	16.2 ± 0.3	42.1 ± 1.2	20.9 ± 0.2**	1254.8 ± 67.1**a	11.4 ± 0.5**
	Control + Vehicle	8.1 ± 0.2	14.5 ± 0.4 iii	43.1 ± 2.0 ii	17.9 ± 0.3 ii	707.6 ± 108.2	9.6 ± 0.4
	Control + SERT	8.1 ± 0.4	14.6 ± 0.5 ii	38.5 ± 1.5 iii	20.4 ± 0.5 bb	728.2 ± 124.3	12.3 ± 0.6** bb
		F=1.9, ns	F=9.3, p<0.001	F=11.7, p<0.001	F=9.5, p<0.001	F=8.5, p<0.001	F=10.5, p<0.001

ANEXO I - Tabla 3. Efectos del estrés por inmovilización sobre parámetros hematológicos. Valores plasmáticos obtenidos en animales de ambos sexos 10 minutos después de la exposición al estrés por inmovilización (**IMO**) (RS, Control+ vehículo, Control+ SERT) o no expuestos a IMO (**No-IMO**).Abreviaturas: MCH, concentración de hemoglobina corpuscular media; MPV, volumen plaquetario medio. ¥, $p < 0,05$, indica diferencias significativas entre machos y hembras; ***, $p < 0,001$, **, $p < 0,01$ y *, $p < 0,05$, indican una diferencia significativa cuando se comparan con los valores de los animales No-IMO; a, $p < 0,05$, indica un efecto del estrés por restricción (diferente de los controles); bb, $p < 0,0$, indica un efecto de la SERT (diferente del vehículo); iii, $p < 0,001$, ii, $p < 0,01$, y i, $p < 0,05$, indican un efecto del procedimiento intraperitoneal (diferente de los controles). Medias ± SEM en los diferentes grupos experimentales (N = 6 por grupo y sexo).

4.1.2.5.2. Bioquímica del suero

Como se muestra en la Tabla 4, la condición de estrés agudo (**IMO**) aumenta los niveles de casi todos los parámetros metabólicos estudiados en el suero de ambos sexos. Aun así, las proteínas albúmina, bilirrubina y las enzimas LDH y GOT incrementaron significativamente en los animales previamente estresados (ya sea como consecuencia de la restricción o por efecto de la inyección i.p. con vehículo), en comparación con los animales sin perturbaciones (Anexo I, Tabla 4); el tratamiento con SERT contrarresta, en parte, esos efectos. No se observaron diferencias con el sexo en los parámetros estudiados, pero en los machos en particular, el **RS** exacerbó los efectos del estrés por **IMO**, arriba descritos (Tabla 4).

Experimental groups		T-pro g/dl	Alb g/dl	T- bil mg/dl	GOT IU/L	GPT IU/L	LDH IU/L
Females	No- IMO	6.0 ± 0.2	3.3 ± 0.1	0.30 ± 0.1	54.6 ± 8.7	27.7 ± 5.8	423.4 ± 122
	Control	6.2 ± 0.1	3.5 ± 0.1	0.60 ± 0.1	107.9 ± 8.2**	37.5 ± 6.8	2589.2 ± 350***
	Restraint stress	6.7 ± 0.1	3.8 ± 0.1 **	0.55 ± 0.1	129.4 ± 8.1***	29.2 ± 4.7	3579.7 ± 247***a
	Control + Vehicle	6.1 ± 0.2	3.8 ± 0.1**	0.43 ± 0.1	117.8 ± 6.8***	22.9 ± 4.3	3324.2 ± 230***
	Control + SERT	5.3 ± 0.2	3.2 ± 0.1 bb	0.42 ± 0.1	122.4 ± 8.1**	18.4 ± 4.8	1694.8 ± 271 b
		F=6.1, p=0.001	F=8.4, p<0.001	F=1.7, p=ns	F=18.4, p<0.001	F=2.9, ns	F=18.8, p<0.001
Males	No- IMO	6.2 ± 0.2	3.4 ± 0.1	0.30 ± 0.1	68.2 ± 9.1	29.7. ± 5.8	485.7 ± 242
	Control	6.2 ± 0.1	3.4 ± 0.1	0.36 ± 0.0	101.8 ± 8.5	34.7 ± 4.2	1614.9 ± 263 *¥
	Restraint stress	6.6 ± 0.2	3.8 ± 0.1 a	0.70 ± 0.1* a	136.5 ± 7.1*** a	31.2 ± 5.7	3081.7 ± 237***a
	Control + Vehicle	6.2 ± 0.2	3.7 ± 0.1	0.37 ± 0.1	109.5 ± 7.5*	37.2 ± 6.0 ¥	3314.2 ± 260***ii
	Control + SERT	5.5 ± 0.1	3.4 ± 0.0	0.28 ± 0.1	106.4 ± 8.0	18.5 ± 4.8 b	1564.8 ± 272 bb
		F=3.2, p=0.02	F=3.4, p=0.02	F=4.7, p=0.003	F=8.2, p<0.001	F=4.2, p=0.006	F=12.5, p<0.001

ANEXO I - Tabla 4. Parámetros séricos analizados en los diferentes grupos de estudio. *****p < 0,001, ** p < 0,01 y * p < 0,05, indican una diferencia significativa en comparación con los animales **No-IMO; a p < 0,05, indica el efecto del estrés por restricción (diferente de los controles); bb, p < 0,01 y b, p < 0,05, indican el efecto de SERT (diferente de Control + vehículo); ii, p < 0,01, indica el efecto del procedimiento intraperitoneal (diferente de los controles); ¥, p < 0,05, indica diferencias significativas entre machos y hembras. Valores medios ± S.E. (N = 6 por grupo y sexo). Abreviaturas: T-Pro, proteína total; Alb, albúmina; T-bil, bilirrubina total; GOT, Glutamato-oxalacetato transaminasa; GPT, Glutamato-piruvato-transaminasa; LDH, lactato deshidrogenasa.

4.1.2.5.3. Análisis del leucograma

Como se muestra en la Tabla 5 del ANEXO I, la **IMO** no afectó el perfil de leucocitos en los animales Control. Sin embargo, en animales que experimentaron un factor estresante previo, se observó leucopenia en ambos sexos. Esta modificación fue más evidente en los animales sometidos al procedimiento de inyección diaria, efecto que no fue revertido por la SERT. En ambos sexos el número total de linfocitos fue significativamente menor en los animales inyectados i.p. que en los controles (Tabla 5). En las otras células leucocitarias analizadas (granulocitos, basófilos y eosinófilos) no se encontraron diferencias por efecto de los tratamientos (datos no mostrados). Por otra parte, no se encontraron diferencias dependientes

del sexo en ningún otro parámetro estudiado, aunque el **RS** afecta más los machos que a las hembras.

Experimental Groups		Leukocytes (10^3/ µ l)	Neutrophils (10^3/ µ l)	Lymphocytes (10^3/ µ l)	Monocytes (10^3/ µ l)
Females	No-IMO	4.8 ± 0.4	0.9 ± 0.1	3.5 ± 0.4	0.08 ± 0.01
	Control	5.2 ± 0.4	0.8 ± 0.1	4.0 ± 0.3	0.07 ± 0.01
	Restraint stress	3.2 ± 0.7	0.4 ± 0.1	2.7 ± 0.5	0.04 ± 0.01
	Control + Vehicle	1.5 ± 0.4***iii	0.4 ± 0.4**ii	0.9 ± 0.7**iii	0.03 ± 0.01*ii
	Control + SERT	1.1 ± 0.7**iii	0.4 ± 0.3*i	0.5 ± 0.5**iii	0.05 ± 0.01*
		F=13.2, p<0.001	F=7.2, p<0.001	F=11.5 p<0.001	F=5.6, p=0.001
Males	No-IMO	5.9 ± 0.5	0.9 ± 0.1	3.9 ± 0.2	0.09 ± 0.01
	Control	5.8 ± 0.5	0.9 ± 0.1	4.7 ± 0.3	0.10 ± 0.01
	Restraint stress	3.5 ± 0.6*a	0.6 ± 0.1	2.7 ± 0.4	0.03 ± 0.01*aa
	Control + Vehicle	2.6 ± 0.5**iii	0.7 ± 0.1	1.8 ± 0.8*iii	0.05 ± 0.01 i
	Control + SERT	1.7 ± 0.6***iii	0.4 ± 0.1	0.6 ± 0.6**iii	0.06 ± 0.01*ii
		F=11.3, p<0.001	F=2.9, p=0.031	F=9.4, p<0.001	F=7.6, p<0.001

ANEXO I - Tabla 5. Efectos del estrés inducido por inmovilización sobre la fórmula leucocitaria en los animales de estudio. Medias ± SE**M** en los diferentes grupos experimentales (N = 6 por grupo). ***p < 0,001, ** p < 0,01 y * p < 0,05, indican una diferencia significativa en comparación con los animales No-IMO; aa, p < 0,01, a, p < 0,05, indica el efecto del estrés por restricción en los machos (diferente de los Control); iii, p < 0,001, ii, p < 0,01 y i, p < 0,05, indican el efecto del procedimiento intraperitoneal (diferente de los Control).

4.2. Animales adultos sometidos a estrés prenatal y tratados con Sertralina durante adolescencia

4.2.1. Efecto del estrés prenatal sobre el peso corporal durante la infancia

Las ratas recién nacidas (P1) hembras que sufrieron estrés prenatal presentan un menor peso corporal con relación a las que no sufrieron **PS** (p<0,01). En los machos, no hay diferencias entre el peso de los animales expuestos al **PS** y sus controles. Las diferencias ponderales desaparecen al destete, pero a los 28 días de edad (P28) el efecto se revierte, y las hembras que

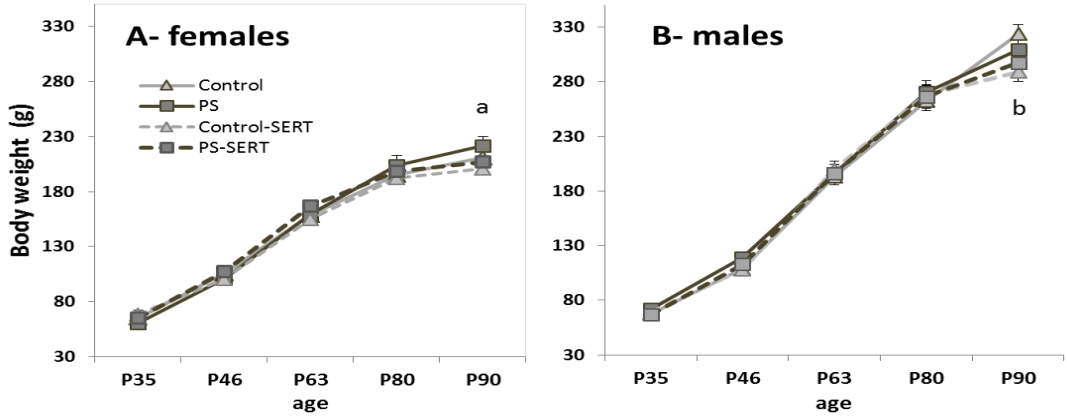
sufrieron **PS,** exhiben un mayor peso que sus controles (63,5± 2,46 vs 57,9± 2,24). (ANEXO II, Tabla 1).

	Females		Males	
	Control	Prenatal stress	Control	Prenatal stress
P1	6.4 ± 1.1	5.9 ± 1.5^{aa}	6.7 ± 1.3	6.2 ± 1.6
P21	40.8 ± 2.4	39.8 ± 2.1	39.5 ± 2.2	43.2 ± 2.7
P28	57.8 ± 2.9	64.8 ± 2.7^{a}	68.2 ± 2.5	66.2 ± 2.8

ANEXO II- Tabla 1. Efectos del estrés prenatal sobre los valores de peso corporal.
Peso corporal de los animales antes del inicio del tratamiento farmacológico: al nacer (P1), en el destete (P21) y a los 28 días de edad (P28) (N= 18-20 animales por grupo y sexo). aa, $p < 0,01$, y a, $p < 0,05$, indican un efecto del estrés prenatal (Medias ± SEM).

4.2.2. Efectos del estrés prenatal y de Sertralina sobre el peso corporal desde la adolescencia hasta la edad adulta

Durante la fase de adolescencia y una vez iniciado el tratamiento con SERT, es evidente la ganancia de peso con la edad ($p < 0,001$), y la gran diferencia en la tasa de crecimiento entre machos y hembras ($p < 0,001$). Analizando las diferencias de peso en conjunto, se aprecia que -mientras al inicio de esta fase no se encuentren diferencias por efecto del estrés precoz ni del fármaco- a los 90 días de edad y tras 2 meses de tratamiento con SERT, el peso en los animales tratados disminuye, siendo significativo el efecto en los machos (ANEXO II, Figura 1 B). En las hembras, se pone de manifiesto además un efecto del estrés precoz, afectando a la ganancia del peso; las hembras **PS** ganan más peso que sus controles y la SERT lo revierte (ANEXO II, Figura 1 A).



ANEXO II- Figura 1. Efectos del estrés prenatal y del tratamiento con SERT sobre el aumento de peso corporal. Valores medios (± SEM) del peso de los animales desde la adolescencia hasta la edad adulta. (A) hembras; (B) machos. (N= 9-11 animales por grupo y sexo). a, $p < 0,05$, indica el efecto del estrés prenatal; b, $p < 0,05$, indica un efecto de la SERT en los animales control

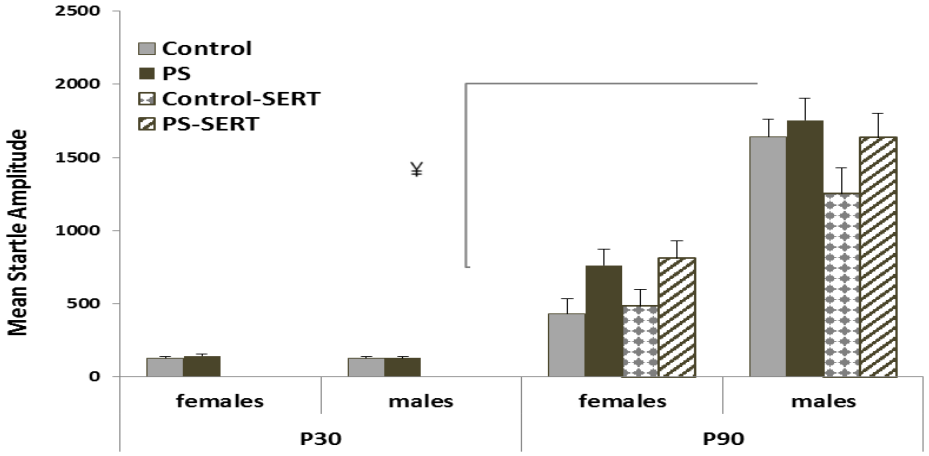
4.2.3. Evaluación de la amplitud del RAS y de su habituación

En general, se observa un incremento significativo de amplitudes del RAS en todos los animales desde la edad P30 hasta P90 ($F_{1,77}$ =312,3), p<0,001. El incremento no depende del grupo experimental ($F_{3,77}$ =2,06), aunque, como cabría esperar, sí depende del sexo ($F_{1,77}$ =69,6), p<0,001.

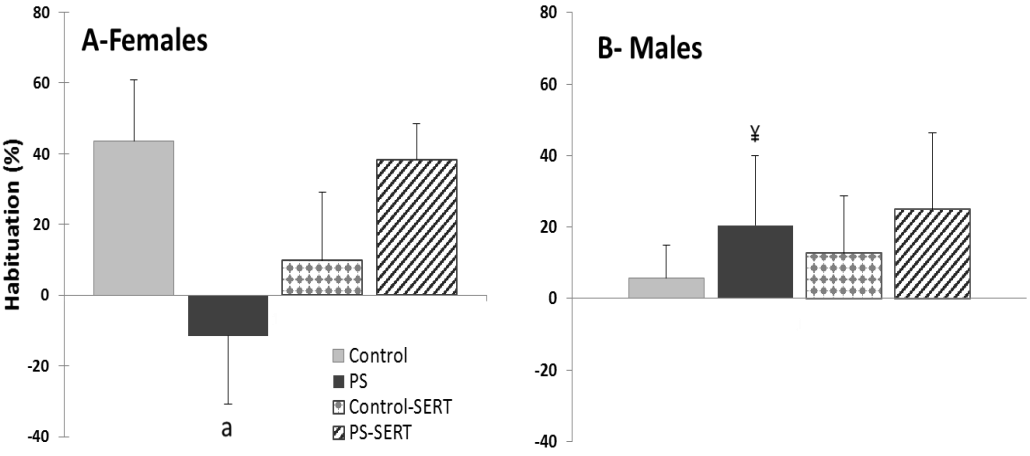
Mientras que a los 30 días de edad aún no se encuentran diferencias entre machos y hembras, éstas se manifiestan en el día P90. En los machos, la amplitud de respuesta al RAS es significativamente superior en todos los grupos experimentales (p<0,01). El estrés prenatal no influye sobre este parámetro a la edad P30 ($F_{3,77}$ = 2,06, p=0,113), pero a los 90 días de edad, ya se comienzan a apreciar ligeras diferencias entre los grupos control y estresado, presentando las ratas estresadas de ambos sexos mayores valores del RAS, independientemente de si éstas recibían o no SERT, pero sin significación estadística ($F_{3,77}$ = 2,67, p=0,058) (ANEXO II, Figura2). Los resultados indican que el tratamiento con el fármaco SERT no induce cambios en el RAS.

Cuando se analiza la habituación a la respuesta de sobresalto intra-sesión (análisis de la diferencia entre el primero y el último bloque, expresada en porcentaje), se observan diferencias entre los diferentes grupos experimentales. El estrés prenatal disminuye la habituación en las hembras (p = 0,034) (ANEXO II, Figura 3), manteniéndose la respuesta de

sobresalto a lo largo de la sesión. Este efecto se revierte por la SERT. Los machos expuestos a **PS** no manifiestan esta modificación en la habituación, por lo que se diferencian significativamente de las hembras de su grupo (p = 0,03).



ANEXO II- Figura 2. Efecto del estrés y de la Sertralina sobre la amplitud del RAS. Valores de RAS expresado en unidades arbitrarias en los días posnatales P30 y P90 en los animales de ambos sexos sometidos o no al estrés prenatal (PS y CONTROL), y sometidos o no a estrés prenatal y tratados con SERT (Control-SERT y PS-SERT) (5 mg/kg/día). Medias ± SEM (en el P30, N = 18-20 animales por grupo y sexo; en el P90, N = 9-11 por grupo y sexo). ¥, p < 0,05, indica un efecto de sexo en todos los grupos experimentales.

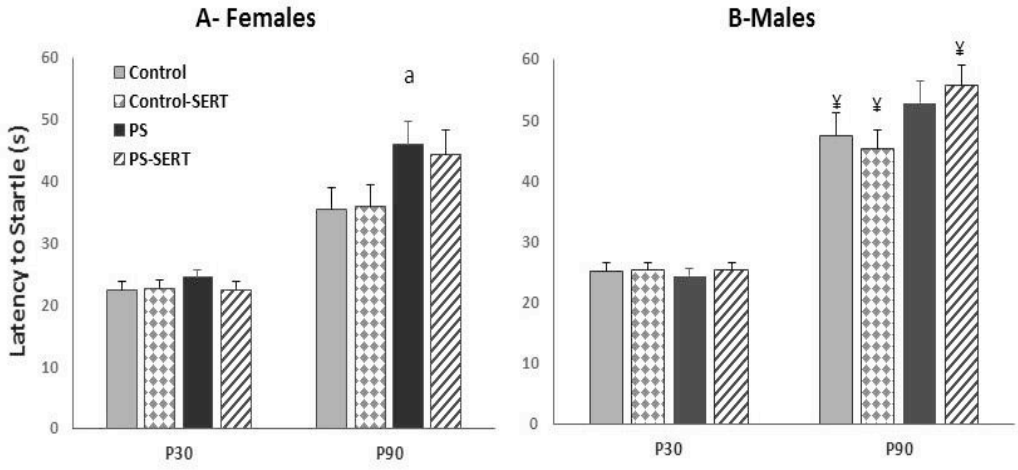


ANEXO II- Figura 3. Efecto del estrés prenatal y del tratamiento con Sertralina sobre la habituación del RAS. Valores de habituación del RAS expresados en % en las hembras (A) y machos (B) en el día posnatal P90. a, p < 0,05, indica el efecto del estrés prenatal; ¥, p < 0,05, indica un efecto del sexo. Medias ± SEM

4.2.4. Latencia del RAS

El estrés prenatal incrementa ligeramente la latencia de respuesta del RAS (ANEXO II, Figura 4), lo que provoca que las hembras que sufren **PS** muestren respuestas más lentas en relación a sus controles (p=0,046), efecto que la SERT no modifica.

También encontramos diferencias en la latencia de respuesta del RAS entre machos y hembras en todos los grupos, excepto el grupo de animales que sufren **PS**, siendo los machos los que presentan mayores valores de latencia del RAS (son los más lentos en responder) que las hembras (p < 0, 001). Por otra parte, se observa una fuerte correlación entre la amplitud del RAS y su latencia (r=0,74, p<0,001), lo que implica que los animales más lentos en responder, son también los que responden con mayor intensidad.



ANEXO II- Figura 4. Latencia del RAS en los diferentes grupos experimentales.
Valores de latencia del RAS en la edad de P30 y P90 en hembras (A) y machos (B). Medias ± SEM. a, p < 0,05 indica el efecto del estrés prenatal en las hembras; ¥, p < 0,05, indica el efecto del sexo en todos los grupos excepto los animales PS

4.2.5. Prueba de Campo Abierto

4.2.5.1. Actividad exploratoria total

La actividad exploratoria total se evaluó, sumando la actividad exploratoria externa (**OA**), con la actividad exploratoria interna (**IA**) y la exploración en vertical (***rearing***). Existe una alteración significativa de la actividad total con la exposición repetida a la prueba (en los 3 días consecutivos) ($F_{2,49}$ = 4,94, p=0,009), sin influencia del grupo o del sexo. La actividad exploratoria total no se encuentra significativamente alterada entre los diferentes grupos experimentales

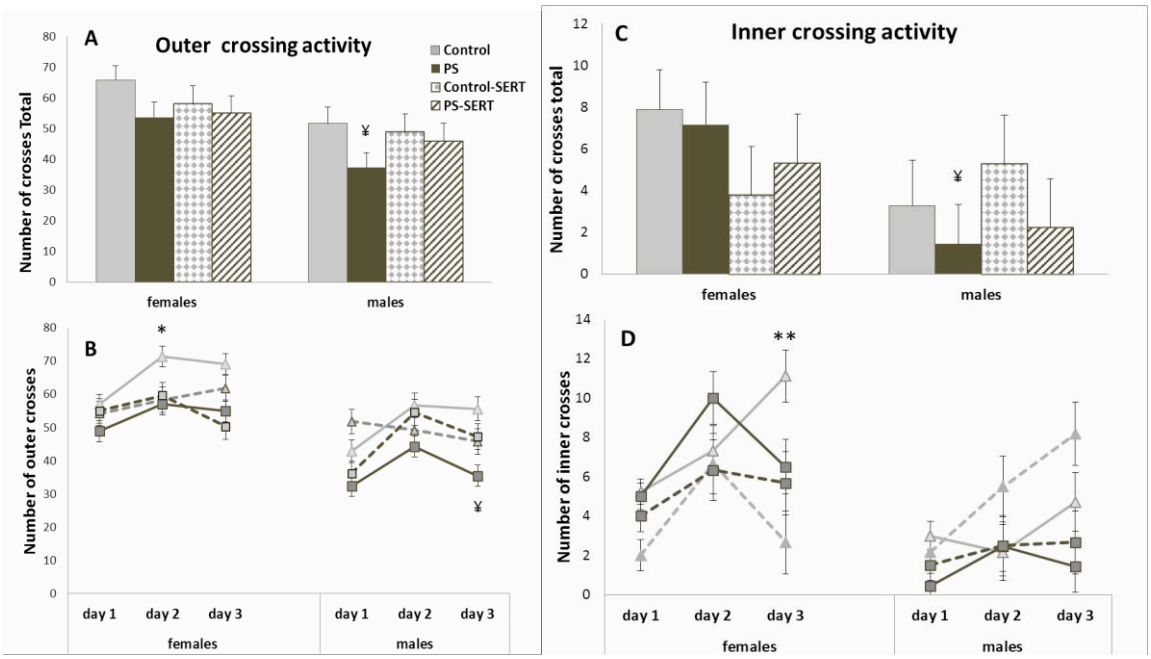
($F_{3,49}$ = 1,74, p=0,84), pero se presentan diferencias entre los dos sexos ($F_{1,49}$ =11,1 p=0,002); las hembras son, en general, más activas que los machos, siendo la diferencia significativa entre los animales control y los sometidos a estrés prenatal. En los animales tratados con SERT, las diferencias entre machos y hembras no resultaron significativas.

4.2.5.2 Actividad exploratoria externa (OA)

No se aprecian diferencias en la actividad exploratoria externa entre los grupos, aunque los animales estresados de ambos sexos presentan una ligera disminución de la actividad en relación a sus controles (PS vs. CONTROL: -12,15± 6,8 número de cruces en las hembras; y -14,38 ± 7,2 en los machos) (ANEXO II, Figura 5A). Ratas machos y hembras sometidas a estrés prenatal manifiestan un comportamiento exploratorio diferente (p=0,02); los machos que sufren **PS** exploran significativamente menos la zona exterior del CA que las hembras del mismo grupo (ANEXO II, Figura 5B).

4.2.5.3. Actividad exploratoria interna (IA)

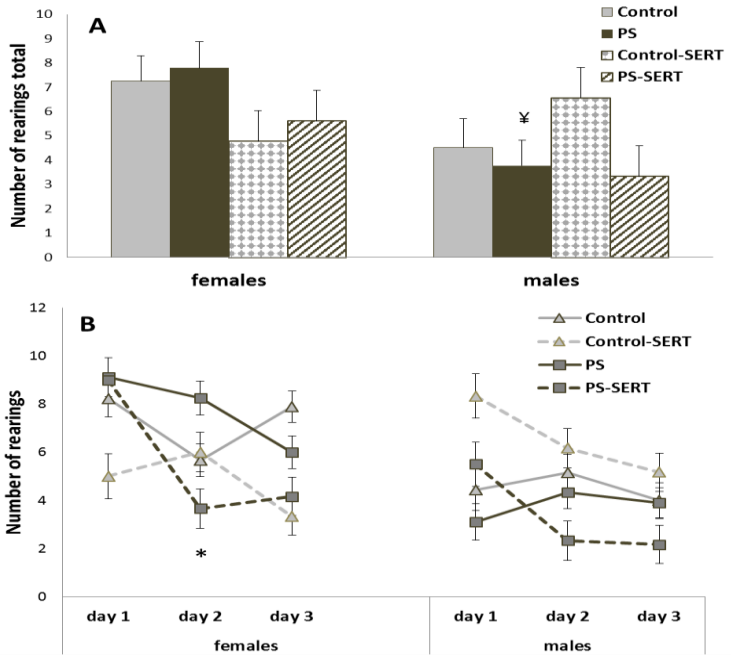
No se aprecian diferencias entre controles y animales sometidos a estrés prenatal ($F_{3,49}$ = 0,26, p=0,81) en la **IA**, pero sí un efecto marginal del sexo, (p=0,058) debido a los diferentes valores entre machos y hembras estresados; los machos sometidos a **PS** exploran significativamente menos el interior del CA que las hembras del mismo grupo (-5,73 ± 1,9 cruces totales, p= 0,045). Las diferencias son significativas los dos primeros días (p= 0,019 y p= 0,049 respectivamente) (ANEXO II, Figura 5C). La administración de SERT modula este parámetro de forma diferente en machos y hembras, disminuyendo la capacidad de exploración interna en las hembras controles (Control-SERT: 3,78± 2,3 vs. Control: 7,89 ± 1,9) y estresadas (PS-SERT: 5,33 ±2,3 vs. PS: 7,16 ± 2,0). En los machos, la SERT no afecta la actividad exploratoria interna y parece ser que es el **PS** el factor que afecta dicha actividad (ANEXO II, Figura 5D).



ANEXO II- Figura 5. Efectos del estrés prenatal y del tratamiento con SERT en la prueba de campo abierto. Valores de actividad exploratoria horizontal donde se muestra el número de cruces externos_ A y B) y de cruces internos (C y D) observados, durante las sesiones de 3 minutos en 3 días consecutivos. Medias ± SEM (N = 9 por grupo y se**xo).** ¥, p < 0,05, indica un efecto del sexo en los animales PS. *, p < 0,05, y **, p < 0,01, indica un aumento significativo en el número de cruces externos e internos en las hembras Control.

4.2.5.4. Actividad exploratoria vertical (*rearings*)

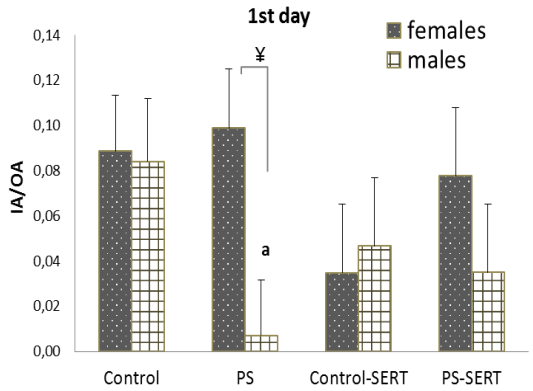
No se aprecian grandes diferencias en la actividad exploratoria vertical entre los grupos de estudio (p=0,63), siendo de nuevo el sexo el único factor influyente (p=0,035), especialmente en los animales del grupo PS, donde los machos muestran valores significativamente menores de ***rearings*** que las hembras (-4,1 ± 1,1 p=0,01) (ANEXO II, Figura 6).



ANEXO II- Figura 6. Efectos del estrés prenatal y del tratamiento con SERT en el número de *rearings* en la prueba de campo abierto. Se muestran el número de *rearings* total (A) y en cada uno de los tres días de la prueba (B) en los diferentes grupos experimentales (medias ± SEM). ¥, $p < 0,05$, indica un efecto del sexo en los animales PS. *, $p < 0,05$, indica una disminución significativa en el número de *rearings* desde el día 1 al día 2 de prueba, en las hembras PS-SERT.

4.2.5.5. Cociente *IA/OA*

Calculado el cociente entre la actividad exploratoria interna y la actividad exploratoria externa (***IA/OA***), globalmente los 3 días de estudio en el CA y de forma individual cada día, se aprecia que los machos sometidos al estrés prenatal muestran señales de ansiedad, presentando dificultades para explorar el centro del Campo desde el primero día (ANEXO II, Figura 7), efecto que la SERT revierte.



ANEXO II- Figura 7. Relación entre la actividad exploratoria interna y externa (IA/OA) el primer día en el campo abierto a, p < 0,05, indica un efecto del estrés prenatal en los machos; ¥, p < 0,05, indica diferencias por efecto del sexo.

4.2.6. Estudio del leucograma

La fórmula leucocitaria se modifica por el estrés gestacional, aunque no lo hace en función del sexo (ANEXO II, Tabla 2). El análisis cuantitativo del recuento de leucocitos indicó una pronunciada leucopenia en animales previamente estresados. El tratamiento con SERT no afectó a la fórmula leucocitaria en los animales del grupo control, pero en los animales estresados, revirtió los valores leucocitarios a niveles normales.

Group	Leukocytes ($10^3/\mu l$)	Neutrophils ($10^3/\mu l$)	Lymphocytes ($10^3/\mu l$)	Monocytes ($10^3/\mu l$)	Basophils ($10^3/\mu l$)
Control	5.45 ± 0.6	0.86 ± 0.1	4.34 ± 0.3	0.09 ± 0.01	0.01 ± 0.001
Control-SERT	4.78 ± 0.7	1.16 ± 0.2	3.53 ± 0.6	0.07 ± 0.02	0.02 ± 0.003
PS	1.85 ± 0.5 aa bb	0.63 ± 0.1	0.93 ± 0.4 aa b	0.06 ±0.01	0.003 ± 0.002 a
PS-SERT	4.43 ± 0.6	1.00 ± 0.2	3.22 ± 0.6	0.07 ± 0.02	0.01 ± 0.003
	F=16.3, p<0.001	F=1.9, p=0.14	F= 18.3, p<0.001	F= 0.8, p=0.49	F= 7.2, p<0.01

ANEXO II- Tabla 2. Valores de varios parámetros plasmáticos en los grupos experimentales. Indicadores plasmáticos obtenidos en la sangre arterial en animales de ambos sexos en el día P98. **Medias ± SEM** (N= 6 por grupo). a, p < 0,05 y aa, p < 0,001, indican un efecto del estrés prenatal; b, p < 0,05 y bb, p < 0,001, indica el efecto de SERT.

4.3. Evaluación de la Conducta tras someter a los animales de ambos sexos a estrés prenatal y tratarlos con Sertralina, después de una sesión de choques inescapables

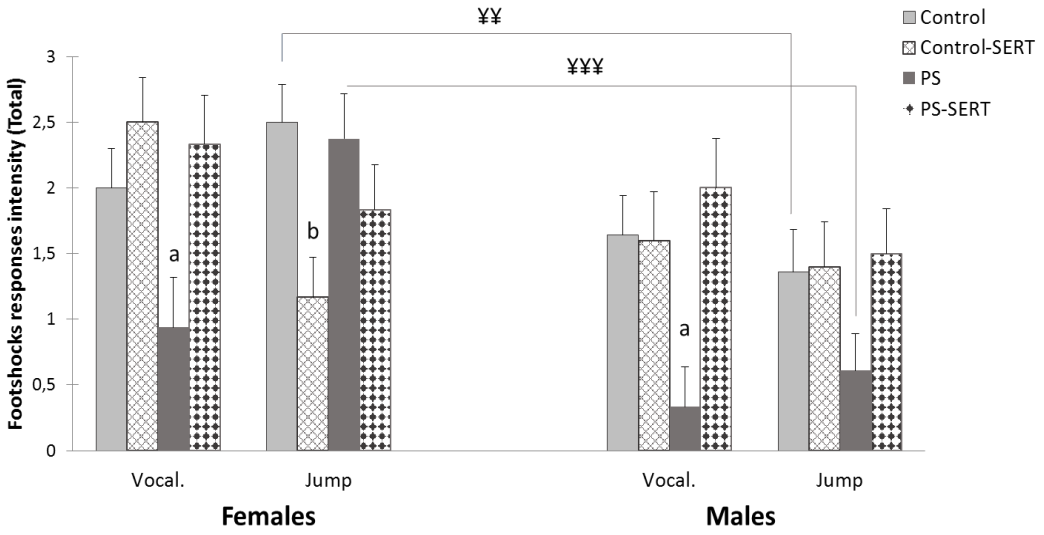
4.3.1. Choques Inescapables (IS)

4.3.1. 1. Reacciones a los choques

La cuantificación de la sensibilidad de los animales estudiados a los choques inescapables (**IS**) a los 88 días de edad (P88), muestra diferencias en función del grupo y del sexo en cuanto a las respuestas de **saltos** (saltos involuntarios), **vocalizaciones** (chillidos) y el porcentaje de tiempo de permanencia en ***freezing***.

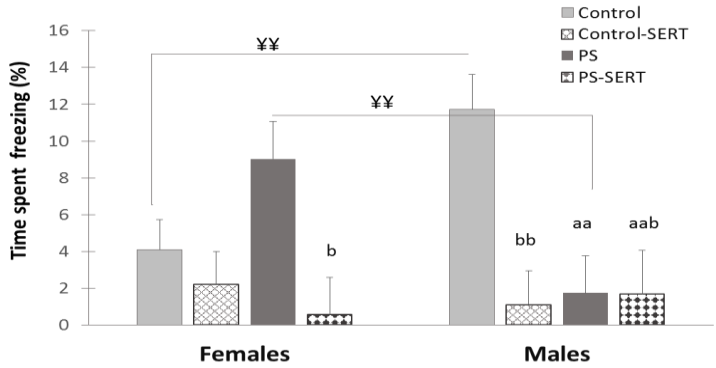
Como se aprecia en la Figura 1 (ANEXO III), el número e intensidad de las **vocalizaciones** resulta afectado por el estrés prenatal en ambos sexos - los animales estresados chillan menos y con menor intensidad en respuesta a los choques eléctricos que sus controles- y estas diferencias son significativas entre las hembras no tratadas (**PS** vs. **Control**: -1,51 ± 0,53, p= 0,036). Por otra parte, la administración de SERT revierte el efecto del estrés sobre estos parámetros (número e intensidad de los chillidos).

En relación a los saltos, existen diferencias en cuanto al género; en los machos, una vez más, el estrés prenatal afecta la respuesta a los **IS** disminuyéndola (-0,75± 0,47, n.s.) y la SERT lo revierte. Por el contrario, en las hembras, la administración del antidepresivo es el único factor que modifica la conducta de salto, disminuyendo su número e intensidad en reacción a cada choque (ANEXO III, Figura 1). Las hembras controles que no tomaron el fármaco muestran una conducta de salto más aumentada que los machos (Control: +1,42 ± 0,47, p=0,004; PS: +1,74 ± 0,46, p<0,001).



ANEXO III- Figura 1. Efectos del estrés prenatal y del tratamiento con Sertralina sobre las reacciones a los choques inescapables. Número e intensidad total de vocalizaciones y de saltos en los animales de los diferentes grupos experimentales de ambos sexos (0 indica que no responde; 3 indica que responde con intensidad máxima a los 3 *footschoks*). a, $p < 0,05$, indica el efecto del estrés prenatal; b, $p < 0,05$, indica el efecto de SERT; ¥¥, $p < 0,01$; ¥¥¥, $p < 0,001$, indican un efecto del sexo en animales no tratados. Cada barra representa la suma de la intensidad de respuesta por presentación de los 3 choques, expresada en medias+ SEM.

Analizando el tiempo en el cual los animales permanecen en estado de ***freezing*** durante la sesión de choques, se aprecia un efecto importante de la SERT, que induce una disminución de ese parámetro en ambos sexos. Puede observarse también un efecto del **PS** actuando de forma opuesta, tanto en machos como en hembras. En los machos que sufren PS el tiempo que permanecen en estado de ***freezing*** disminuye, mientras que en las hembras sometidas a PS aumenta (ANEXO III, Figura 2).



ANEXO III- Figura 2. Efectos del estrés prenatal y del tratamiento con Sertralina sobre el tiempo que las ratas permanecen en estado de *freezing* durante la sesión de choques inescapables. aa, $p < 0,01$, indica un efecto del estrés prenatal en los machos; bb, $p < 0,01$; b, $p < 0,05$, indican un efecto de la SERT; aab, $p < 0,01$, indican diferencias entre los animales PS-SERT y los controles. ¥¥, $p < 0,01$, indica un efecto del sexo en los animales no tratados (Medias + SEM).

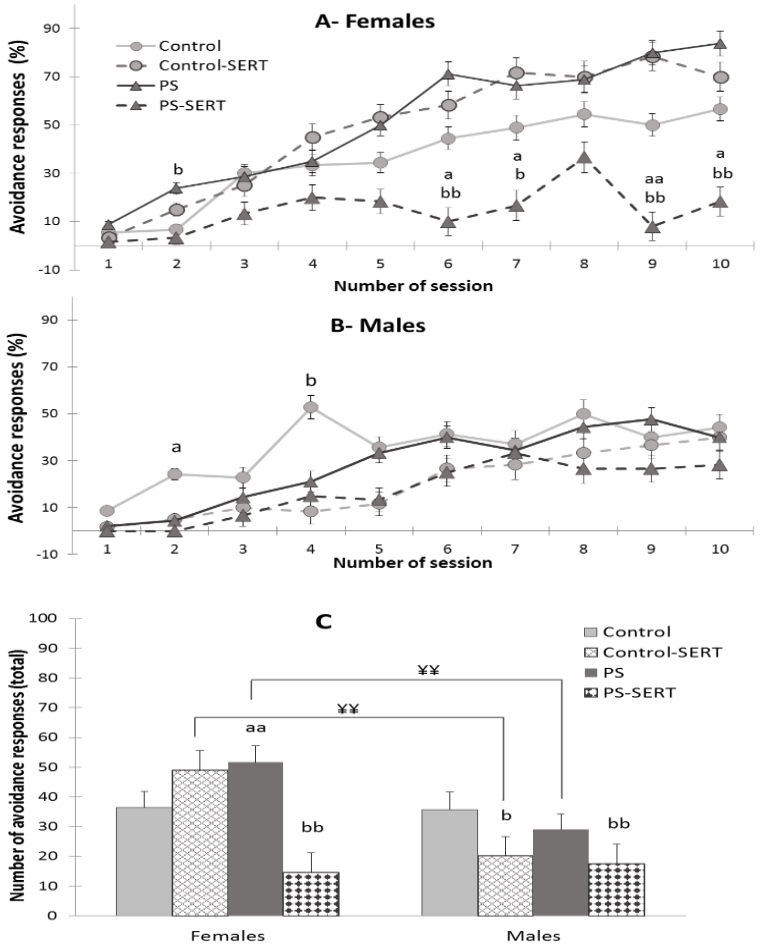
4.3.2. Choques Escapables

4.3.2.1. Respuesta de evitación

Al someter los animales a **IS**, detectamos diferencias grandes en función del sexo y del grupo en cuanto a las respuestas de evitación. En los machos, un bajo porcentaje de animales logró superar los criterios de condicionamiento propuestos en las pruebas estandarizadas (el 22% de los machos PS; 14% de los machos Control; 16% de los machos PS-SERT y 16% de los machos Control-SERT)**.**

Entre los grupos de hembras, encontramos grandes diferencias en la capacidad de evitación ($p = 0,001$): las hembras estresadas tuvieron la mayor tasa de éxito y no parece que la exposición previa a los **IS** produzca ninguna afectación (el 87,5% de las hembras PS cumplieron los criterios de condicionamiento propuestos frente al 44,4% de hembras controles); también, el fármaco parece revertir el efecto de los **IS** en las hembras controles, ya que el 66,6% de las hembras Control-SERT logró evitar los choques; en las hembras estresadas y tratadas (PS-SERT) apenas el 16% aprende a evitar los choques. Se constata, además, un adelantamiento de las hembras PS en el tiempo necesario para que se condicionen (ANEXO III, Figura 3B).

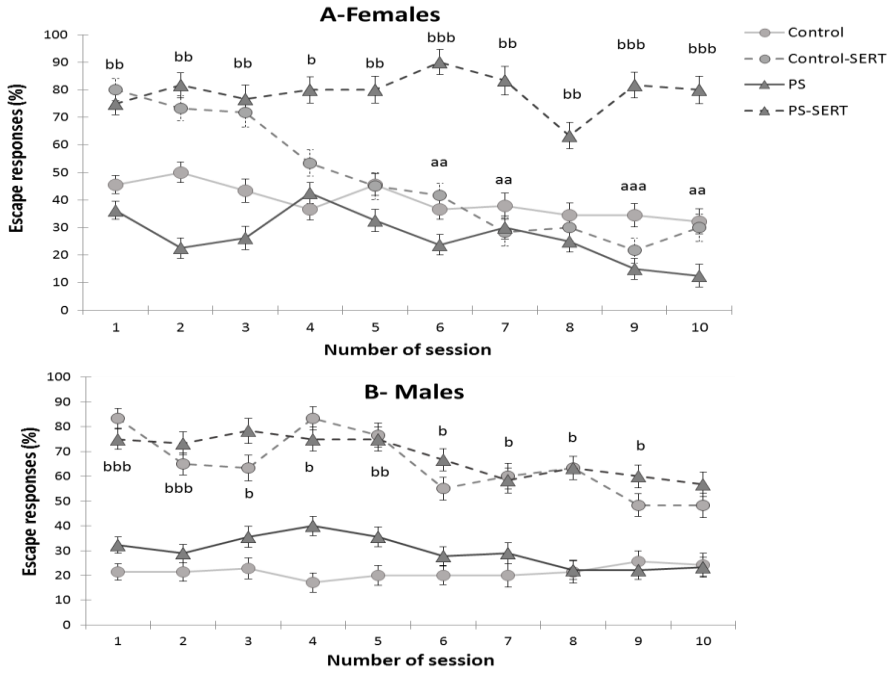
El efecto del sexo en la capacidad de evitación, arriba apreciado, es debido a la proporción superior de ratas hembras que cumplen los criterios de condicionamiento en relación a los machos. Cuando se analizan las diferencias entre sexo para cada grupo, las pruebas *post-hoc* señalan un número superior de respuestas de evitación en las hembras que en los machos, específicamente en los grupos PS (+ 22,6 ± 3,9%, $p < 0,01$) y Control-SERT (+ 28,8 ± 4,6%, $p < 0,01$).



ANEXO III- Figura 3. Respuestas de evitación entre sexos y grupos.
Valores de evitación en sesiones diarias en hembras (A) y machos (B) y (C) comparación entre machos y hembras en cada uno de los grupos experimentales (cada barra representa la suma de las respuestas de evitación en las diez sesiones diarias). N = 8 por grupo y sexo. a, $p < 0,05$, y aa, $p < 0,01$, indican un efecto del estrés prenatal; b, $p < 0,05$, y bb, $p < 0,01$, indican un efecto de la SERT; ¥¥, $p < 0,01$; indica un efecto del sexo (Medias ± SEM).

4.3.2. 2. Respuesta de escape

El análisis del porcentaje de respuestas de huida permite demostrar marcadas diferencias entre los grupos estudiados, $F_{3,49}$ = 23,2 p < 0, 001. Como se aprecia en la Figura 4, ANEXO III, la SERT induce un aumento del número de respuestas de huida, en ambos sexos. Entre los machos, la diferencia es significativa cuando se comparan los animales tratados con SERT y los no tratados, los machos que tomaron SERT presentan una conducta de huida persistentemente superior a la que exhiben los que no tomaron el fármaco (p<0,001). También, se diferencian las hembras que tomaron SERT de las que no la tomaron, siendo esta diferencia estadísticamente significativa para las hembras estresadas (p<0,001). En ambos sexos, los animales PS-SERT mantienen un nivel de respuestas de huida elevado (entre los 55,3 ± 9,6% y los 90 ± 9,1%), desde la primera hasta la última sesión, siendo ésta la respuesta predominante en este grupo.



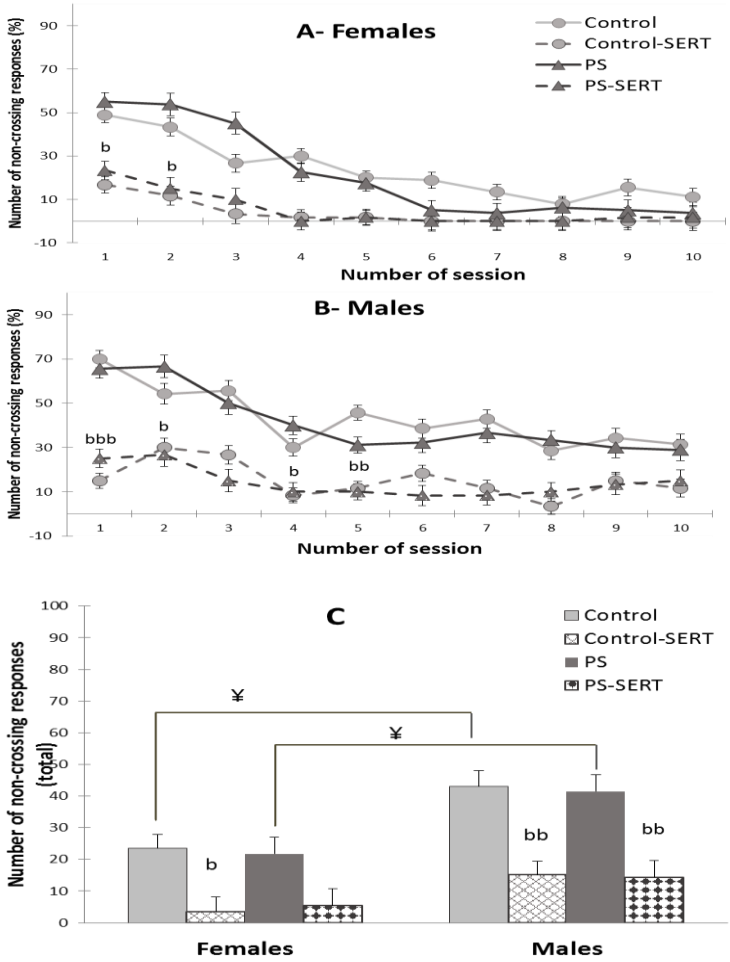
ANEXO III- Figura 4. Comparación de las respuestas de escape entre los diferentes grupos experimentales. Valores de las respuesta de escape expresado en % a lo largo de las sesiones diarias en las hembras (A) y machos (B), en cada grupo experimental. aa, p < 0,01 y aaa, p < 0,001, indican el efecto del estrés prenatal; b, p < 0,05, bb, p < 0,01 y bbb, p < 0,001, indican el efecto de la SERT (valores medios ± SEM).

4.3.2.3. Déficit de escape

El análisis del porcentaje de respuestas nulas es un indicador del desamparo que pueda padecer un animal; los animales que presentan déficit en la respuesta de escape, no evitando ni huyendo de los choques muestran una actitud de desamparo y viceversa. En la Figura 5, ANEXO III, se puede observar el efecto de la SERT en ambos sexos a lo largo de las sesiones de estudio. Los animales tratados con SERT (**Control-SERT** y **PS-SERT**) presentan desde la primera sesión muy pocas respuestas nulas (ANEXO III, Figura 5A y 5B). En ambos sexos, la SERT actúa ayudando a que los animales escapen de los choques, independientemente de la condición de estrés anterior.

Los animales no tratados (**Control** y **PS**), inician la prueba de aprendizaje sin conseguir alcanzar las respuestas adecuadas, pero, con el seguimiento de las sesiones, mientras las hembras sustituyen las respuestas nulas por respuestas de evitación o de huida, los machos no tratados (estresados o no) siguen presentando el déficit de escape y no responden. En ambos grupos de animales no tratados con el antidepresivo, los machos presentan significativamente más respuestas nulas que las hembras (+19,6 ± 6,6%, p=0,005, en los animales Control; y + 19,7 ± 6,4%, p =0,003, en los animales PS).

Evaluado el porcentaje de animales que presentan 100% de respuestas nulas en 3 sesiones consecutivas de estudio (como indicador de respuesta conductual extrema), comprobamos que los machos expuestos al **PS** fueron el único grupo que presentó tal conducta (30% de los machos PS, datos no mostrados).



ANEXO III - Figura 5. Efectos de la SERT sobre la conducta de escape. Valores de escape en los animales estudiados, hembras (A) y machos (B) en cada grupo experimental; y (C) comparación entre machos y hembras en cada uno de los grupos experimentales (cada barra representa la suma de las respuestas nulas en las diez sesiones diarias). n = 8por grupo y sexo. b, $p < 0,05$ y bb, $p < 0,01$, indican un efecto de la SERT. ¥, $p < 0,05$, indica un efecto del sexo en los animales no tratados (Medias ± SEM).

4.3.2.4. Actividad locomotora durante los intervalos entre ensayos

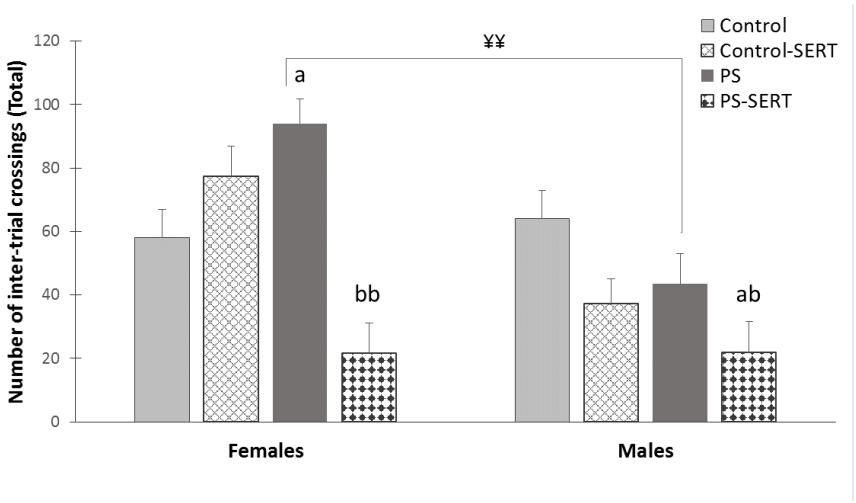
Al medir la actividad locomotora antes del inicio de cada ensayo (**AAE**) y la actividad entre ensayos (IEE) se verifica que la **AAE** no está influida por el grupo o sexo de los animales, cosa que sí sucede en la **IEE**, observándose la influencia del grupo (p=0,009) y del sexo (p=0,015). Analizando cada uno de los grupos, se apreciaron diferencias en ambos sexos entre

los valores estimados para la actividad **IEE** por efecto de la SERT, disminuyéndola específicamente en los animales sometidos a estrés prenatal (ANEXO III, Figura 6).

Las hembras, sin embargo, también mostraron diferencias por efecto del estrés prenatal, siendo las hembras **PS** más activas que sus controles (p=0,04). Esta mayor actividad está correlacionada con el porcentaje de evitaciones obtenido (r=0,91*), o sea, las ratas más activas durante la prueba, al ir pasando de un compartimento al otro, por ensayo y error, van aprendiendo a asociar los estímulos. Por contraste, la **IEE** está negativamente relacionado con el déficit de escape (r= -0,6**).

Una vez más, se demuestran diferencias en la actividad motora entre machos y hembras sometidos a PS (p=0,007). Las hembras sometidas a **PS** hacen más cruces que los machos del mismo grupo (+47,1 ± 16,8).

Aunque existe una correlación positiva entre la actividad motora demostrada en el CA (Actividad exploratoria total) y la **IEE** en esta prueba (r= 0,36**), esta correlación no se mantiene entre la Actividad exploratoria total y la **AAE** (r=0,14).



ANEXO III - Figura 6. Actividad inter-ensayo de los animales de ambos sexos en cada grupo experimental. a, p < 0,05 indica un efecto del estrés prenatal; ab, p < 0,05, indica las diferencias entre los machos PS-SERT y Control; bb, p < 0,01, indica el efecto de la SERT; ¥¥, p < 0,01, indican un efecto del sexo en los animales estresados (PS). Cada barra representa la suma de cruces inter-ensayos durante las diez sesiones de choques escapables (Medias ± SEM).

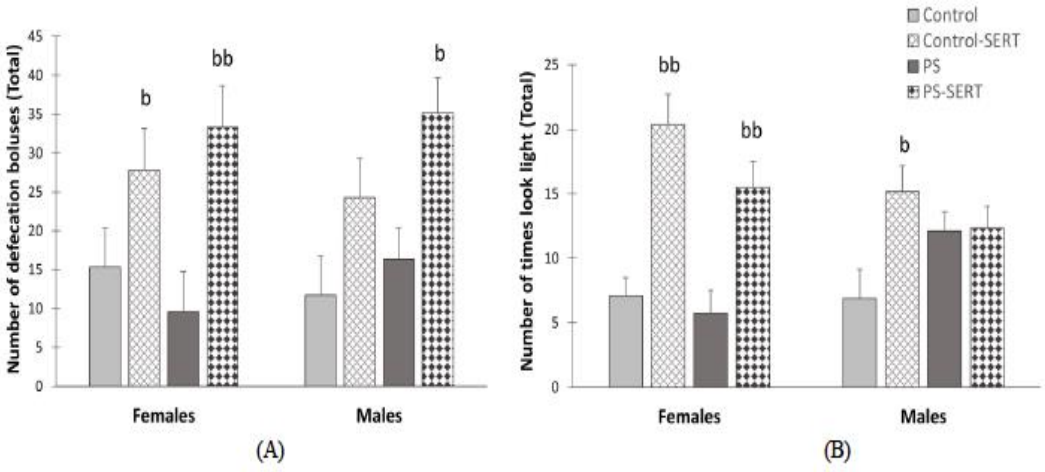
4.3.2.5. Análisis de la emotividad

En cada exposición a la caja de choques se contabiliza el número de **bolos fecales** para cada animal, encontrando una diferencia significativa en los mismos entre animales tratados y no tratados de ambos sexos ($p < 0,001$) (ANEXO III, Figura 7). El número de bolos fecales, cuyo análisis permite evaluar la ansiedad, está correlacionado con el índice de huidas que caracterizó a este grupo animal (r=0,69**).

No existen diferencias en la manifestación de las reacciones a los choques (**saltos y vocalizaciones**) entre grupos, y tampoco hay diferencias según el sexo. Como sería de esperar, el número e intensidad de los saltos en respuesta a los choques disminuye gradualmente desde la 1ª hasta la última sesión (datos no mostrados). Lo que es más sorprendente es que esta disminución ocurre igual en los animales que no logran aprender y no evitan los choques, no estando este parámetro correlacionado con el aprendizaje (r=-0,24).

Como cabría esperar, mientras los animales van asociando el **EC** (luz) con el **EI** (choques), van dejando de mirar la luz y van actuando, siendo la disminución de respuestas de orientación a la luz significativa, $F_{9,441}= 54,3$, $p < 0,001$ y correlacionada con las respuestas de evitación (r=-0,31*). Pero esta disminución es dependiente del tratamiento con SERT, $F_{27,441} =2,67$ $p<0,001$. Una vez más, hay diferencias entre los animales tratados con SERT y los no tratados en ambos sexos ($p < 0,001$), los animales tratados presentan más veces las respuestas de orientación al EC (ANEXO III - Figura 7).

En ambos sexos, los animales estresados y tratados con SERT no logran evitar los choques (apenas huyen cuando se aplican). Esto no puede explicarse porque no aprendan o no entiendan el significado de los estímulos que los preceden, ya que miran constantemente la luz y se ponen en la puerta esperando el choque (datos no mostrados), estando estos parámetros positivamente correlacionados (respuestas de huida y Luz, r= 0,6**). El número de defecaciones está positivamente correlacionado con el número de respuestas a los estímulos, como la orientación hacia la luz (r=0,62**) y las vocalizaciones audibles (r=0,32*), y está negativamente correlacionado con la actividad motora durante los ensayos (**IEE**, r=0,38**; **AEE**, r=0,26*).

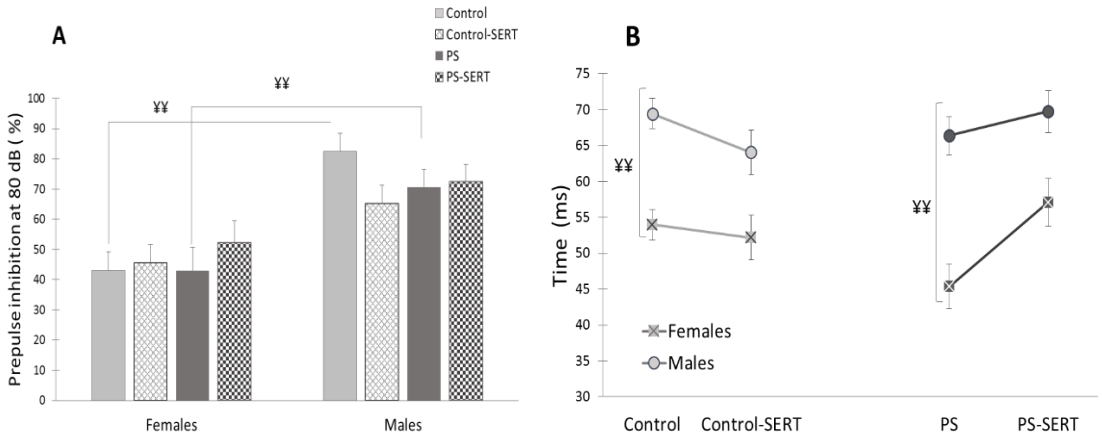


ANEXO III - Figura 7. Análisis del número de defecaciones. Se evalúan los efectos del estrés prenatal y del tratamiento con SERT (5 mg/kg/día) en las medidas de emocionalidad en los animales de ambos sexos, cuando se exponen repetidamente a la caja de choques previamente emparejada con choques inescapables. Cada barra representa la suma de los bolos (A) y las respuestas de orientación a la luz (B) en las diez sesiones diarias (valores de media + SEM). bb, $p < 0,01$ y b, $p < 0,05$, indican un efecto de la SERT.

4.4. Evaluación de los efectos del estrés prenatal y del tratamiento con Sertralina tras someter a condiciones de re-estrés a los animales adultos de ambos sexos

4.4.1. Evaluación de la inhibición prepulso y su latencia

En el día posnatal 90 (P90), cuando se mide la **PPI**, verificamos que no hay diferencias ni en los valores de inhibición de prepulso ni en la latencia de la PPI entre los grupos experimentales (ANEXO IV – Figura 2). El sexo del animal sí afecta los valores de PPI y su latencia; los machos presentan valores de PPI superiores a las hembras ($p<0,01$) y su latencia es mayor en los grupos de animales no tratados. La SERT anula este dimorfismo sexual.



ANEXO IV – Figura 2. Comparación entre los valores de PPI y su latencia en los diferentes grupos experimentales. (A) Valores de la PPI y (B) de la latencia de la PPI (estímulos 80dB), en el día posnatal P90 en los animales de ambos sexos, anteriormente estresados por estrés prenatal o no (PS y Control) y tratados con SERT (5 mg/kg/día) o no (PS-SERT y Control de-SERT). N = 8 animales por grupo y sexo (Medias ± SEM). ¥¥, p < 0,01, indica un efecto del sexo en los animales no tratados.

4.4.2. Cambios en la respuesta condicional emotiva (Prueba del CER)

4.4.2.1. Postura de miedo o defensa (DEF)

El análisis del porcentaje del tiempo utilizado por cada animal en cada una de las posturas asociadas a conductas defensivas o de miedo (*freezing y crouching* respectivamente), muestra una correlación muy fuerte entre ellas en todos los momentos de la prueba (antes de los choques r=0,6, p<0,001; en el día de los choques r=0,844, p<0,001; y después de los choques r=0,52 p<0,001), por lo cual, preferimos analizarlas en conjunto y llamar a este nuevo parámetro postura defensiva (**DEF)**.

Se aprecian fuertes diferencias en la adopción de posturas defensivas en cada momento según el género; en los animales **Control** no existe dimorfismo sexual ni antes ni después de los **IS**, pero en los animales **PS**, las diferencias entre sexos son evidentes antes y en el día de **IS**, estando los machos más tiempo inmóviles y alertas que las hembras (+3,90 ± 1,6, p=0,03), diferencias que dejan de observarse tras los **IS**. Al contrario, los animales tratados con SERT de ambos sexos no se diferencian antes de los **IS**, pero tras este proceso, muestran grandes diferencias en el condicionamiento del miedo y su extinción.

4.4.2.1.1. Habituación al contexto de estudio

La primera vez que se introduce a las ratas en la caja experimental, antes de los choques, el acto no tiene todavía valor aversivo, los animales demuestran poco comportamiento defensivo (día-1 y día-2), y el porcentaje de tiempo **DEF** fue comparable para todos los animales (ANEXO IV, Figura 3). Hay diferencias entre los sexos en los animales **PS**; los machos PS pasan más tiempo inmóviles y alertas que las hembras (p=0,03).

4.4.2.1.2. Sesión de Choques

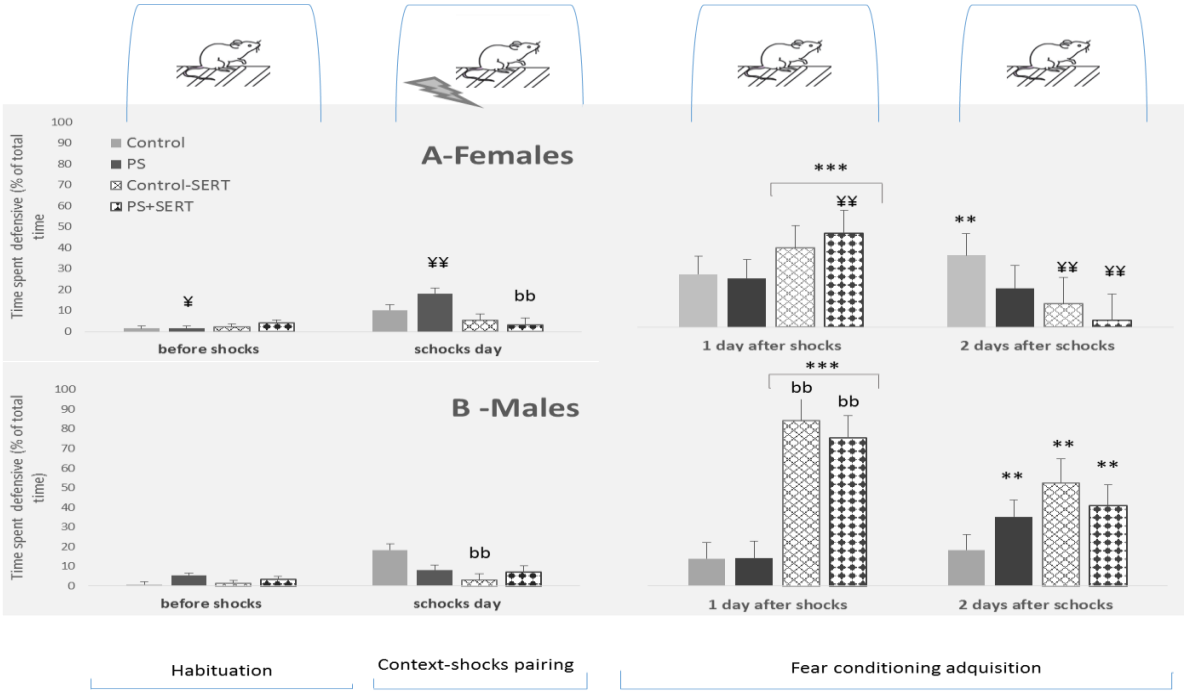
Durante la sesión de choques, se asocian los choques (**EI**) con el contexto (**EC**), y el miedo a la caja (tiempo **DEF**) aumentó significativamente cuando se compara con el periodo de habituación ($p < 0,001$). La SERT disminuye la respuesta defensiva a los choques en ambos sexos ($p < 0,01$).

4.4.2.1.3 Condicionamiento del miedo

Cuando se vuelven a colocar en la caja asociada a los choques eléctricos, los animales de todos los grupos experimentales, de ambos sexos, aumentó el comportamiento de **DEF** ($F_{1,49} = 65,6$, $p<0,001$).

Para explorar el efecto del fármaco y del estrés en la adquisición de la respuesta condicionada de miedo para cada sexo, se analizaron por separado los días 1 y 2 post-choque. Tras un día de la administración de los choques, al re-exponer las ratas a la caja, apreciamos grandes diferencias en el comportamiento **DEF** entre los animales de los diferentes grupos experimentales ($p<0,001$) y entre los sexos ($p=0,034$). En ambos sexos, los animales tratados con SERT presentan un fuerte condicionamiento contextual al miedo (ANEXO IV, Figura 3). En estos animales, el miedo a la caja está exponencialmente aumentado, independientemente de que hayan sido o no estresados durante el desarrollo prenatal ($p<0,001$).

Cuando analizamos el día 2 post-choque, observamos diferencias entre machos y hembras que toman SERT ($P<0.01$); las hembras tratadas con el antidepresivo empiezan a extinguir el comportamiento de miedo y disminuyen el comportamiento **DEF** (ANEXO IV, Figura 3). Por otra parte, en los animales no tratados, la **DEF** no cambia o sigue aumentando.

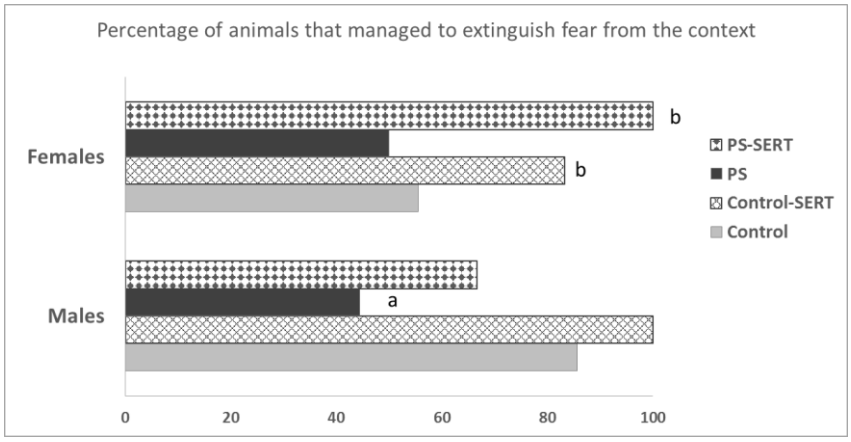


ANEXO IV- Figura 3. Efectos del estrés y de la Sertralina sobre la respuesta defensiva.
Efectos del estrés prenatal y del tratamiento con SERT (5 mg/kg/día) en la respuesta defensiva (**DEF**) (expresada en porcentaje) durante la prueba del **CER** en cada una de sus fases: de habituación (dado por la medias ± SEM de los días 1 y 2 antes de los choques), de emparejamiento de los choques con el contexto (día de los choques) y de adquisición del miedo condicionado (día 1 y 2 después de los choques), en las hembras (A) y los machos (B). bb, $p < 0,01$, indica las diferencias significativas por efecto de la SERT; ¥, $p < 0,05$, y ¥¥, $p < 0,01$, indican las diferencias entre sexos; **, $p < 0,01$, indica el cambio significativo cuando comparado con la sesión de choques (Medias ± SEM).

4.4.2.1.4 Extinción del miedo.

Analizando el tiempo del comportamiento **DEF** el último día de prueba (4ª sesión post-Choques), observamos que no se aprecia efecto del grupo, ni del sexo. Pero, si comparamos dentro de cada grupo el porcentaje de animales que presentan extinción (menos del 10% del tiempo DEF en la 4ª sesión post IS), se observan diferencias entre grupos y sexos (ANEXO IV, Figura 4). En general, el fármaco induce una mejoría en la extinción del miedo condicionado (X^2 = 7,99, p=0,046), con la única excepción de los machos **PS**, en los cuales la SERT no ejerce efecto. En las hembras, el fármaco SERT es el único factor que modula la extinción del miedo (p=0,014); en los machos, también interviene el estrés prenatal, encontrando machos

estresados que tienen dificultades para extinguir el miedo en comparación con los controles (X^2 = 7,9, p = 0,046), efecto que la SERT no revierte.



ANEXO IV- Figura 4. Efectos del estrés y de la Sertralina sobre el porcentaje de animales que logran extinguir el miedo. Cada barra representa el porcentaje de animales que exhiben menos del 10% del tiempo defensivo en la 4ª sesión después de los **IS** en cada grupo experimental. a, p < 0.05, indica las diferencias como efecto del estrés prenatal; b, p < 0.05, indica diferencias significativas como efecto de SERT.

4.4.2.2. Exploración vertical *(rearings)*

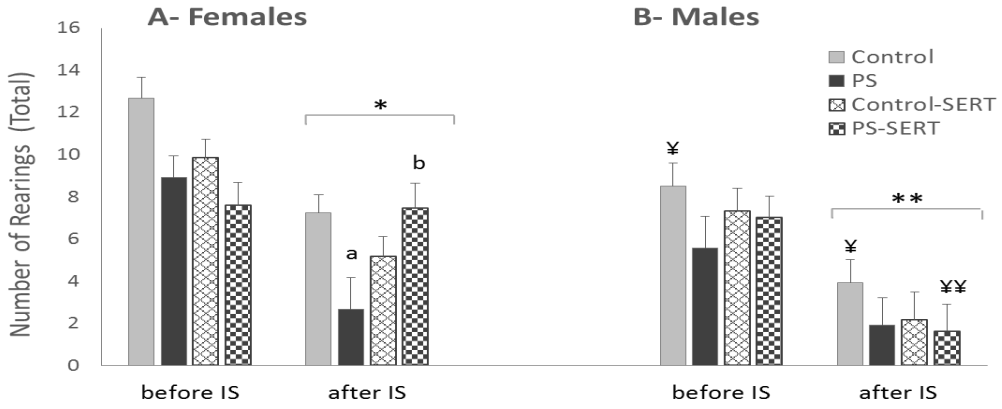
Antes de los choques no hay diferencias en el número de *rearings* entre los grupos experimentales, en ambos sexos (ANEXO IV,Figura 5).

Como se puede apreciar en la Figura 5 (ANEXO IV,) La exposición de los animales a los IS indujo una disminución general en el número de *rearings* en todos los grupos, F_{349} = 55.1, p < 0.001, con influencia diferencial entre los dos sexos ($F_{1, 49}$ = 17,41, p < 0.001) En la mayoría de los animales la tendencia a la disminución del número de *rearings* se invierte, mientras disminuye el miedo de la caja (existe una correlación entre la actividad exploratoria y el tiempo DEF, r=- 0,41**), sin embargo, en los animales expuestos al PS esto no pasa (Figura 6 A y B,ANEXO.IV).

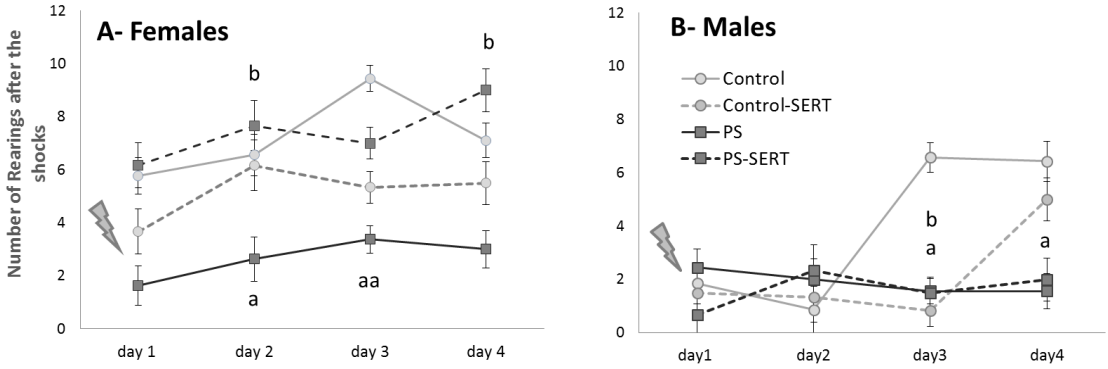
Como consecuencia, después de expuestos a los IS, las diferencias entre grupos fueron evidenciadas, en ambos sexos. Los análisis *Post-hoc* muestran el efecto del estrés prenatal afectando a la actividad exploratoria- después de expuestos a IS, los animales PS de ambos sexos muestran significativamente menos *rearings* que los controles (p < 0.05). En las hembras

PS, el tratamiento con SERT invierte los efectos de los choques, en los machos PS eso no sucedió (Figura 5 y 6).

Por otra parte, se demuestra un fuerte efecto del sexo sobre la conducta de *rearing*, las hembras presentan mayor actividad de exploración vertical que los machos. Estas diferencias son significativas en el grupo Control y se evidencian antes de los IS (p=0,031) y después de los IS (p=0,04); y en los animales estresados y tratados con SERT, el dimorfismo sexual es evidenciado tras los IS (p=0,041), consecuencia de la falta de respuesta al fármaco en los machos PS.



ANEXO IV- Figura 5. Efectos de la aplicación de choques inescapables (antes y después) sobre la conducta de *rearing* en los grupos de estudio. Número de *rearings* (media ± SEM). Comparación entre las hembras (A) y machos (B) de los diferentes grupos experimentales. Antes de **IS**: cada columna representa la media + SEM en las dos sesiones antes de los IS; Después de IS: cada columna representa la media + SEM en las cuatro sesiones después de los IS. a, $p < 0,05$, indica las diferencias como efecto del estrés prenatal; y b, $p < 0,05$, indica las diferencias como efecto de SERT en las hembras; ¥, $p < 0,05$, y ¥¥, $p < 0,01$, indican las diferencias entre sexos; **, $p < 0,01$, y *, $p < 0,05$, indican la disminución significativa en el número de *rearings* por efecto de los IS en todos los grupos experimentales.



ANEXO IV- Figura 6. Efectos que induce la aplicación de choques inescapables sobre la conducta de rearing en los grupos de estudios. Efectos de los choques inescapables (**IS**) en el número de *rearings* (media ± SEM) en las hembras (A) y machos (B) de los diferentes grupos experimentales. aa, $p < 0,01$, a, $p < 0,05$, indican las diferencias como efecto de estrés prenatal; y b, $p < 0,05$, indica las diferencias como efecto de la SERT.

4.4.2.4. Conducta de acicalamiento (*grooming*)

Los **IS** influyen en el número de *groomings,* p = 0, 024. Al comparar los efectos de los choques en los diferentes grupos de animales estudiados, se aprecia una reducción en este tipo de comportamiento solamente en los machos **PS** (p = 0, 012) (datos no mostrados). Una vez más, el número de menor *groomings* demuestra que los machos **PS** adoptaron un comportamiento más pasivo que sus controles.

4.4.3. Evaluación de parámetros fisiológicos

4.4.3.1 Análisis del peso corporal

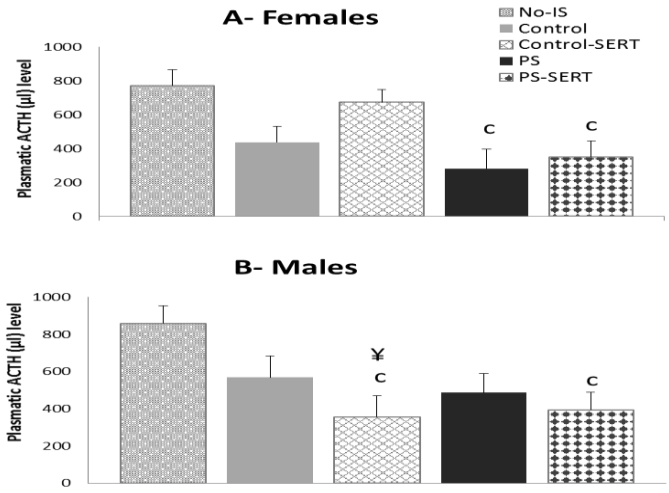
En el día posnatal 120 (P120), los tratamientos aplicados inducen diferencias en el peso corporal entre los animales de ambos sexos. La aplicación de **IS** tiene un efecto ligero en la ganancia de peso (Tabla 1), sin que la SERT lo revierta. En los machos, las diferencias son significativas entre los grupos, observando que la SERT induce una pérdida de peso en comparación con los animales **No-IS**, independientemente de la condición de estrés anterior (p < 0, 05) (Tabla 1).

Groups	Females	Males
No-IS	244.8± 4.5	390.2 ± 10.6
Control	227.1± 4.4	358.2 ± 11.5
Control-SERT	226.3± 4.8	340.6± 10.2 c
PS	242.9± 4.4	357.5± 9.3
PS-SERT	224.1± 5.2	335.5± 9.6 cc

ANEXO IV- Tabla 1. Comparación de los valores de peso corporal entre los diferentes grupos experimentales y animales que no sufren choques inescapables. Valores de peso corporal al día posnatal 120 en los animales de ambos sexos de todos los grupos experimentales (**Control, Control-SERT, PS y PS-SERT**) comparados con un grupo de animales no perturbados (**No-IS**). Valores de las medias ± SEM. (N = 7 por grupo y sexo). cc, $p < 0,01$ y c, $p< 0,05$ indican el efecto de la SERT en los machos (diferente de los **No-IS**).

4.4.3.2. Niveles plasmáticos de ACTH en respuesta al estrés.

Los niveles de ACTH en respuesta al estrés por IMO fueron modulados por los diferentes procedimientos a que los animales fueron sometidos ($p = 0,001$). En general, los niveles plasmáticos de ACTH, después del período de recuperación, fueron menores en los animales, de ambos sexos, sometidos a los IS que en los animales No-IS (Figura 7). En las hembras, el PS incrementó el efecto de los IS, y las diferencias alcanzaron significación cuando se comparan con las hembras no perturbadas (PS y PS-SERT vs. No-IS, $p < 0,05$), un efecto que la SERT no revierte. En los machos, la toma de SERT redujo significativamente los niveles de la hormona ACTH ($p < 0,05$) (ANEXO.IV,Figura 7).



ANEXO IV- Figura 5. Niveles plasmáticos de ACTH. Niveles plasmáticos de ACTH (pg/µl) obtenidos en la sangre arterial en las hembras (A) y machos (B) de todos los grupos experimentales, 10 minutos después de la exposición al estrés por **IMO** en comparación con un grupo de animales no perturbados (**No-IS**). Valores medios + SEM (N = 7 por grupo y sexo). c, $p < 0,05$, indica las diferencias significativas en comparación con los **No-IS**; ¥, $p < 0,05$ indica diferencias significativas entre machos y hembras.

4.4.3.3 Efectos de estrés en los parámetros hematológicos

Tras el estrés por IMO, los valores hematológicos obtenidos diez minutos después de la prueba muestran valores mayores que los valores los observados en condiciones basales en los animales No-IS (Tabla 2). Comparativamente, los animales previamente sometidos a los IS (PS o Controles), muestran alteraciones en los parámetros hematológicos en comparación con los animales No-IS ($p<0.05$) de ambos sexos. En estos animales, los valores plasmáticos de hemoglobina, MCH y VPM, fueron significativamente más bajos que en los animales No-IS.

Por otra parte y de manera importante, el tratamiento con SERT revirtió las alteraciones sobre los parámetros hematológicos causados por los factores estresantes previos y, los animales tratados con el fármaco mostraron una recuperación de los indicadores hematológicos estudiados equiparable a los animales No-IS.

Se encontró además una fuerte leucopenia en los animales prenatalmente estresados de ambos sexos (Tabla 2), un efecto invertido por la administración con SERT.

Groups		Leukocytes (10^3 /μ l)	Erythrocytes (10^6 /μ l)	Hemoglobin (g/dl)	Hematocrit (%)	MCH (pg)	MPV fL(μm^3)
Basal values		*3.3 – 8.7*	*5.5 -9.3*	*10.6 – 15.6*	*32.7 – 44.8*	*15.8-19.9*	*5.4 -9.2*
Females	No-IS	4.9 ± 0.6	7.9 ± 0.2	16.0 ± 0.3	44.9 ± 1.0	19.8 ± 0.2	10.6± 0.2
	Control	3.4 ± 0.6	8.1 ± 0.3	14.6 ± 0.5	41.5± 1.7	18.3 ± 0.7 **c**	8.8 ± 0.4 **c**
	PS	1.4 ± 0.5 **aa**	7.8 ± 0.2	14.3 ± 0.3	44.0± 1.1	18.5 ± 0.4 **cc**	7.5 ± 0.3 **ccc**
	Control- SERT	5.3 ± 0.3	7.9 ± 0.2	16.0 ± 0.5	43.9 ± 1.7	20.2 ± 0.4 b**b**	11.3 ± 0.4 **bb**
	PS- SERT	4.1 ± 0.5 **bb**	8.0 ± 0.5	16.1 ± 0.5 **b**	45.2 ± 1.7	20.2 ± 0.4 **b**	10.6 ± 0.5 **bbb**
		F=8.2, p<0.001	**F=0.1, ns**	**F=4.2, p=0.006**	**F=0.95, ns**	**F=7.9, p<0.001**	**F=22.5, p<0.001**
Males	No-IS	5.8 ± 0.3	8.5 ± 0.2 **¥**	17.1 ± 0.3 **¥**	47.8 ± 1.0 **¥**	19.9 ± 0.2	11.0 ± 0.3
	Control	3.1 ± 0.3 **c**	8.1 ± 0.1	15.1 ± 0.5 **c**	42.5 ± 1.7	18.1 ± 0.4 **cc**	7.4 ± 0.4 **¥ ccc**
	PS	1.9 ± 0.5 **a**	8.6 ± 0.3 **¥**	15.3 ± 0.3 **¥ cc**	44.5 ± 1.0	17.9 ± 0.2 **ccc**	8.2 ± 0.3 **ccc**
	Control-SERT	4.2 ± 0.3 **b**	8.3 ± 0.3	16.6 ± 0.5	46.7 ± 1.7	20.0 ± 0.4 **b**	10.6 ± 0.4 **bbb**
	PS- SERT	4.7 ± 0.5 **bb**	8.5 ± 0.3	16.4 ± 0.4	44.4 ± 1.5	19.7 ± 0.4 **bb**	10.7 ± 0.4 **bbb**
		F=7.8, p<0.001	**F=0.5, ns**	**F=6.3, p<0.001**	**F=2.3, p=0.04**	**F=12.5, p<0.001**	**F=22.9, p<0.001**

ANEXO IV- Tabla 2. Efectos de la Sertralina sobre parámetros hematológicos en los diferentes grupos de estudio. Los valores de los parámetros hematológicos se obtienen en sangre arterial 10 minutos después de terminada la exposición al estrés por **IMO** en los animales de ambos sexos que fueron expuestos a choques inescapables (**IS**) o no expuestos a choques inescapables (**No-IS**). Valores medios ± SEM en los diferentes grupos experimentales (N = 7 por grupo y sexo). Abreviaturas: MCH, concentración de hemoglobina corpuscular media; MPV, volumen plaquetario medio. aa, p < 0,01, indica el efecto del estrés prenatal (diferente del grupo control); bb, p < 0,01 y b, < 0,05 indican el efecto de SERT (diferente del grupo control); ccc, p < 0,001, cc, p< 0,01 y c, p < 0,05, indican las diferencias significativas en comparación con el grupo No-IS; ¥, p < 0,05 indica las diferencias significativas entre machos y hembras.

V. DISCUSIÓN

5.1. Consideraciones metodológicas

5.1.1. Elección del animal de experimentación.

En este estudio, elegimos como animal de experimentación ratas de la cepa Wistar (*Rattus norvegicus*), ya que hemos realizado estudios previos en nuestro laboratorio sobre la administración crónica con otros fármacos en estas ratas, y disponemos de una amplia base de datos sobre resultados de evaluación de la PPI en esta misma cepa (Castellano y cols., 2009). Además, la cepa Wistar es uno de los animales más utilizados en otros laboratorios para estudios conductuales (Lehmann y cols., 2000; Hauser y cols., 2006; Leventopoulos y cols., 2009). Otras ventajas, como el bajo coste y la gran facilidad de manejo, hacen que la rata albina sea el animal experimental idóneo para este trabajo.

5.1.2. Elección del tipo de estrés empleado.

Hoy en día se sabe que la elección del tipo de estrés, su duración e intensidad, y otros factores como la edad o el género de los sujetos sometidos a estrés tienen efectos marcadamente distintos.

Hemos elegido el "**Modelo de estrés por restricción**" por ser un paradigma de estrés muy sencillo y práctico por sus características (no requiere una respuesta física ni causa dolor) (Bowman y cols., 2009), es éticamente bien aceptado como modelo de estrés prenatal y es el paradigma de estrés más extensamente usado en los estudios con ratas gestantes (en particular por el grupo de Stefania Maccari, desde hace 20 años).

Elegimos un protocolo de restricción de 7 días de duración, inducido en períodos de 45 minutos y 3 veces al día, por ser el protocolo utilizado rutinariamente en los trabajos que investigan los efectos del estrés prenatal en la conducta (Maccari y Morley-Fletcher, 2007). Este modelo ha sido empleado también en ratas adultas no gestantes (Bowman y cols., 2009), y parece ser lo suficiente potente para inducir las alteraciones observadas con paradigmas de restricción más prolongados (hasta 21 días). La premisa ampliamente aceptada de que la rata sufre fotofobia, fue la razón por la cual también usamos una luz sobre la rata, a una altura de 30 cm (Archer, 1973).

5.1.2.1 Protocolo de estrés prenatal

En los humanos, la exposición al estrés en edades muy tempranas, es un factor que potencia la susceptibilidad de desarrollar psicopatologías en la vida futura (Fumagalli y cols., 2007; Gunnar y Quevedo, 2007). Sin embargo, en la rata, hasta las dos primeras semanas de vida, las crías no responden, o responden de forma muy débil al estrés; la respuesta adrenocortical es marcadamente reducida al nacer, la concentración de corticosterona (el GC más abundante en la rata) cae dramáticamente a 1-3 µg/100 ml. Este período de inactividad se conoce como "período de no respuesta al estrés" (Sapolsky y cols., 2000).

El período prenatal es diferente; las ratas gestantes estresadas en la última semana de gestación responden fuertemente al estrés, y presentan elevados niveles de GCs (Barbazanges y cols., 1996; Lemaire y cols., 2000), lo que influye en el desarrollo de las crías. Además, en la rata, el feto a partir del día 13 de vida prenatal segrega su GC específico en respuesta a una gran variedad de estresores (Patin y cols., 2002), y los receptores de glucocorticoides se expresan ya en el hipocampo, hipotálamo e hipófisis (Matthews, 2000; Edwards y Burnham, 2001). Todo ello indica que, en roedores, hay una ventana de susceptibilidad al estrés durante la última semana de gestación (Kofman, 2002), motivo por el cual elegimos ese período para someter las ratas gestantes al protocolo de estrés utilizado. A pesar de que los primeros trabajos que emplearon el modelo de PS sugirieran un efecto directo del **PS** sobre una psicopatología específica, hoy en día se explora el hecho de que más que un efecto directo, el **PS** puede originar una susceptibilidad general a desarrollar psicopatologías y alteraciones psicosomáticas en la edad adulta (Lemaire y cols., 2000; Darnaudéry y Maccari, 2008; Clinton y cols., 2008; Leventopoulos y cols., 2009; Cherian y cols., 2009).

5.1.3. Elección del tipo y duración de la administración del fármaco

En el **grupo Piloto** se empleó la inyección intraperitoneal para administrar la SERT. Este tipo de vía de administración es muy usada en investigación animal, especialmente en tratamientos con drogas sistémicas por su facilidad de administración. Pensamos que sería adecuada la administración diaria durante 8 días, ya que el perfil farmacológico de la SERT

permite la dosificación diaria, y los niveles de la droga en plasma se equilibran en 1 semana (Shelton, 1994).

La primera y última administración de la droga se hace una hora antes de la evaluación conductual (RAS y PPI), ya que se pretende observar los efectos del fármaco en el momento de su máxima actividad, lo que según Sokolowski y Sieden (1999) y Nowakowska y cols. (2000) sucede 60 min después de la administración del ISRS.

En el **grupo Estrés Prenatal,** la administración de fármaco se realizó por vía oral, utilizando el agua de bebida como vehículo. Se eligió la vía oral ya que representa la forma menos cruenta de administración farmacológica en administraciones prolongadas y en estudios conductuales. También, porque es el método que se asemeja más a la realidad clínica, y proporciona niveles plasmáticos adecuados y mantenidos del fármaco (Murdoch y Mc Tavish, 1992). El hecho de ser la vía de administración menos estresante, tiene importancia añadida en nuestro estudio.

5.1.4. Elección de las pruebas comportamentales

La selección y empleo de las pruebas de evaluación de la conducta fué de considerable interés en nuestro trabajo, donde valoramos la interacción entre el estrés precoz y la exposición a estresores intensos en la edad adulta (Prut and Belzung, 2003; Louvart y cols., 2009) y su reversión por la administración de SERT.

Por una parte, valoramos los efectos del **PS** en condiciones basales, para lo cual usamos la medición del RAS y sus modulaciones, así como la prueba de CA. Por otra parte, sometimos los animales a eventos aversivos, una vez evidenciada la relación entre el estrés y alteraciones psicopatológicas como ansiedad, TEPT o depresión (Swaab y cols., 2005; Pinheiro y cols., 2007; Cirulli y cols., 2009). Los eventos aversivos se basaron en la exposición de los animales a condiciones estresantes (situaciones de amenaza potencial o real) (Palanza, 2001; Vermetten y Bremner, 2002; Schweizer y cols., 2009), para poder evaluar si existe correlación con alteraciones psicopatológicas y otras variaciones en parámetros fisiológicos. El uso de diferentes pruebas conductuales nos permitió obtener un mayor volumen de información en el momento de medir las respuestas comportamentales que reflejan mecanismos tan complejos como la reactividad emocional y la vulnerabilidad al estrés.

5.2. Discusión de los Resultados

5.2.1. Grupo piloto

Empezamos nuestro estudio con un experimento piloto que pretende demostrar la hipótesis de trabajo según la cual, el estrés por restricción altera a los animales, evaluando los efectos del mismo en ratas jóvenes de ambos sexos.

El experimento piloto confirma que 7 días de estrés por restricción, son suficientes para inducir cambios en el comportamiento de los animales, y también, en algunos parámetros fisiológicos. Nuestros datos indican que el parámetro sexo es un factor significativamente predisponente en cuanto a la sensibilidad de los animales frente al estrés por restricción, demostrándose que los machos son más sensibles que las hembras a las secuelas provocadas por el paradigma de estrés empleado en este trabajo.

El procedimiento de inyectar repetidas veces a las ratas con vehículo, fue suficiente para alterar la capacidad sensoriomotora de los animales, y afectó además, parámetros metabólicos y hematológicos en los animales de ambos sexos. La administración i.p. de SERT durante 8 días (sub-crónica) revertió la mayoría de los efectos de los factores estresantes, pero sin embargo, fue incapaz de normalizar la desregulación del sistema inmune inducida por el estrés. Por otro lado, la eficacia del fármaco en la estabilización de los parámetros comportamentales ha dependido en gran medida del sexo del animal, siendo más eficaz en las hembras (ANEXO I).

5.2.1.1. Efectos sobre la modulación del RAS y su PPI

Diez días después de finalizar el procedimiento de estrés por restricción, los machos **RS** exhibieron un aumento en la amplitud del sobresalto en comparación con los valores basales, lo que significa que, al contrario de lo que sucede con los animales controles, no se habituaron a los sobresaltos inducidos por sonidos inesperados (ANEXO I).

Nuestros datos muestran que a los 90 días de edad la PPI permaneció estable en todos animales, con la excepción de los animales que recibieron inyecciones repetidas con salino, en las cuales siguió aumentando (ANEXO I). El incremento de los valores de la PPI después de someter a las ratas a inyecciones diarias, está directamente relacionado con dicho procedimiento, lo cual, podría inducir una modulación en los mecanismos de atención y

vigilancia del animal, como consecuencia de su exposición a dicha condición adversa (Ishii y cols., 2010). Este efecto fue revertido por la administración de SERT, de forma más eficiente en las hembras (ANEXO I).

En los estudios del RAS, se puso de manifiesto una disminución significativa tanto de la latencia como de la PPI, debido únicamente al procedimiento de inyección (ANEXO I). La disminución de los valores de latencia para RAS y PPI sugiere que los animales se tornan más reactivos y más alertas ante un estímulo auditivo y tal vez, hasta más temerosos (Tang y cols., 2011). La reversión en la disminución de los valores de latencia causada por la SERT, podría interpretarse como un efecto positivo del fármaco sobre la reactividad del animal, que resulta alterada como consecuencia de su exposición a un procedimiento traumático. Es de interés mencionar que el fármaco actúa de la manera antes señalada sólo en las hembras y no en los machos.

5.2.1.2. Cambios en los parámetros fisiológicos con los diferentes paradigmas de estrés

En nuestro estudio, verificamos que el estrés por **IMO** induce una respuesta de estrés agudo (Bierhaus y cols., 2006; von Kanel y cols., 2009), con un aumento significativo en los valores del hematocrito, de plaquetas; del peso de hemoglobina en cada eritrocito (del MCH) y del volumen plaquetario (MPV) (Patterson y cols., 1995; Ozdemir y cols., 2004). En los animales previamente sometidos a inyecciones repetidas, los cambios en los valores hematológicos fueron más bien moderados (ANEXO I), efecto que la SERT revierte, restableciendo la respuesta normal de estrés.

Por otra parte, nuestros datos muestran la reversión que induce la SERT sobre el fuerte aumento de las enzimas GOT y LDH en los animales sometidos a condiciones de estrés-re-estrés, lo que demuestra que el fármaco entre otros efectos, también juega un papel importante en la preservación de la integridad tisular, lo cual es un resultado de gran importancia (ANEXO I).

Constatamos además, que la condición de someter a los animales a estrés repetido (por restricción o por inyecciones) provoca leucopenia. Esta disminución de leucocitos posterior a episodios de estrés, ha sido confirmada en varias especies (Steplewski y Vogel, 1986; Dhabhar,

2014), produciéndose una recuperación lenta y completa una vez finalizado el factor estresante (Dhabhar, 2007). Tras el estrés, nosotros no encontramos cambios en los animales expuestos a estrés por IMO, en comparación con los animales no perturbados (ANEXO I); sin embargo, los animales que fueron expuestos a condiciones estresantes antes del estrés agudo por IMO (sea inyecciones repetidas o restricción), mostraron una caída persistente en el recuento total de leucocitos. En este caso, el tratamiento previo con SERT durante 8 días no fue suficiente para normalizar la inmunosupresión inducida por el estrés.

5.2.2. Grupo estrés prenatal

Las principales conclusiones de los estudios publicados en los ANEXOs II, III y IV indican que el estrés por restricción aplicado a las ratas gestantes durante la última semana de gestación produce alteraciones fisiológicas y comportamentales en las crías, que persisten en la edad adulta. Hay diferencias notables en los efectos del estrés prenatal y en la respuesta al tratamiento con SERT en función del sexo.

5.2.2.1. Los efectos del estrés prenatal y de la SERT fueron más evidentes tras someter los animales a los choques inescapables

Cuando se evalúan los efectos del estrés prenatal en condiciones basales, no se detectan grandes alteraciones en los parámetros comportamentales - en la amplitud del RAS, en la conducta en el CA (ANEXO II) o en la PPI (ANEXO IV)-, aunque sí se demuestran algunos cambios fisiológicos (los animales sometidos a **PS** presentan leucopenia y las hembras con PS presentan problemas en la ganancia de peso corporal desde el nacimiento).

Sería de esperar que las crías de madres estresadas tuviesen respuestas de sobresalto incrementadas, déficits en la PPI y cambios en la prueba del CA. Aunque en los machos estresados se detectan algunos comportamientos de ansiedad (en la prueba del CA exhiben menos entradas centrales y mayor relación de EI/EE), y las hembras presentan la habituación del RAS afectada, los efectos no fueron muy visibles. Sin embargo, cuando los animales se enfrentaron a una situación estresante (los choques Inescapables), los efectos del PS se pusieron de manifiesto.

Hay referencias que afirman que las alteraciones por estrés durante el neurodesarrollo pueden no ser suficientes para que se manifieste su efecto (White y Birkle, 2001; Kjær y cols., 2010), siendo necesaria una combinación de los mismos, con una exposición del animal al estrés en la edad juvenil o adulta (Barros y cols., 2006; Darnaudéry y Maccari, 2008; Louvart y cols. 2009; Nadal y Armario, 2010; Choy y cols., 2009). Nuestro trabajo corrobora dicha hipótesis, que el procedimiento traumático (los **IS**) en las ratas jóvenes que sufrieron estrés prenatal, pone de manifiesto las alteraciones inducidas durante el desarrollo fetal.

Los animales estresados durante el desarrollo fueron los más vulnerables al estrés durante la edad adulta, y demostraron cambios de comportamiento exploratorio, peor adaptación ante un ambiente adverso con una mayor pasividad y dificultades en la extinción del miedo, menor reacción a los choques, desamparo en los machos**,** etc. También, se presentaron cambios fisiológicos**,** como leucopenia, cambios metabólicos y alteraciones en la respuesta somática y endocrina al estrés, lo que está de acuerdo con lo descrito por numerosos autores (Belda y Armario., 2004; Mueller y Bale, 2006; Fumagalli y cols., 2007; Merlot y cols., 2008).

En relación al efecto de la SERT, en nuestro estudio, la administración de SERT no altera la emotividad o la ansiedad demostrada en condiciones basales, pero sí mejora la capacidad cognitiva y psicomotora en los efectos que se observan tras someter las ratas a los IS. Estos resultados sugieren la importancia del sistema serotoninérgico en la mediación del efecto del estrés precoz, revirtiendo muchos de los efectos de los IS en el comportamiento y en las alteraciones fisiológicas subsiguientes, aunque con algunas diferencias dependientes del sexo.

5.2.2.2. Los efectos del estrés prenatal dependen del sexo del animal

Desde el nacimiento, se ponen de manifiesto las diferencias entre sexos en los efectos del estrés sobre el desarrollo fetal. Mientras que en los machos, el **PS** no influye sobre el crecimiento, en las hembras, el estrés sobre las madres afectó a la ganancia del peso desde el nacimiento hasta la edad adulta. Las hembras PS desarrollaron un tipo de "crecimiento compensatorio" (*catch-up growth*), un síndrome con conocida implicación en la salud en humanos debido a que aumenta el riesgo de obesidad y los trastornos metabólicos (ANEXO II).

En la prueba del CA, pudimos apreciar que, de forma general, las hembras se mostraron más activas en comparación con los machos, siendo el dimorfismo todavía más evidente entre los animales PS, debido a la baja actividad demostrada entre los machos de este grupo.

Con la exposición a los **IS**, se demostraron todavía más las diferencias entre sexos en los efectos del **PS**; los machos sometidos a PS fueron los más susceptibles a desarrollar el comportamiento de desamparo (LH) (ANEXO III), además de ser el único grupo en el cual encontramos animales que presentaron una respuesta conductual extrema (más de 3 sesiones consecutivas mostrando respuestas nulas). Al contrario de lo que pasó con los machos, las hembras sometidas al PS no parecen tener afectada la capacidad de aprender en un ambiente hostil. Es más, las hembras PS fueron las más capaces de aprender a evitar los choques, tras haber sido sometidas a choques inescapables, diferenciándose de sus controles.

También, la respuesta adrenocortical al estrés en los machos y hembras sometidos al estrés prenatal es distinta; mientras que en los machos los niveles plasmáticos de ACTH no se modifican significativamente, sí lo hacen en las hembras PS tras los IS, existiendo por tanto, una sensibilidad de respuesta endocrina al estrés diferente entre los dos sexos (ANEXO IV).

5.2.2.3. Tras un evento traumático en la edad adulta (IS), los efectos de la SERT en los parámetros comportamentales dependen del sexo del animal

En ratas, la exposición crónica a una dosis baja de SERT (5 mg/kg/día) desde la adolescencia hasta la edad adulta no afectó al comportamiento locomotor, ni a la ansiedad en la prueba del CA, y tampoco influyó en la coordinación psicomotora de las ratas, antes de los **IS** (ANEXO II). Este tratamiento fue seguro y efectivo para revertir algunos de los efectos nocivos del **PS**. Sin embargo, tras someter las ratas a un evento traumático, los **IS**, se pone de manifiesto que la eficacia de la SERT no ha sido total, manifestándose algunas diferencias en función del sexo.

De forma resumida (descrito más ampliamente en el ANEXO III), podemos afirmar que el fenómeno del "Desamparo aprendido" (**LH**) inducido por los **IS** fue sensible al tratamiento crónico con SERT (ANEXO III). La administración de SERT ayudó a los animales de ambos sexos a escapar de los choques, y resultó fundamental para revertir la anhedonia demostrada en los

machos, lo que está de acuerdo con lo descrito previamente para este tipo de antidepresivos (Maier y Watkins, 2005; Zazpe y cols., 2007; Bilge y cols., 2008). Sin embargo, la SERT sólo fue eficaz para restituir los déficits de aprendizaje inducidos por los **IS** en las hembras no estresadas, sin ejercer ningún efecto sobre los machos.

Las diferencias de efectividad de la SERT en función del sexo, pueden apreciarse también en el proceso de extinción del miedo y en la recuperación de la actividad exploratoria tras los IS. En las hembras, la SERT resultó ser eficaz para revertir los efectos del PS sobre la extinción del miedo, y en la recuperación de la actividad exploratoria, efecto que no se ejerce sobre los machos (ANEXO IV). Sorprendentemente, tras someter las ratas machos a los IS, el tratamiento con SERT, además de no revertir los efectos de la experiencia traumática, aparentemente les incrementó la ansiedad. La administración de SERT durante 3 meses afectó la ganancia de peso en los machos (los machos Control-SERT y PS-SERT presentan un menor peso con relación a los machos no tratados) e incrementó la recuperación de los niveles de ACTH a sus valores basales, posiblemente por volverse la respuesta endocrina más reactiva (Liberzon y cols., 1997).

5.2.2.4. Los efectos de la SERT en la respuesta hematológica al re-estrés no dependen del sexo del animal

Contrariamente a los efectos duales en los parámetros comportamentales y neuroendocrinos, la administración crónica de SERT en ambos sexos resultó efectiva sobre las alteraciones hematológicas, normalizando los valores alterados (hematocrito, hemoglobina corpuscular media y el volumen plaquetario) inducidos por los **IS** y la inmunosupresión encontrada como efecto del estrés prenatal (ANEXO IV).

Finalmente, no quisiéramos concluir la redacción de este trabajo sin mencionar, por un lado, la utilidad del modelo experimental y de las pruebas propuesta en el presente estudio, así como la trascendencia de nuestros resultados (expuestos a continuación en las Conclusiones), que podrían sentar las bases para pensar en extrapolar hallazgos experimentales hacia la esfera clínica, tanto en el campo diagnóstico como en el terapéutico, específicamente en el área farmacológica, para contribuir a la detección, el seguimiento y el tratamiento de afecciones neuropsicológicas en el humano.

VI. CONCLUSIONES

De forma general, nuestro trabajo corrobora y amplía conclusiones anteriores sobre los efectos del estrés y la respuesta a fármacos antidepresivos. Sin embargo, y de una forma sorprendente, registramos grandes diferencias entre machos y hembras en la susceptibilidad a diferentes tipos de estrés y en respuesta al tratamiento con SERT. Este aspecto es fundamental a la hora de interpretar los efectos que tiene el estrés y las formas de revertirlo.

Primera

El estrés por restricción afecta más a los machos que a las hembras, ralentizando el crecimiento, alterando la habituación a largo plazo de la respuesta auditiva de sobresalto e incrementando les los cambios fisiológicos inducidos por el estrés por inmovilización.

Segunda

Las inyecciones repetidas, independientemente de la sustancia administrada, disminuyen la latencia del RAS y aumentan su PPI y también provocan cambios fisiológicos en ambos sexos, lo cual debe tenerse en cuenta en los estudios farmacológicos que utilicen esa vía de administración.

Tercera

La administración sub-crónica de Sertralina revierte las graves alteraciones que causa el estrés sobre los niveles plasmáticos de GOT y LDH.

Cuarta

El estrés provoca leucopenia en animales de ambos sexos, más pronunciada en animales que han sufrido episodios previos de estrés que revierte con la administración crónica de Sertralina.

Quinta

El estrés por restricción aplicado a las ratas gestantes durante la última semana de gestación produce alteraciones fisiológicas y comportamentales en sus crías que persisten con la edad.

Sexta

El estrés prenatal aumenta significativamente la susceptibilidad de los animales a sufrir cambios comportamentales y fisiológicos cuando se enfrentan nuevamente a situaciones estresantes.

Séptima

Hay diferencias notables en los efectos del estrés prenatal y en la respuesta al tratamiento con Sertralina en función del sexo del animal.

Octava

La administración de Sertralina no altera la emotividad o la ansiedad demostrada en las pruebas de comportamiento, pero sí revierte muchos de los efectos deletéreos causados por el estrés, siendo dicha reversión más efectiva en hembras.

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VIII. ANEXOS

ANEXO I

J. Biomedical Science and Engineering, 2015, 8, 399-419
Published Online July 2015 in SciRes. http://www.scirp.org/journal/jbise
http://dx.doi.org/10.4236/jbise.2015.87038

Scientific Research Publishing

Sex Differences in the Effects of Sertraline and Stressors in Rats Previously Exposed to Restraint Stress

Inês Pereira-Figueiredo[1], Juan Carro[1,2], Luis J. Muñoz[3], Consuelo Sancho[1], Orlando Castellano[1,4], Ricardo Gómez-Nieto[1,4], Dolores E. López[1,4]

[1]Neuroscience Institute of Castilla y León/IBSAL, University of Salamanca, Salamanca, Spain
[2]Department of Basic Psychology, Psychobiology and Methodology, University of Salamanca, Salamanca, Spain
[3]Animal Research Service, University of Salamanca, Salamanca, Spain
[4]Department of Cell Biology and Pathology, University of Salamanca, Salamanca, Spain
Email: lopezde@usal.es

Received 13 May 2015; accepted 27 June 2015; published 1 July 2015

Abstract

The serotonergic system in the brain plays a major role in mood and anxiety regulation when exposed to stress. The aim of the present study was to evaluate the effects of Sertraline administration in coping with stress using the behavioural paradigms of the acoustic startle reflex (ASR) and its prepulse inhibition (PPI) in both sexes. Wistar rats were divided into two groups: intact animals and exposed to restraint stress (RS) 3 times per day during 7 days, which were then subdivided into three other groups: injected with Sertraline (5 mg/kg/day) or the drug vehicle saline for 8 consecutive days, and non-injected. ASR and PPI values were analyzed along 4 sessions to determine behavioral changes. Upon it, we also determine the effects of acute immobilization stress analyzing physiological stress indicators in blood. Our data show sex differences in response to stress paradigms. RS affected more intensely males than females, disturbing the males' growth and the long-term startle habituation that were not affected in females. PPI increased in the vehicle-injected animals when compared to baseline in both sexes, and Sertraline reversed more efficiently it in females. Moreover, despite both sexes exposed to stressful paradigms exhibited a significant increase in serum glutamic-oxaloacetic transaminase and lactate dehydrogenase enzymes when compared with intact controls, as well as leucopenia, some differences according to sex were found in the haemostatic response to stress. Notably, the repeated injections procedure disturbed the early response to stress, which Sertraline only attenuated in both sexes. Our data suggest that 8-day Sertraline administration is effective in reversing stress-induced changes in some physiological parameters, but insufficient to return immunological values to normality.

How to cite this paper: Pereira-Figueiredo, I., Carro, J., Muñoz, L.J., Sancho, C., Castellano, O., Gómez-Nieto, R. and López, D.E. (2015) Sex Differences in the Effects of Sertraline and Stressors in Rats Previously Exposed to Restraint Stress. *J. Biomedical Science and Engineering*, **8**, 399-419. http://dx.doi.org/10.4236/jbise.2015.87038

Keywords

Behavior, Habituation, PPI, Serotonin, Startle

1. Introduction

Our current understanding of the deleterious effects of stress, affecting many other physiological systems besides the brain, is raising increasing concern. Stress is an ambiguous term, as regards its labeling as protective or damaging. The early response to a stressor is protective; the body responds to a sudden unexpected event and many chemical mediators (such as glucocorticoids, catecholamines, and pro and anti-inflammatory cytokines) are released. It is in this way that the organism starts to cope with the situation [1]. However, as is very often the case in life, if the stress condition is prolonged then physiological [2] [3] or psychological changes may be induced [4], as a consequence of the persistent increase in the levels of the same mediators, opening a window which may lead to diseases (among others, coronary heart disease, hypertension, type 2 diabetes, atherosclerosis, obesity), as well as depression, or generalized anxiety disorders [2] [5]-[7]. In light of this, over the past few years several authors have attempted to unveil how organisms manage to mediate the effects of stress [4] [8]-[10].

Both animals and humans are able to develop intrinsic adaptive changes when exposed to stress. A typical form of adaptation is habituation [10]-[12]; this happens when an individual is repeatedly exposed to the same type of stimuli, the responses to these gradually becoming reduced [13]. Another is the ability to achieve irrelevant sensory stimuli, the responses to these gradually being suppressed [14]. A common operational model of this latter is prepulse inhibition (PPI)—a form of plasticity of the startle reflex [15]—in which the startle, induced by an intense startling stimulus that evokes a whole-body reflective response, can be inhibited by prior presentation of a weaker stimulus [16].

According to the literature, both the acoustic startle reflex (ASR) and its modulation (such as the startle habituation and the PPI) are very often used as behavioral tests for the assessment of emotional status in animals and humans [17], and may well be disturbed in many psychiatric disorders [18]-[20] such as anxiety [19], panic disorder [21], or obsessive compulsive disorder [22]. Thus, disruption of the ASR may reflect functional abnormalities in cognitive processes and lead to difficulties in adapting to subsequent stressors [19] [23] [24]. In this respect, the first goal of our work was to check the early and immediate changes induced by restraint stress on disturbing the startle response and it modulation in young rats.

Restraint stress is a very simple animal model extensively used in stress studies with rodents whose effects mimic the above-described pathophysiologic disorders [24]-[27]. The animals must be repeatedly placed in Plexiglas tubes, restricting their movements. The method is straightforward and painless and is assumed to leave no lasting debilitation [28]. However, even though the method is fairly gentle, animals exposed to restraint exhibit alterations in multiple brain structures and behavioral functions that have been proposed to be tightly linked to dysfunctional changes in the serotoninergic system [27] [29] [30]. Among all the monoamines found in the brain, serotonin (5-HT) plays an important role in the processes of the early brain development [31] [32], and it is mainly involved in processes of arousal, vigilance, anxiety, mood and impulsiveness [26]. In this sense, startle and sensorimotor measurements have been suggested to be sensitive to the serotonin balance [33]-[37], which may be disturbed in stressed animals [38]. Currently, the main drugs employed for the management of anxiety disorders are 5-HT reuptake inhibitors [29] [39]. In light of this, we speculated that by dispensing Sertraline (SERT), a selective serotonin reuptake inhibitor (SSRI) to young rats we would be able to mediate stress-induced changes in behavioral models. Also, considering the crucial role of 5-HT in the physiological response to stress [40] [41], our main goal was to examine the effects of SERT treatment (via the intraperitoneal route) in previously restrained animals, when these are subjected to a new stressor, the immobilization stress (IMO). Giving the significant body of evidence regarding stress and sex on later behavior [28] [42]-[44], and the sex-dependent response to antidepressants in several species [45] [46], analyses were made simultaneously for male and female rats.

2. Materials and Methods

2.1. Animal Experiment

Highly outbred male and female Wistar rats of 70 - 72 days of age (respectively weighing 290 - 310 g and 170 -

185 g at the beginning of the experiment) were provided by our own animal facility at the University of Salamanca. Rats were housed and maintained on a 12:12 hour light: dark cycle (lights on at 8 am) in a room with controlled temperature.

For all experiments, the animals were allowed access to food and water ad libitum, and were maintained on a regular light-dark cycle (lights on: 07:00am - 19:00pm) with constant temperature (21˚C). The animals were handled and cared for according to the guidelines of the European Community's Council Directive (2010/63/CE) and current Spanish legislation for the care and use of laboratory animals.

2.2. Determination Startle and Prepulse Inhibition

The acoustic startle reflex was measured in six identical startle-response cages, using the SR-LAB system (SDI, San Diego, CA, USA). Acoustic stimulus intensities and response sensitivities were calibrated (using an SR-LAB Startle Calibration System) so that they would be nearly identical in each of the six SR-LAB systems (maximum variability < 1% of stimulus range and <5% of response ranges). Each testing session consisted of an acclimatization period of ~5 min, followed by 64 trials presented pseudo-randomly, with a mean inter-trial interval of 30 s, as previously described [47]. Briefly, the sessions had four blocks of pulse and prepulse, with prepulse-to-pulse intervals of 50ms. Whole-body movements corresponding to startle responses were recorded and analyzed with the SR-LAB system, providing ASR latencies and amplitudes. The background noise of 65 dB SPL was generated throughout the entire session in order to avoid interference from external noise and to ensure equal experimental conditions. As depicted in the experimental design (**Figure 1**), four sessions of startle and sensory motor gating were performed in all animals. Before testing, the rats were habituated to the experimental conditions, especially regarding their introduction into the ASR device. All testing was carried out between 10:00 and 13:00 hours [48].

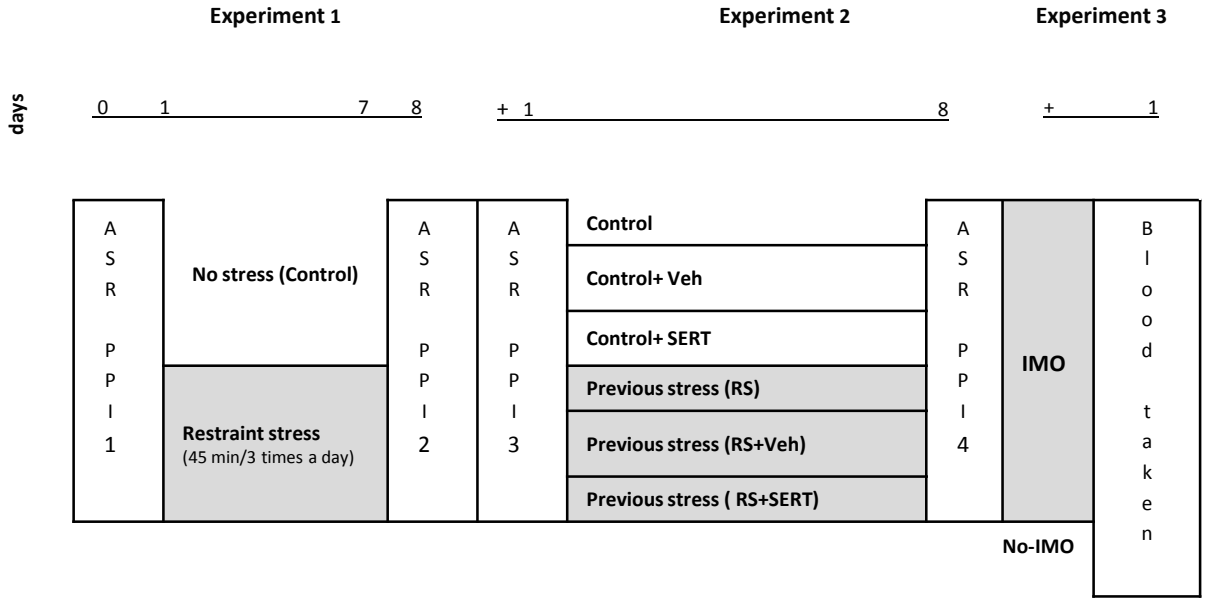


Figure 1. Experimental design used for the present study. Experiment 1: the day before starting the protocol of restraint stress (RS, day 0), all animals were subjected to the first behavioral test (ASR/PPI 1) in which basal values were obtained; and the day after the end of RS, a second behavioral test was performed (ASR/PPI 2). Experiment 2: Control or previously stressed animals of both sexes were subdivided into 6 subgroups according to the treatment applied: the animals subjected to 8 days of intraperitoneal (i.p.) injections with vehicle (Control + Veh, RS + Veh), or SERT (SERT: 5 mg/kg/day) (Control + SERT; RS + SERT); and the animals not injected that remained undisturbed (Control, RS). On the first day of experiment 2, one hour after the i.p. protocol all animals were subjected to the third behavioural test (ASR/PPI 3); and again on the last day, one hour after the i.p. procedure, all animals were subjected to the fourth behavioural test (ASR/PPI 4). Experiment 3: all animals except those subjected to restraint plus the i.p. injections were subjected to a session of immobilization stress (IMO) and 10 min after that, trunk blood samples were taken for comparison with a group of undisturbed controls, subjected only to blood sampling (No-IMO).

In the first experiment, the effect of the stress was indexed by (Average ASR2/ Average ASR1) × 100) − 100), giving the percentage (%) of change in each individual. In the second experiment, long-term change (%) (LTC) in ASR amplitudes as a result of repeated testing were indexed by (Average ASR4/ Average ASR1) × 100) − 100, with body weight as a covariate. Thus, a positive and negative LTC respectively indicate the sensitization or habituation of the startle [11]. Immediately after each session, all animals were weighed and all females were subjected to estrous determination.

2.3. Estrous Cycle Determinations

Vaginal smears were obtained by dipping a sterile swab (0.6 mm diameter, 0.025 in Fischer Scientific) in sterile saline, and then gently swabbing the vaginal lumen. The swabs were smeared onto labeled glass slides that were previously cleaned with 95% ethanol. The cells were fixed with 95% ethanol for 15 minutes and then air-dried before staining with haematoxylin-eosin. The vaginal smears were inspected and the phase of the estrous cycle was determined using an Olympus Microscope (×40), and following previous criteria [49], a 4 to 5 day-cycle was considered (proestrus, estrous, metestrus and diestrus). Rats in proestrus with the highest estrogen levels (with high number of nucleated epithelial cells); by contrast, rats in estrus with the lowest estrogen levels (with anucleate cornfield cells) [24] [49]; rats in metestrus with high number of white blood cells (WBC) and nucleated cornified cells; and rats in diestrus having some epithelial cells and still a predominance of WBC. Females were later distributed in order to have an equal number of animals in each phase in each group.

2.4. Restraint Stress

The day after the first startle/PPI session, animals of both sexes were placed in an isolated room, where they were distributed to either the restraint stress paradigm (N = 21 females and N = 20 males) or were left undisturbed (served as controls in the first experiment) (N = 19 females and N = 22 males). The rats exposed to restraint (RS) were placed daily in small transparent Plexiglas cylinders, three times a day for 45 minutes along 7 days. This device limited their body movements (the rats were not able to move forward or backward) and was positioned directly under a bright light. The length and diameter of the cylinder were based on body size, smaller-diameter cylinders being used in females than in males (70 vs. 80 mm), all with adjustable endplates.

2.5. Intraperitoneal Protocol

On the day after the second behavioral session (ASR/PPI 2) and two days after completion of the RS period, all animals of both sexes were subdivided into six subgroups according to the further treatment to be subjected (see **Figure 1**).

SERT-receiving rats were given Sertraline (Besitran© Pfizer S.A. Madrid, SPAIN) intraperitoneally at 5 mg/kg/day once daily (i.p.) for 8 days. Sertraline was dissolved in 0.9% NaCl vehicle and were administrated in a dose of 1 ml/kg. The dose was based on previous reports [50]. Vehicle-injected animals (Control + Veh and RS + Veh) were given 0.9% saline solution in the same manner. These animals were used in order to control for the stressful effect of the intraperitoneal (i.p.) administration route. Also, two groups of non-injected (RS and Control) animals of both sexes were left undisturbed from the first to the last day of the i.p. procedure, and were used as a control of the experimental conditions.

2.6. Immobilization Stress

On the day after the completion of the behavioural tests, all animals were exposed to immobilization stress (IMO), a procedural variation of restraint. The same cylinders that had been used previously were adjusted to achieve total restriction of movements, taking care not to hurt the animal [51]. Each rat was placed in the cylinder for 30 min, after which it was placed in a new cage and allowed to rest for 10 min. Then, the animal was anesthetized and trunk blood was taken by cardiac puncture [50].

This procedure was carried out simultaneously on three animals from 10:00 h to 13:00 h. A group of undisturbed animals (No-IMO) of the same age was also used in order to achieve the effects of IMO stress. In order to reduce the inherent effects of differences in the estrous cycle, only females that were not in proestrus were used [52].

2.7. Hematological Analyses

For hematological analyses, trunk blood was collected to EDTA (K3)-containing tubes that were freshly processed on an automatic cell counter (ADVIA 120 cytometer, Bayer, Leverkusen, Germany). The obtained hematological parameters were number of erythrocyte, hemoglobin concentration, mean corpuscular hemoglobin (MCH), hematocrit values, platelet number and mean platelet volume (MPV), and the number and type of leukocytes (WBC).

2.8. Biochemical Analyses

For biochemical analyses, blood samples were collected into heparinized tubes, which were then centrifuged at 10,000 × g for 20 min to obtain serum, which was drawn into Eppendorf tubes and used fresh on a SPOTCHEM™ EZ device (QBC Europe). The levels of serum total protein (T-Pro), albumin and bilirubin, and the cytosolic enzymes glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) were measured using the commercial kit (ARKRAY®) according to the manufacturer's directions.

2.9. Statistics

Statistical analyses were performed using IBM® SPSS® software, version 20 (IBM Crp. and SPSS Inc., Chicago, IL, USA, 2011). Differences between groups were analyzed by ANOVA (one, two and three way), followed by the Fisher-PLSD-test for post hoc comparison if appropriate, and ANOVA mixed (or "SPLIT-PLOT") with the Bonferroni-test. Mean differences were subjected pairwise to Student's t-test, using the Levene Test for equality of variances. Pearson's coefficient was used to determine correlations. Differences were regarded as statistically significant when $p \leq 0.05$.

3. Results

3.1. Experiment 1

This experiment aimed at obtaining preliminary data regarding the effects of one week of restraint stress on ASR and PPI, measuring the data one day after completion of the stress protocol.

3.1.1. Startle Amplitude after Stress by Restraint

The three-way (group × sex × day) repeated-measures ANOVA indicated a significant effect of the repeated measurements of startle ($F_{1,74} = 5.30$, $p = 0.02$). Post-hoc analyses revealed that a slight increase in the startle reflex with stress was present ($F_{1,74} = 2.58$, n.s.), but no differences in ASR amplitude between the control and stressed animals of both sexes were significant (**Figure 2(a)**). As depicted in **Figure 2**, **Figure 2(a)** given the huge differences between males and females in this parameter the sex of the animal was a determining factor in the startle response ($F_{1,74} = 4.47$, $p < 0.001$).

When the change of the startle amplitudes between sessions was evaluated (using a two-way ANOVA, with group and sex as factors), the analysis confirmed that startle modulation was not significantly altered by stress ($F_{1,74} = 1.29$, n.s) despite the control males exhibited an habituation trend (**Figure 2(b)**).

3.1.2. Prepulse Inhibition

No differences in PPI levels as effect of stress were found (**Figure 3**) in either sex (**Figure 3(a)**). In overall the males exhibited significantly higher PPI values than the females ($F_{1,74} = 70.60$, $p < 0.001$).

Moreover, the repeated measures ANOVA (group × sex × day) revealed that PPI increased significantly with respect to its basal values ($F_{1,74} = 24.06$ $p < 0.001$), with no interaction with group ($F_{1,74} = 1.89$) or sex ($F_{1,74}$= 3.89). Post-hoc analysis revealed that the difference in males was significant, regardless of the stress treatment, and in females PPI only increased when the repetitive situation of restraint stress was applied (**Figure 3(b)**).

3.1.3. Latencies

The repeated measures of ANOVA showed no significant changes with the stress procedure in the latency of startle and PPI latency ($F_{1,75} = 2.17$ and $F_{1,75} = 0.021$, respectively). On the day after completion of the stress pro-

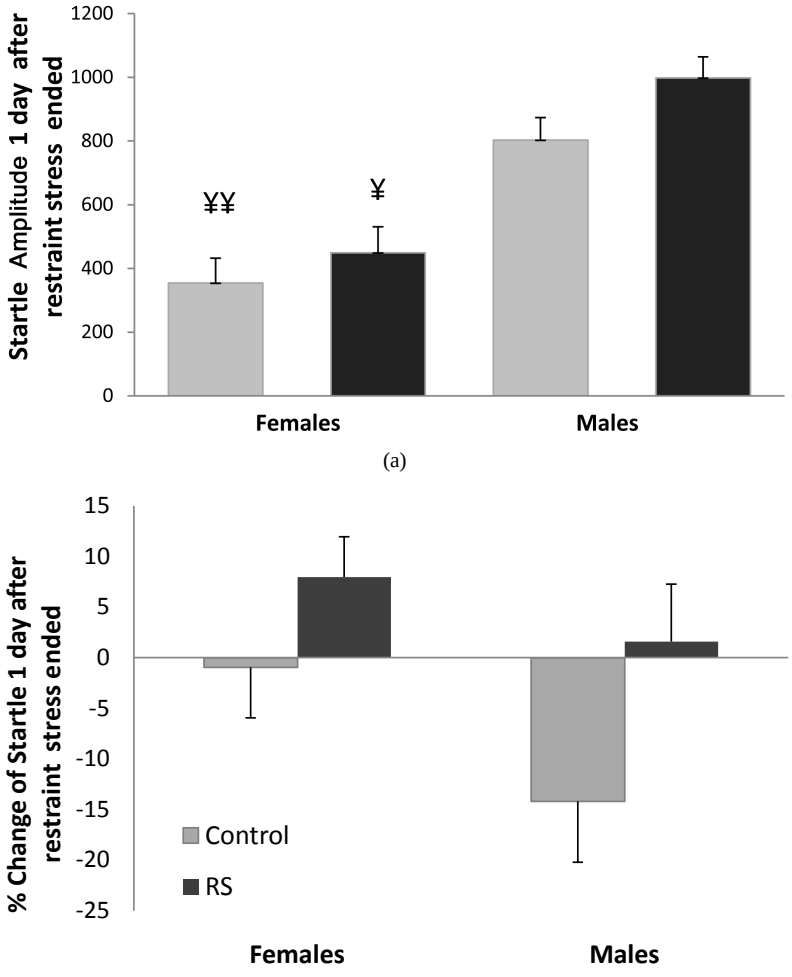
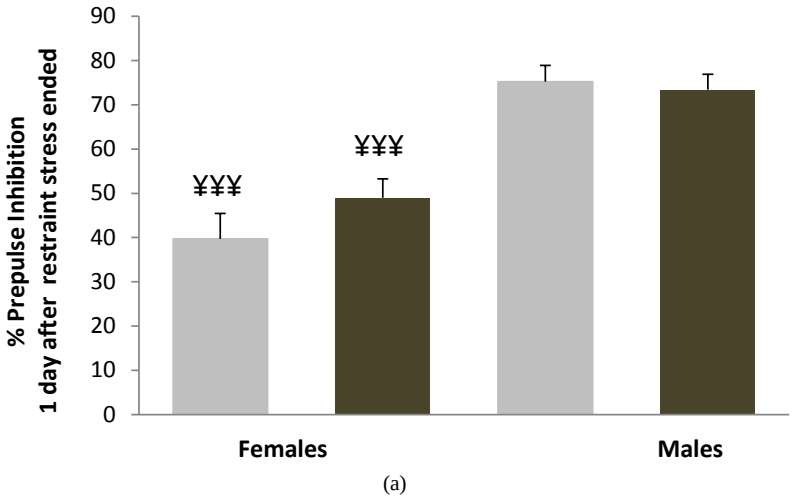
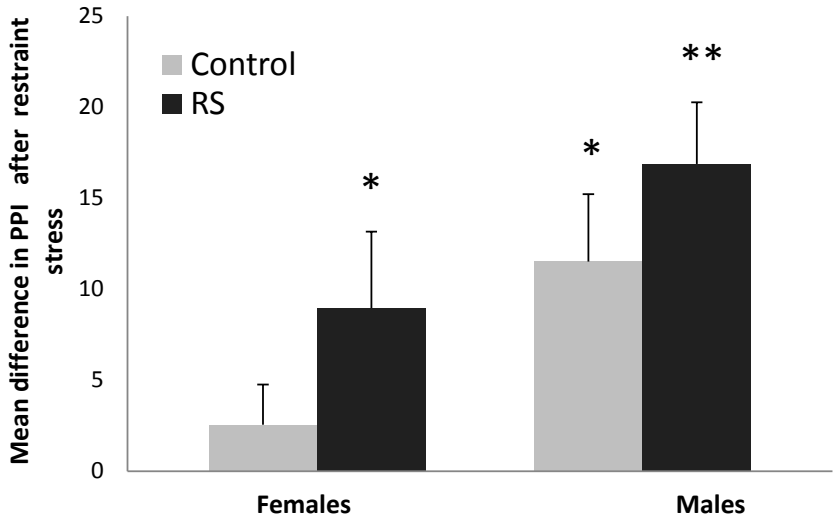


Figure 2. Acoustic startle reflex (ASR) results of experiment 1. (a) ASR amplitude (in arbitrary units) after 1 week of restraint stress (RS) or no stress (Control); (b) Percentage of change in the startle amplitudes induced by stress in female and male animals, when measured one day after it ended. N = 17 - 21 animals per group and sex. Each column represent the means ± standard error (S.E.) $^{¥¥}p < 0.01$ and $^{¥}p < 0.05$ indicate the differences between males and females.





(b)

Figure 3. Percentage of prepulse inhibition (PPI) of experiment 1. (a) Percentage of prepulse inhibition after 1 week of restraint stress (RS) or no stress (Control); (b) The mean differences ± S.E in the PPI values induced by stress in the animals of both sexes, one day after it ended (when related to baseline). ¥¥¥$p < 0.01$ indicate the differences between males and females; $^{**}p < 0.01$ and $^{*}p < 0.05$, indicate a significant increase of the PPI.

cedure, no differences as an effect of stress were found in either sex. However a group × sex interaction was revealed ($F_{1,75} = 6.5$, $p = 0.013$), meaning that there were differences in the response to stress depending on the animal's sex (**Table 1**).

3.1.4. Body Weight Variation

Analyses were made separately for males and females. All animals increased their weight (from PND 70 to PND 78, $p < 0.001$). A two-way ANOVA (group x day) revealed that restraint stress affected body weight gain in both sexes. When the animals were examined the day after completion of the stress protocol, the RS animals were seen to have gained less weight than their non-stressed counterparts (females $F_{1,32} = 9.32$, $p = 0.005$ and males $F_{1,44} = 50.1$, $p < 0.001$) (**Figure 4**). As shown in **Figure 4(a)** and **Figure 4(b)**, although the effect of this type of stress on weight gain in both sexes was clear, the difference only reached significance in males ($F_{1,44} = 11.8$, $p < 0.01$).

3.2. Second Experiment

This experiment had two main objectives: to examine the effects of one week of restraint stress 10 days after completion of the stress protocol, and to examine the effects of repeated injections (either vehicle or SERT) in previously stressed animals.

3.2.1. Startle Amplitudes after the Experimental Paradigms

A three-factor ANOVA with group and sex as between-subject design factors and time as the repeated design factor revealed that a single SERT/vehicle i.p. injection did not affect the startle response ($F_{1,62} = 0.04$, n.s.), and no differences were found as an effect of group or sex (ASR × group, $F_{5,62} = 0.98$, n.s.; ASR × sex, $F_{1,62} = 0.15$, n.s.). Again, 8 days following the i.p. procedure did not affect ASR amplitude ($F_{1,62} = 0.26$, n.s.) Also, when the analyses were split by days, no significant differences in the ASR means were observed as an effect of the combined paradigms of restraint stress plus Sertraline/vehicle procedure in either sex ($p > 0.05$). Moreover, clear sex differences in the ASR were found, regardless of the group, $F_{1,62} = 39.7$, $p < 0.001$(**Figure 5**).

When the long-term change (%) (LTC) of the startle was further analyzed (using a two-way ANOVA), no significant differences between groups were found ($F_{5,73} = 1.22$), however, both sex ($F_{1,73} = 7.43$, $p = 0.008$) and group × sex, ($F_{5,73} = 3.23$, p = 0.01) interactions affected it.

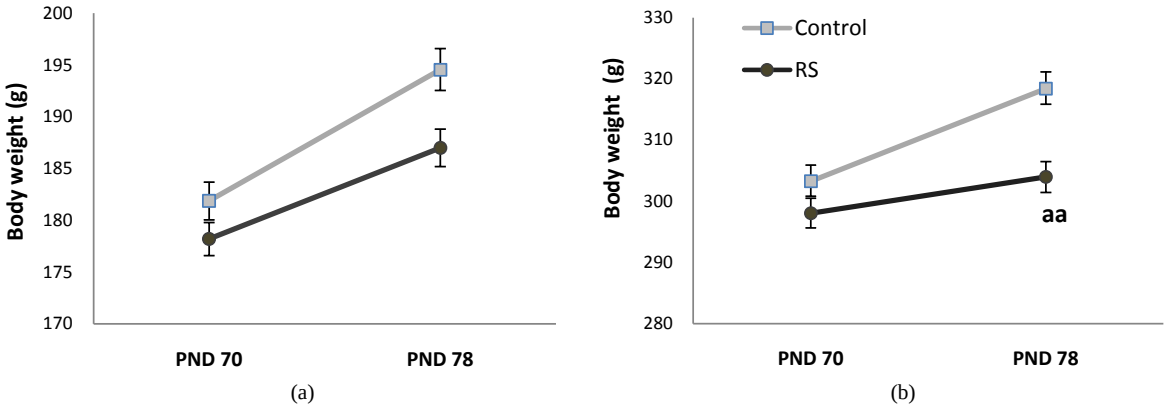


Figure 4. Body weight gain. Effects of one week of restraint stress on the in female and male animals submitted or not to restraint (RS and Control). Abbreviations: PND 70 and PND 78, postnatal days 70 and 78, respectively; N = 17 - 21 animals per group and sex. Mean values ± S.E. aa indicates a main effect of restraint stress in males. (a) Females; (b) Males.

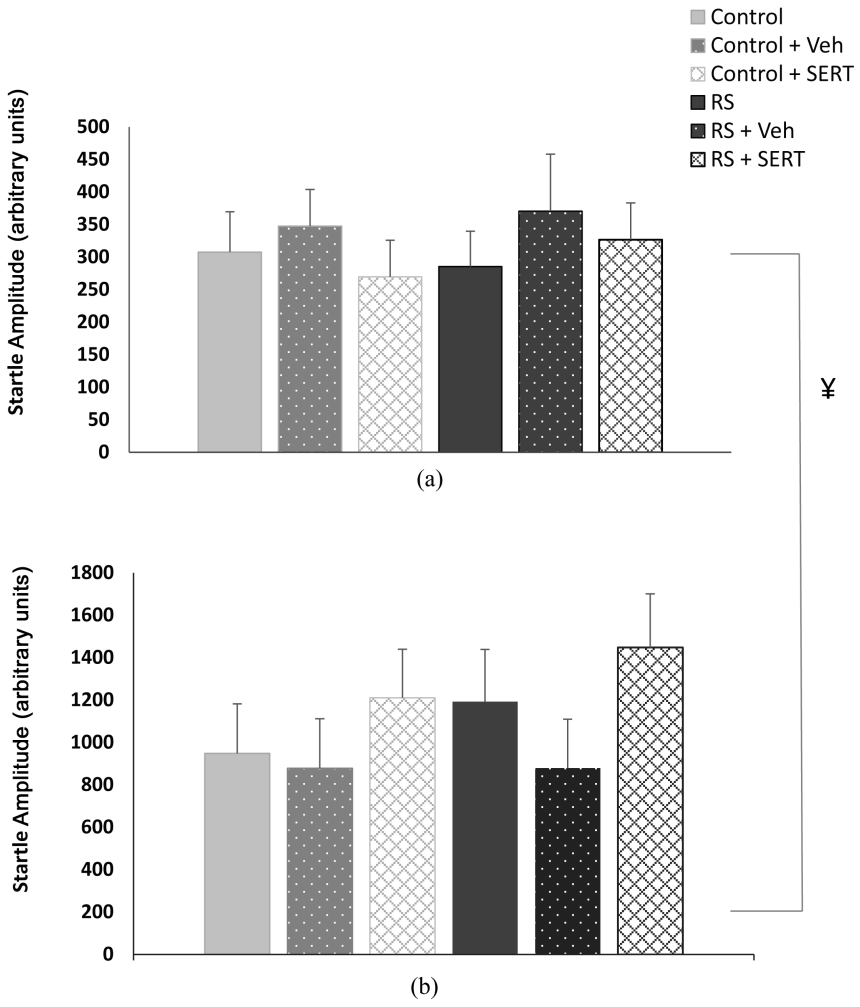


Figure 5. Acoustic startle reflex (ASR) amplitude of experiment 2. Mean ASR amplitude values (in arbitrary units) in (a) females and (b) males previously stressed/or not stressed by restraint that were further subjected to 8 days of intraperitoneal injections with vehicle (RS + Veh; Control + Veh), SERT (5 mg/kg/day) (RS+SERT CC + SERT), or not injected (Control, RS). Body weight was used as a covariate. N = 6 - 8 animals per group and sex. Mean values ± S.E. $^{¥}p < 0.05$ indicates the significant differences between males and females in all the experimental groups. (a) Females; (b) Males.

Table 1. Effects of restraint stress on the acoustic startle reflex (ASR) and prepulse inhibition (PPI) latency one day after the end of the stress procedure.

Startle latency						
Groups	**Females**			**Males**		
	Mean value	**Difference vs. basal**		**Mean value**	**Difference vs. basal**	
Control	31.1 ± 2.1	+0.27	±2.6	35.3 ± 1.8	−3.52	±2.3
RS	30.5 ± 2.3	−1.82	±3.3	36.7 ± 1.8	+0.61	±1.7
PPI latency						
Groups	**Females**			**Males**		
	Mean value	**Difference vs. basal**		**Mean value**	**Difference vs. basal**	
Control	26.9 ± 1.3$^{¥}$	−0.25	±1.6	28.7 ± 1.3	−2.37	±2.1
RS	28.6 ± 2.1	+1.11	±1.4	28.9 ± 1.1	−1.95	±2.2

Mean values ± error standard in the different experimental groups (N = 6 - 8 per group). $^{¥¥}p < 0.01$ and $^{¥}p < 0.05$ indicate significant differences between males and females after the experimental procedure; b indicates the main effect of SERT in males vs. Control; $^{*}p < 0.05$, indicates the significant decrease of the ASR latency as an effect of the intraperitoneal injections with vehicle in males, when compared to baseline.

Split the analyses by sex, post-hoc shows in males that RS induced a significant increase in ASR amplitudes (in %), $F_{5,62} = 2.74$, $p = 0.034$ (**Figure 6**). Previously restrained males exhibited an increase of 65.8% ± 30.7% amplitude relative to baseline ($p = 0.027$), indicating that these animals had difficulty in becoming accustomed to retesting (**Figure 6(b)**). In the females, no significant changes in the ASR as an effect of restraint were found ($F_{5,62} = 0.65$, n.s.), despite, it was marginally affected by the i.p. method, an effect that was inverted by SERT administration (**Figure 6(a)**).

3.2.2. Prepulse Inhibition after the Experimental Paradigms

As seen above, a single i.p. injection did not change PPI ($F_{1,61} = 1.92$, n.s.). However, repeated SERT/vehicle intraperitoneal injections induced a significant effect on PPI ($F_{1,61} = 19.92$, $p < 0.001$), with a group × sex $F_{5,58} = 7.2$, $p < 0.001$ interaction. Post-hoc analysis revealed that while PPI remained virtually unchanged in the Control and RS animals of both sexes, PPI increased in both groups of vehicle-injected animals (Control + Veh and RS + Veh), regardless of the stress treatment, and SERT reversed this effect (**Figure 7**) more efficiently in females than in males. Also, a main effect of sex, $F_{1,58} = 122.9$, $p < 0.001$ was observed.

3.2.3. Variation of the Startle Reflex and PPI Latencies

ASR latency did not change significantly in comparison with the baseline, although the latency of startle × group × sex interaction ($F_{5,62} = 2.58$, $p = 0.035$) was observed and, again, differences between the sexes were observed ($F_{1,62} = 22.2$, $p < 0.001$). Regarding each sex, no significant differences in startle latency were found between the groups ($F_{5,62} = 0.09$, n.s.); however, a different trend was revealed when its values after the experimental paradigms were compared to baseline figures. Post-hoc analysis revealed that the daily-injection procedure shortened the latency to respond. In males, this effect reached significance, regardless of previous stress, and SERT treatment reversed it in both sexes (**Table 2**).

Overall, PPI latency did not change significantly when compared with the baseline, $F_{1,65} = 1.56$, n.s., but again, differences depending on sex were apparent, $F_{1,65} = 14.3$, $p < 0.001$. As shown in **Table 2**, a decrease (although not significant) in PPI latency was observed in previously injected animals of both sexes, an effect that was not reversed by SERT in males. When the analyses were made split by sex and day, after the treatments a main effect of group ($F_{5,65} = 4.5$, $p = 0.001$) was revealed in males, probably as effect of SERT administration, whereas in females differences between the experimental groups reached no significance.

Moreover, an overall dimorphism was observed in both the startle reflex and PPI latency values in the vehicle-injected and restrained animals (data not shown), again indicating a different response to the adverse procedures by each sex.

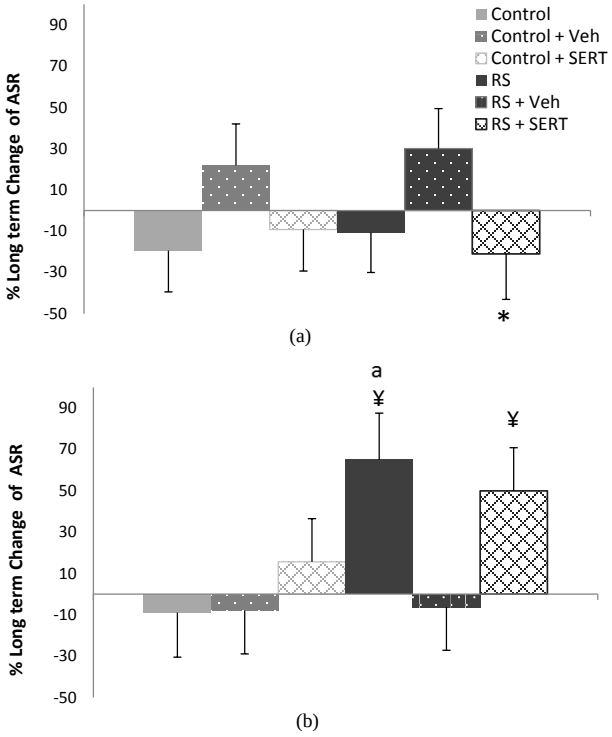


Figure 6. Long term change of the acoustic startle reflex (ASR) amplitude. Percentage of long-term Change in the startle amplitude induced by the experimental paradigms in (a) females and (b) males. N = 6 - 8 animals per group and sex. Means ± S.E. $^{*}p < 0.05$, indicates a significant decrease of the startle reflex; a $p < 0.05$, indicates the main effect of restraint stress in males (RS vs. Control). $^{¥}p < 0.05$ indicates significant differences between males and females. (a) Females; (b) Males.

Table 2. Effects of restraint stress + SERT/Vehicle intraperitoneal treatment on the acoustic startle reflex (ASR) and prepulse inhibition (PPI) latency).

Startle latency				
Experimental groups	**Females**		**Males**	
	Mean values ± s.e.		**Mean values ± s.e.**	
Control	30.1	±3.3	37.2	±3.1
Control + Veh	26.8	±3.2$^{¥}$	35.7	±3.0*
Control + SERT	32.6	±3.3	37.9	±3.1
RS	29.5	±3.1$^{¥}$	40.7	±3.3
RS + Veh	31.0	±4.5	34.6	±3.0*
RS + SERT	33.7	±3.3	40.2	±3.1
PPI latency				
Experimental groups	**Females**		**Males**	
	Mean values ± s.e.		**Mean values ± s.e.**	
Control	31.1	±2.5	34.1	±2.1
Control + Veh	26.7	±2.1	28.4	±2.1
Control + SERT	30.7	±2.3	25.6	±2.3^{b}
RS	27.2	±2.1$^{¥¥}$	35.2	±1.8
RS + Veh	24.7	±3.2$^{¥}$	32.6	±3.2
RS + SERT	28.6	±2.3	24.9	±2.1^{b}

Mean values ± error standard in the different experimental groups (N = 6 - 8 per group). $^{¥¥}p < 0.01$ and $^{¥}p < 0.05$ indicate significant differences between males and females after the experimental procedure; ^{b}indicates the main effect of SERT in males vs. Control; $^{*}p < 0.05$, indicates the significant decrease of the ASR latency as an effect of the intraperitoneal injections with vehicle in males, when compared to baseline.

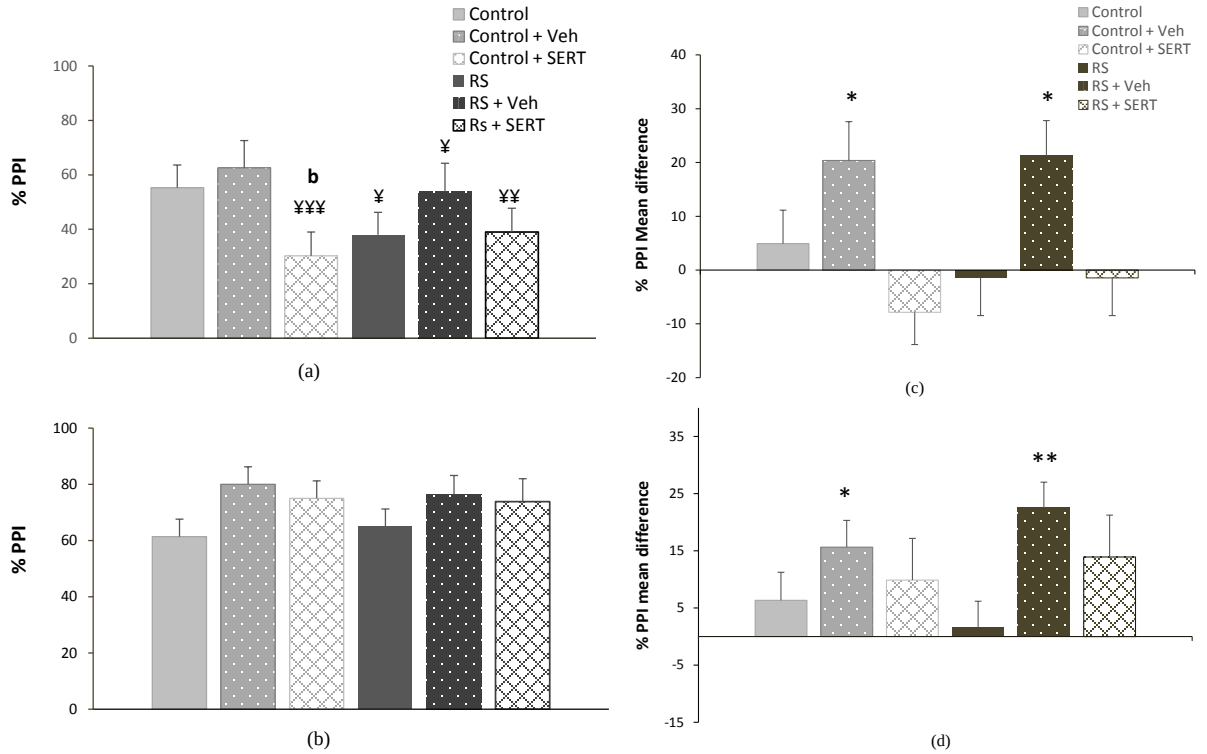


Figure 7. Percentage of prepulse inhibition (PPI) of experiment 2. Percentage of prepulse inhibition in females (a) and males (b) for the 6 experimental groups: previously stressed/not stressed and not injected (RS; Control); and previously stressed/not stressed and intraperitoneal (i.p.) injected with SERT (5 mg/kg/day) (RS + SERT Control + SERT), or vehicle (RS + Veh; Control + Veh) for 8 days. $^{¥¥¥}p < 0.001$, $^{¥¥}p < 0.01$ and $^{¥}p < 0.05$ indicate the significant differences between males and females; $^{b}p < 0.05$, indicates the main effect of Sertraline in females. Mean differences ± S.E. in the PPI values for females (c) and males (d) induced by the experimental paradigms. $^{**}p < 0.01$ and $^{*}p < 0.05$, indicate the significant increase of the PPI as effect of the i.p. injections with vehicle in both sexes. (a) Females; (b) Males; (c) Females; (d) Males.

3.2.4. Variations in Body Weight

The body weights of all the animals increased along the 18 days of the experiment ($F_{1,39} = 406.7$, $p < 0.001$, in females and, $F_{1,44} = 704.01$, $p < 0.001$ in males). I.p. injections with SERT or saline for 8 days did not induce significant changes in the growth of the animals of either sex ($F_{1,39} = 0.11$, and $F_{1,40} = 2.17$). However, following SERT administration (regardless of stress) in the males from both groups, the drug affected body weight gain slightly (Control + SERT and RS+SERT males respectively lost 7.4 ± 3.2 g and 6.3 ± 2.9 g in comparison with their controls).

Furthermore, ten days after completion of the restraint stress protocol the body weight gain of previously stressed males was still affected, $F_{1,44} = 4.9$, $p = 0.03$, (stressed males lost 8.5 ± 3.8 g when compared to their controls, data not shown).

3.3. Third Experiment

Given the large differences found in the behavioral parameters of each sex in response to each type of stressful condition and to SERT treatment, in this experiment our main goal was to extend the results by comparing the effects of each paradigm on later stress responsiveness.

Thus, one day after the last test ended the animals of both sexes subjected to each type of paradigm-restraint stress, i.p. injections with SERT or vehicle, or controls—were exposed to a new stressor, IMO. Blood samples were extracted and compared with those of a group of undisturbed animals (NO-IMO).

3.3.1. Hematological Analyses

A main effect of group was found in both sexes for all parameters ($p < 0.001$), except in erythrocytes (**Table 3**). A significant increase in the amount of hemoglobin (g/dl), hematocrit (%) and platelet volume (MPV) in the

Table 3. Hematological data.

Experimental groups		Erythrocytes (10^6/µl)	Hemoglobin (g/dl)	Hematocrit (%)	MCH (pg)	Platelet (10^3/µl)	MPV fL (µm^3)
Females	**No-IMO**	7.8 ± 0.1	14.9 ± 0.3	40.4 ± 0.7	19.6 ± 0.2	914.1.5 ± 60.4	7.9 ± 0.2
	Control	7.8 ± 0.3	15.7 ± 0.3$^{¥}$	45.4 ± 1.6$^{**¥}$	19.9 ± 0.7	1036.3 ± 62.8	10.9 ± 0.3**
	Restraint stress	7.9 ± 0.3	16.6 ± 0.4	44.0 ± 1.6	20.7 ± 0.4	1411.2 ± 71.1^{**a}	11.0 ± 0.5*
	Control + Vehicle	7.4 ± 0.2$^{¥}$	13.8 ± 0.6 iii	37.9 ± 0.9 ii$^{¥}$	17.8 ± 0.6^{**iii}	771.6 ± 88.2	10.1 ± 0.5*
	Control + SERT	7.0 ± 0.5	15.2 ± 0.6	39.6 ± 1.7	20.6 ± 0.5^{bb}	866.2 ± 128.3	10.5 ± 0.7*
		F = 1.3, ns	**F = 11.3, $p < 0.001$**	**F = 64, $p < 0.001$**	**F = 12.9, $p < 0.001$**	**F = 10.4, $p < 0.001$**	**F = 5.5, $p < 0.001$**
Males	**No-IMO**	8.2 ± 0.2	15.4 ± 0.2	40.7 ± 0.5	18.7 ± 0.1	908.5 ± 61.1	7.7 ± 0.1
	Control	8.4 ± 0.2	17.2 ± 0.2	47.6 ± 1.0***	19.7 ± 0.3	927.3 ± 62.8	10.8 ± 0.3**
	Restraint stress	7.7 ± 0.3	16.2 ± 0.3	42.1 ± 1.2	20.9 ± 0.2**	1254.8 ± 67.1^{**a}	11.4 ± 0.5**
	Control + Vehicle	8.1 ± 0.2	14.5 ± 0.4^{iii}	43.1 ± 2.0^{ii}	17.9 ± 0.3^{ii}	707.6 ± 108.2	9.6 ± 0.4
	Control + SERT	8.1 ± 0.4	14.6 ± 0.5^{ii}	38.5 ± 1.5^{iii}	20.4 ± 0.5^{bb}	728.2 ± 124.3	12.3 ± 0.6^{**bb}
		F = 1.9, ns	**F = 9.3, $p < 0.001$**	**F = 11.7, $p < 0.001$**	**F = 9.5, $p < 0.001$**	**F = 8.5, $p < 0.001$**	**F = 10.5, $p < 0.001$**

Plasma values obtained in the arterial blood in the animals of both sexes 10 minutes after exposure to IMO stress (Restraint stress, Control + Vehicle, Control + SERT) or not exposed to IMO (No-IMO). Mean values ± S.E. in the different experimental groups (N = 6 per group and sex). Abbreviations: MCH, mean corpuscular hemoglobin concentrations; MPV, mean platelet volume. $^{¥}p < 0.05$ indicates significant differences between males and females; $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$, indicate a significant difference when compared to No-IMO; a $p < 0.05$, indicates the main effect of restraint stress (different from Controls); $^{bb}p < 0.01$ indicates a main effect of SERT (different from vehicle); iii p < 0.001, $^{ii}p < 0.01$ and $^{i}p < 0.05$, indicate a main effect of the intraperitoneal procedure (different from controls).

animals subjected to acute stress (IMO) was found as compared to undisturbed control animals (No-IMO).

As shown in **Table 3**, differences were found according to the type of the previous stressful paradigm. Whereas the previously restrained animals responded intensely when exposed to the new stressor, in the animals subjected to daily injections the concentration of hemoglobin, hematocrit, MCH, and platelet values did not increase. This suggests that in these animals the stress response is disturbed, an effect that was not fully reversed by SERT treatment. Moreover, no differences in the hematological values according to sex were found in undisturbed animals. However, differences were found in both the animals subjected to IMO and in the animals previously subjected to a repeated i.p. procedure, the males exhibiting higher values of erythrocytes, hemoglobin and hematocrit in blood plasma.

3.3.2. Metabolic Analyses

As shown in **Table 4**, the acute stress condition (IMO) increased the release of almost all metabolic parameters studied in blood serum in both sexes. Even so, albumin and bilirubin and the cytosolic enzymes LDH and GOT were significantly increased in the previously stressed animals (whether with restraint or i.p. vehicle) in comparison with the undisturbed animals (**Table 4**); SERT-treatment partly counteracted this effect. No sex differences were seen in the parameters studied, but in males in particular, restraint stress proved to exacerbate the effects of the IMO stress.

3.3.3. Leukogram Analysis

As shown in **Table 5**, the acute condition of IMO stress did not affect the leukocyte profile in Controls. Differently, in animals that underwent a prior stressor, leukopenia was observed in both sexes. This condition was more prominent in the animals subjected to the daily injection procedure, an effect that was not reversed by SERT. In both sexes the total number of leukocytes, of neutrophils, lymphocytes and monocytes were significantly lower in vehicle-injected animals than Controls (**Table 5**). In the other leukocyte cells analyzed (basophil's and eosinophil's granulocytes) no differences were found as effect of the treatments animals experienced. Moreover no sex-dependent differences were found in none parameter measured, but again restraint proved to affect more the males than the females.

Table 4. Biochemical serum values.

Experimental groups		T-pro (g/dl)	Alb (g/dl)	T-bil (mg/dl)	GOT (IU/l)	GPT (IU/l)	LDH (IU/l)
Females	No-IMO	6.0 ± 0.2	3.3 ± 0.1	0.30 ± 0.1	54.6 ± 8.7	27.7 ± 5.8	423.4 ± 122
	Control	6.2 ± 0.1	3.5 ± 0.1	0.60 ± 0.1	107.9 ± 8.2**	37.5 ± 6.8	2589.2 ± 350***
	Restraint stress	6.7 ± 0.1	3.8 ± 0.1**	0.55 ± 0.1	129.4 ± 8.1***	29.2 ± 4.7	3579.7 ± 247^{***a}
	Control + Vehicle	6.1 ± 0.2	3.8 ± 0.1**	0.43 ±0.1	117.8 ± 6.8***	22.9 ± 4.3	3324.2 ± 230***
	Control + SERT	5.3 ± 0.2	3.2 ± 0.1^{bb}	0.42 ± 0.1	122.4 ± 8.1**	18.4 ± 4.8	1694.8 ± 271^{b}
		F = 6.1, *p* = 0.001	**F = 8.4, *p* < 0.001**	**F = 1.7, *p* = ns**	**F = 18.4, *p* < 0.001**	**F = 2.9, ns**	**F = 18.8, *p* < 0.001**
Males	No-IMO	6.2 ± 0.2	3.4 ± 0.1	0.30 ± 0.1	68.2 ± 9.1	29.7. ± 5.8	485.7 ± 242
	Control	6.2 ± 0.1	3.4 ± 0.1	0.36 ± 0.0	101.8 ± 8.5	34.7 ± 4.2	1614.9 ± 263$^{*¥}$
	Restraint stress	6.6 ± 0.2	3.8 ± 0.1^{a}	0.70 ± 0.1^{*a}	136.5 ± 7.1^{***a}	31.2 ± 5.7	3081.7 ± 237^{***a}
	Control + Vehicle	6.2 ± 0.2	3.7 ± 0.1	0.37 ± 0.1	109.5 ± 7.5*	37.2 ± 6.0$^{¥}$	3314.2 ± 260^{***ii}
	Control + SERT	5.5 ± 0.1	3.4 ± 0.0	0.28 ± 0.1	106.4 ± 8.0	18.5 ± 4.8^{b}	1564.8 ± 272^{bb}
		F = 3.2, *p* = 0.02	**F = 3.4, *p* = 0.02**	**F = 4.7, *p* = 0.003**	**F = 8.2, *p* < 0.001**	**F = 4.2, *p* = 0.006**	**F = 12.5, *p* < 0.001**

Mean values ± S.E. in the different experimental groups (N = 6 per group and sex). Abbreviations: T-Pro, total protein; Alb, albumin; T-Bil, total bilirubin; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvic transaminase; LDH, lactate dehydrogenase. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$, indicate a significant difference when compared to No-IMO; a $p < 0.05$, indicates the main effect of restraint stress (different from Controls); $^{bb}p < 0.01$ and $^{b}p < 0.05$ indicates the main effect of SERT (different from Control+ Vehicle); $^{ii}p < 0.01$ indicates a main effect of the intraperitoneal procedure (different from controls); $^{¥}p < 0.05$ indicates significant differences between males and females.

Table 5. Leukogram.

Experimental groups		Leukocytes (10^3/µl)	Neutrophils (10^3/µl)	Lymphocytes (10^3/µl)	Monocytes (10^3/µl)
Females	No-IMO	4.8 ± 0.4	0.9 ± 0.1	3.5 ± 0.4	0.08 ± 0.01
	Control	5.2 ± 0.4	0.8 ± 0.1	4.0 ± 0.3	0.07 ± 0.01
	Restraint stress	3.2 ± 0.7	0.4 ± 0.1	2.7 ± 0.5 i	0.04 ± 0.01
	Control + Vehicle	1.5 ± 0.4^{***iii}	0.4 ± 0.4^{**ii}	0.9 ± 0.7^{**iii}	0.03 ± 0.01^{*ii}
	Control + SERT	1.1 ± 0.7^{**iii}	0.4 ± 0.3^{*i}	0.5 ± 0.5^{**iii}	0.05 ± 0.01*
		F = 13.2, *p* < 0.001	**F = 7.2, *p* < 0.001**	**F = 11.5 *p* < 0.001**	**F = 5.6, *p* = 0.001**
Males	No-IMO	5.9 ± 0.5	0.9 ± 0.1	3.9 ± 0.2	0.09 ± 0.01
	Control	5.8 ± 0.5	0.9 ± 0.1	4.7 ± 0.3	0.10 ± 0.01
	Restraint stress	3.5 ± 0.6*a	0.6 ± 0.1	2.7 ± 0.4	0.03 ± 0.01^{*aa}
	Control + Vehicle	2.6 ± 0.5^{**iii}	0.7 ± 0.1	1.8 ± 0.8^{*iii}	0.05 ± 0.01^{i}
	Control + SERT	1.7 ± 0.6^{***iii}	0.4 ± 0.1	0.6 ± 0.6^{**iii}	0.06 ± 0.01^{*ii}
		F = 11.3, *p* < 0.001	**F = 2.9, *p* = 0.031**	**F = 9.4, *p* < 0.001**	**F = 7.6, *p* < 0.001**

Plasma values obtained in the arterial blood in female and male animals 10 minutes after exposure to IMO stress. Mean values ± S.E. in the different experimental groups (N = 6 per group). $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$, indicate a significant difference when compared to No-IMO; $^{aa}p < 0.01$, a $p < 0.05$, indicates the main effect of restraint stress (different from IMO); $^{iii}p < 0.001$, ii p < 0.01 and $^{i}p < 0.05$, indicate a main effect of the intraperitoneal procedure (different from IMO).

4. Discussion

In agreement with previous authors, the first experiment vouches that 7 days of restraint stress was enough to induce changes in later behavior and physiological blood parameters [25] [28] [43]. Our data indicate that sex was a main factor in this stress-type sensitivity, males being more affected by restraining than females.

Repeated injections of vehicle were sufficient to affect sensorimotor gating, and hematological and metabolic parameters in both sexes. Using the commonly prescribed antidepressant SERT, we were able to prevent almost all the deleterious effects of the stressful procedures. However, again a different sensitivity to SERT treatment was found in each sex, the drug being more effective in stabilizing the behavioral parameters in females, which partly corroborates the clinical findings regarding gender differences in the antidepressant treatment with such drug, in human beings [46]. Furthermore, SERT administration i.p. for 8 days was unable to normalize the deregulation of the immune system induced by stress. Thus, our data extend previous findings concerning the effects of stress and the antidepressant response, by evaluating male and female rats at the same time that confirmed the differences across the sexes.

4.1. Body Weight Changes with Stress

Our data show that one-week of restraint stress was sufficient to affect growth in young rats, an effect that was more marked in males. It is assumed that the decrease in body weight is a good physiological marker of stress, and hence the intensity and duration of the stressor would be determining factors in the results. With more intense stressors, such as IMO for 14 days [53] or 8 hours restriction/day plus the use of a variable stress paradigm [3], more spectacular results have been achieved. However, in the present work we failed to detect significant differences in weight gain in the restrained animals subjected later to the i.p. procedure or in those treated with SERT.

4.2. The Effects of Stress on Startle and PPI Modulation

It is well known that the startle response is very sensitive to stress and anxiety both in humans and animals [11] [23] [52] [54]. In the present work, our results indicated there were no differences in ASR amplitude means in either sex as a function of stress paradigm or SERT treatment. However, we found that long-term habituation was disrupted as an effect of RS, in males.

In control animals of both sexes, startle amplitude decreased slightly each time it was examined (data not shown), indicating that a kind of habituation had occurred [11]. Previous works have confirmed the existence of acoustic startle habituation both between consecutive trials during a single session [11] [23] [36] [50] and between the ASR sessions [13] [55]. Nevertheless, ten days after the end of the restraint stress procedure, the RS males exhibited an increase in startle amplitude (when compared to the baseline). These results are partly in agreement with what has been reported in humans with psychopathological symptoms [19] [23]. These authors reported that their patients did not become habituated easily during the startle probe, increasing startle or maintaining it at the same levels in each exposure to the test.

When we analyzed PPI we observed a significant increase in PPI up to an age of 12 weeks in the animals of both sexes regardless of treatment, as we previously found in our laboratory, in males (unpublished data). At that moment, in the control animals PPI remained stable among sessions, as expected [36] [55]. According to the literature, stress affects PPI. PPI may be enhanced by fear or in response to emotional conditions [14] [55] [56]. Our data show that, alone, one week of restraint stress was not sufficient to induce changes in PPI. Also, a single i.p. injection with vehicle had no effects on either the startle reflex or PPI. However, when the animals were exposed to repeated injections with saline, a significant increase in PPI was observed in both sexes, an effect that was counteracted by SERT, although more efficiently in females. The PPI enhancement after daily injections could be interpreted in the sense that this injection procedure might have facilitated attention and vigilance after the animals had been exposed to an adverse condition [14] [55] [57]. In fact, other authors have reported that epeated injections [58], or even blood sampling [52] [59], are sufficient to increase corticosterone (CORT) levels (an indicator of stress response) and to increase the startle reflex (in the first presentation trials) [58] and PPI in rodents [52]. However, although it has been reported that changes in the environment are sufficient to enhance PPI in both humans and rodents [18] [55] [57] [60]—in humans PPI may even be increased if the prepulse stimulus has an affective component [60]—the route through which the drugs are supplied is not normally taken into account and its aversion value is not considered [13] [35] [37] [58].

Furthermore, the importance of taking the time to respond to the startle stimulus as an adaptive ability has recently been reported [61]. This is meaningful, considering the protective function of the startle reflex; when animals are first challenged by unexpected loud noises, a faster startle response is expected because the environment is potentially unsecure. Then, after repeated and inconsequential encounters with the same stimulus sequences, the startle should slow down, due to habituation. With repeated exposure to the test sessions (from ASR1 to ASR4) we found that whereas restraint stress did not affect ASR or PPI latency in either sex, the procedure of repeatedly injecting the rats induced a significant decrease in latencies. These animals speeded up their startle response, in contrast to habituation. This suggests that they became more reactive and fearful [61], an effect that SERT-pretreatment totally reversed in females but not in males.

4.3. The Sensitivity to Stress Is Sex-Dependent

In comparison with the amount of work conducted on the effects of stress and their modulation in startle and sensorimotor gating mechanisms in males, the respective literature addressing female responses is fairly limited. Here, we found that sex was a major factor affecting the results.

At the beginning of the experiment, the males exhibited higher startle amplitudes and PPI levels than the females, in agreement with the previous data [18]. In contrast to other authors [62] [63], Lehmann's group described the existence of consistent differences between male and female Wistar rats in sensorimotor gating mechanisms [18]. It is now well established that these differences also exist in humans [36] [64].

The fact that males reach higher startle amplitudes than females is evident, since in rats the ASR is measured based on acceleration [18]. With regard to PPI, this physical law does not apply since the PPI is calculated as a percentage of reduction in ASR and not as an absolute value. Accordingly, body weight is not a determining factor. In fact, our data show that when the animals reached adulthood (at 12 weeks of age) the differences in startle habituation and PPI values between the sexes were no longer found between controls, but were found among the animals that experienced stressful paradigms. Apparently, females were somewhat more sensitive to the injection procedure and males were more sensitive to restraint. To the best of our knowledge, the differences in our data concerning startle and PPI modulation due to the type of the stressor in each sex have never been considered. Previously, some studies reported different results with physical vs. emotional stressors [14] [56]; however, most of those studies were conducted in males.

The reported sex-differences could be due to changes in reproductive hormones, in particular estrogen (E2) [4] [62]. Nevertheless, when the differences at E2 levels throughout the estrous cycle were considered in females [49], neither PPI nor the startle values were changed, as we did not find any correlation between E2 levels and PPI, or between E2 levels and startle at any time point. Moreover, recently, it has been reported [48] [65], the estrous cycle had no effects on ASR amplitude and PPI when tested in random phases of the estrous cycle.

When we examined the physiological analyses, whereas no sex differences were found in the biochemical parameters or leukogram, some differences between the males and females were found in the haemostatic response to stress. In agreement with our results whereas under basal conditions, no differences between the sexes in chemical or hematological parameters are expected [66], several authors have reported differences in the stress response [42] [67]-[69].

4.4. SERT Administration Counteracts the Effects of Stress on Startle and PPI

According to the literature, the startle reflex and its modulations are sensitive to 5-HT levels and to the administration of SSRIs, such as SERT [34] [35] [37]. In the present study, SERT did not act in control animals; but in the animals exposed to stressful conditions SERT counteracted the effects of stress, although more efficiently in females. Results were only seen after 8 days of SERT administration; a single administration of SERT did not change any of the behavioral measures. This agrees with previous works, in the sense that antidepressants (such as SSRIs) may normalize or stabilize serotonin function and restore stress-induced behavioral changes [1]. Only repeated treatment with SSRIs affords the desired effects by blocking the harmful effects of stress [29] [30] [54] [70] [71] and no effects have been reported with acute treatment [35]. Nevertheless, in our study, the effects of SERT in modulating startle and PPI were different according to sex; SERT treatment was slightly more effective in female rats than in males. In fact, sex differences in 5-HT levels have been reported in many structures of the corticolimbic region known to regulate stress and emotion processing [72] [73]. Moreover, the spontaneous firing rate of dorsal raphe neurons (DRN) in males is more than 40% higher than in females [73]. Considering

these sex differences, it could be suggested that the already increased DRN 5-HT activity in males could be related to the observed differences of SERT in reversing anxiety-related behaviors [74]. The serotonergic signaling can either facilitate or attenuate anxious states, depending on the site of action and the specific serotonin receptor subtype involved [37]. However, the present study, using the same administration route and treatment duration, highlights the differences between the sexes in the antidepressant response, with regard to anti-anxiety effects, *i.e.* modulating the startle reflex and PPI in response to stressors.

4.5. Changes in Physiological Parameters with Stress

We found that physiological parameters, together with our behavioral observations, were useful for monitoring the stress responsiveness and the role of SERT protecting it. After the animals had been exposed to IMO stress, changes in the hematological and metabolic values were found in all the experimental groups in comparison with the undisturbed controls.

When animals are exposed to an acute stressor physiological changes in the animals' body are expected in response to the strong increase in glucocorticoids (GCs) and catecholamines secreted by the adrenal glands [1]. Blood coagulation accelerates, eliciting hemoconcentration [2]; the catabolic characteristics of GCs produces a rapid mobilization of amino acids and lipids [4] [75] and the immune response is faster, enhanced by catecholamines (increasing pro-inflammatory cytokine production) but soon suppressed if GCs remain at high levels [1] [5] [7] [76]. Together, these conditions have evident advantages for the animal's short-term survival, which are of utmost importance when it must face a potential danger [7] [12]. Thus, and in agreement with previous studies, after being exposed to acute stress the young rats in our study exhibited a significant increase in hematocrit, hemoglobin and platelet numbers [2] [12] [77]. Surprisingly, in the animals previously exposed to daily injections, the stress-response was less evident.

Although there are contradictory results in humans in regard to physiological habituation to stress [12] [78], in agreement with our data, it has been reported that subjects displaying negative affect showed consistently reduced coagulation activation in response to acute psychosocial stress [79], and that this was the exact opposite of what they expected in their first hypothesis. Also, in rodents, it was reported that rats exposed to an early type of stress (social deprivation) exhibited failure in fear potentiation [14] and, specifically, the same authors stated that previously injected rats exhibited signs of learned helplessness. As those rats had lost, somewhat, the ability to attend to ecologically important sensory signals. Moreover, when animals are subjected to inescapable stress conditions, the ascendant activity of the serotonergic system may become deregulated [80], the involvement of the serotoninergic system in regulating the autonomous functions having been suggested [40] [41]. The present study shows that SERT administration attenuates the changes induced by the i.p., affecting the hemostatic response to stress.

Moreover, our data also point to a significant increase in cytoplasmic enzymes, (GOT), and lactate dehydrogenase (LDH) in response to IMO stress in the animals of both sexes, as expected [75]. Even so, it almost doubled the intensity in previously stressed animals as compared to controls, indicating potential tissue-damage in previously stressed animals [81]. Rats exposed to either restraint stress or vehicle injections plus IMO had twice the absolute values of LDH and GOT enzymes in comparison with animals only subjected to IMO. Importantly, SERT administration prevented the prior stress-induced increase in enzyme levels, showing that the drug plays an important role in the preservation of cellular integrity. Nevertheless, recent reports have associated the use of SERT with acute liver failure [82] [83]. Despite intensive investigation using SERT in both animals and humans, the hepatotoxicity of Sertraline remains to be fully elucidated. The toxicity of higher doses, collateral factors, and the method used to study it (in vitro) are major factors that can affect the effects of SERT [83]. Moreover when the impact of SERT on post-myocardial infarction was studied [84] [85], it was observed that besides being safe, this antidepressant drug was efficacious in patients with unstable ischemic heart disease, decreasing the incidence of severe adverse cardiovascular events [85].

4.6 The Effects of Stress and SERT on Immune Function

In the present study, stress induced a dramatic decrease in the total number of leukocytes in rats of both sexes. The significant decrease in the number of leukocytes and lymphocytes after stress exposure has already been reported in several species [86] [87] and should be followed by a slow and complete recovery once it ends [76]. In fact, we found no changes in the animals exposed to IMO when compared to the undisturbed animals (after

the recovery period had ended). By contrast, the animals that were exposed to stressful conditions prior to IMO exhibited a persistent fall in the leukocyte count. Even at 10 days after the stress had ended, the animals exhibited a decrease in the total WBC concentration. This prolonged effect is consistent with recent findings reported from our laboratory, where differences were found in prenatally stressed offspring when they were studied in adulthood [50]. We also reported that by administering SERT for two months during adolescence it was possible to reverse the deleterious effects of prenatal stress on the leukocyte count. In the present study, pretreatment with SERT i.p. for 8 days was not sufficient to normalize stress-induced immunosuppression. The inhibition of the reuptake and synthesis of 5-HT does not occur in an immediate way [29] [71] [88] [89]. One week of administration of the drug is sufficient to maintain plasma drug levels steady [90], but normally the antidepressant effects can only be reached after a few weeks of treatment [46]. It could be surmised that this period of SERT treatment would be sufficient to lead to an increase in the basal levels of 5-HT in the brain [71] [88] [91], but insufficient to induce the adaptive changes in monoaminergic neurotransmission or in neurotrophic factors, and as consequence, unable to modulate the immune reactivity and possibly the central actions of the cytokines [9].

5. Conclusion

The main results of our study suggest there are sex-dependent differences both in the behavioral modulation (in startle, PPI and the latencies of both) and in the physiological response to a new stressor depending on the type of the previous stress condition (restraint vs. daily injections). Moreover, we tested the hypothesis that by administrating SERT, a well-known modulator of the neuronal circuitry involved in anxiety regulation, we could modulate the stress-elicited effects in young rats of both sexes. In fact, SERT has been shown to be able to modulate the startle and the PPI both in vehicle-injected and restrained animals, although more efficiently in females than males. The present study also shows that previous treatment with SERT attenuates some of the biochemical and hematological parameter changes induced by stress; however, SERT administration for 8 days does not reverse the deleterious effects of stress on the immune profile.

Acknowledgements

This work was supported by the University of Salamanca Research Support Grant 2015 to Dr. Dolores E. López.

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ANEXO II

frontiers in
BEHAVIORAL NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 06 August 2014
doi: 10.3389/fnbeh.2014.00260

The effects of sertraline administration from adolescence to adulthood on physiological and emotional development in prenatally stressed rats of both sexes

Inês Pereira-Figueiredo[1], Consuelo Sancho[1,2,3], Juan Carro[1], Orlando Castellano[1,2,4] and Dolores E. López[1,2,4]*

[1] *Neuroscience Institute of Castilla y León (INCYL), University of Salamanca, Salamanca, Spain*
[2] *Institute of Biomedical Research of Salamanca (IBSAL), University of Salamanca, Salamanca, Spain*
[3] *Department of Physiology and Pharmacology, University of Salamanca, Salamanca, Spain*
[4] *Department of Cell Biology and Pathology, University of Salamanca, Salamanca, Spain*

Edited by:
Regina M. Sullivan, Nathan Kline Institute and NYU School of Medicine, USA

Reviewed by:
Gregg Stanwood, Vanderbilt University, USA
Manuel Portavella, University of Seville, Spain

***Correspondence:**
Dolores E. López, Neuroscience Institute of Castilla y León (INCYL), University of Salamanca, C/ Pintor Fernando Gallego 1, 37007 Salamanca, Spain
e-mail: lopezde@usal.es

Sertraline (SERT) is a clinically effective Selective Serotonin Reuptake Inhibitor (SSRI) known to increase and stabilize serotonin levels. This neurotransmitter plays an important role in adolescent brain development in both rodents and humans, and its dysregulation has been correlated with deficits in behavior and emotional regulation. Since prenatal stress may disturb serotoninergic homeostasis, the aim of this study was to examine the long-lasting effects of exposure to SERT throughout adolescence on behavioral and physiological developmental parameters in prenatally stressed Wistar rats. SERT was administered (5 mg/kg/day p.o.) from the age of 1–3 months to half of the progeny, of both sexes, of gestating dams stressed by use of a restraint (PS) or not stressed. Our data reveal that long-term SERT treatment slightly reduced weight gain in both sexes, but reversed the developmental disturbed "catch-up" growth found in PS females. Neither prenatal stress nor SERT treatment induced remarkable alterations in behavior and had no effects on mean startle reflex values. However, a sex-dependent effects of PS was found: in males the PS paradigm slightly increased anxiety-like behavior in the open field, while in females, it impaired startle habituation. In both cases, SERT treatment reversed the phenomena. Additionally, the PS animals exhibited a disturbed leukocyte profile in both sexes, which was reversed by SERT. The present findings are evidence that continuous SERT administration from adolescence through adulthood is safe in rodents and lessens the impact of prenatal stress in rats.

Keywords: behavior, habituation, open field, restrain stress, serotonin, startle

INTRODUCTION

Sertraline (SERT) is a clinically effective Selective Serotonin Reuptake Inhibitor (SSRI) that increases serotonin (5-hydroxytryptamine, 5-HT) levels in the brain (Koe et al., 1983; Byerley et al., 1987; Manfridi et al., 1992) and hence plays an important role in stabilizing nervous activity.

Even though there are many children and adolescents with psychiatric disorders (Emslie and Mayes, 2001), decisions regarding the use of antidepressants in young people (such as the SSRIs) are still largely based on data from adults (Manfridi et al., 1992; de Jong et al., 2006). Efficacy measurements in humans recommend SSRIs as the initial medication of choice for young individuals in depression and for improving obsessive-compulsive disorder (OCD; Doogan and Caillard, 1988; Alderman et al., 1998; Emslie and Mayes, 2001; Moreno et al., 2006). SERT seems to be well tolerated in both children and adolescents, with adverse effects similar to those previously reported by adult patients (Alderman et al., 1998; Cook et al., 2001; Skaer et al., 2009). However, side effects with impact on later development have not yet been fully elucidated.

The serotoninergic system is highly complex, as evidenced by the great diversity of subtypes of receptors on which this neurotransmitter acts (at least 14 different subtypes) and the variety of functions regulated by each receptor subtype (O'Leary et al., 2013). 5-HT presynaptic receptors are located in the dorsal raphe nuclei (DRN) and postsynaptic 5-HT receptors (5-HTR) are largely present in the limbic system (Newport et al., 2001; Hensler, 2003; Leventopoulos et al., 2009). Thus, disturbing serotoninergic homeostasis during its development may result in a changed framework of brain connections and permanent alterations may be induced in adult behavior (Morley-Fletcher et al., 2003; Whitaker-Azmitia, 2005; Ansorge et al., 2008).

It is known that prenatal stress disturbs serotoninergic metabolism (Peters, 1986; Hayashi et al., 1998; Maccari and Morley-Fletcher, 2007) and is responsible for several psychiatric

disorders and negative affective states later in life, such as anxiety and depression (Green et al., 2011). These disorders have previously been related to low 5-HT levels (Koe, 1990; Graeff, 2004; Ansorge et al., 2008), and more recently such low levels have been implicated in developmental perturbations, both in laboratory animals (Kay et al., 1998; Coe and Lubach, 2005; Götz and Stefanski, 2007) and in humans (Cottrell and Ozanne, 2008; O'Connor et al., 2013). This early type of stress can affect the loss of synapses and dendritic arborization, which normally occurs from puberty to adulthood (Barros et al., 2006; Zhang et al., 2013), and induces a decrease in the levels of 5-HT in the brains of young individuals (Hayashi et al., 1998).

It has also been suggested that anxiety and depression-like effects could be counteracted by treating prenatally stressed offspring with antidepressants that affect the serotonin system, such as SSRIs (Matar et al., 2006; Van den Hove et al., 2005). Nevertheless, few studies have addressed the impact of these treatments during the developmental stage of adolescence.

The acoustic startle reflex (ASR) and its habituation, both considered good tools for the investigation of emotional status and the brain mechanisms involved in behavioral plasticity, are often used in pharmacological animal models (Davis, 1980; Dulawa and Geyer, 2000; Quednow et al., 2004; Jensen et al., 2007). In view of the involvement of this (5-HT) transmitter system in the descending pathways modulating the startle reflex (Geyer et al., 2001; Quednow et al., 2004), it seem seems quite clear that its manipulation would alter the startle response.

Thus, to evaluate the long-lasting effects induced by both early stress and exposure to antidepressants during youth, our main goal was to determine the neurobiological changes in previously stressed rats subjected to SERT treatment. Considering during this developmental period, in humans, antidepressant treatments could last for years (O'Leary et al., 2013), SERT was given from the beginning of adolescence of the animals until the end of the experiments, when they were fully grown. Along the experiment, physiological (body weight gain, appetite, thirst, and immunological function) and behavioral measurements (anxiety-related behaviors using the open field and the ASR paradigm) were taken.

To best of our knowledge, few data on the long-term effects of pharmacological therapy with SERT during adolescence in normal (control) or previously disturbed (PS) subjects of both sexes are currently available. Thus, we hope present findings help to further our understanding of the long-term effects of antidepressants during this critical window of brain development.

MATERIALS AND METHODS

ANIMALS

Virgin female Wistar rats CLS:WI(HAN) ($n = 12$) weighting 250 g were obtained from outbred rats from our own animal facility at the University of Salamanca. Vaginal smears were collected daily for 8 days before mating to determine the stage of the estrus cycle and the day of conception. On the day of proestrus, sexually experienced male Wistar rats were introduced for mating. The day the spermatozoa were found in the smear was designated as day 1 of pregnancy.

The animals were housed randomly and maintained under a normal 12/12 h light/dark cycle (lights on at 08:00 h) in a temperature- and humidity-controlled environment. The rats were given *ad libitum* access to food and water along the study period. The experiments were conducted in compliance with the guidelines for the use and care of laboratory animals of the European Communities Council Directive (2010/63/EU), the current Spanish legislation (RD 1201/05), and with those established by the Institutional Bioethics Committee. All efforts were made to minimize the number of animals used.

EXPOSURE TO PRENATAL STRESS

Pregnant female rats were randomly assigned to the stress or control groups ($n = 6$ per group) and housed individually in plastic breeding cages. Stress consisted on placing the females in the third trimester of gestation (days 15–21) on transparent cylinder restrainers (7 cm diameter, 19 cm long); under a bright light directed onto the surface of the restrainer for 45 min three times a day (at 9 am, 12 pm, and 4 pm) (adapted from Lemaire et al., 2000). Control mothers were only subjected to routine changes (handling them the less as possible), as were the stressed females. All stress and control mothers delivered normally and only offspring from litters containing 9–13 pups were used in the experiments. Offspring were weighted at birth and weaned at 21 days of age, after which they were separated into group cages housing four animals of the same sex and treatment. Then, the pups were tail-marked and body weights were recorded weekly.

Thirty days after birth, pups from one of the two groups, Control vs. PS, depending on the previous treatment, were subdivided, to receive either chronic treatment with SERT (Control-SERT and PS-SERT) or not (Control, PS). This resulted in equal number of animals in each condition ($n = 9$–11 per sex and group). To avoid the effect of the dams, care was taken so that groups included no more than two pups from the same litter, in agreement with the protocols and results of previous authors (Bowman et al., 2004; Estanislau and Morato, 2006; Van den Hove et al., 2014). Additionally, a cursory analysis revealed no differences in litter sizes, the male-to-female ratio of the offspring, or pre-weaning-mortality.

DRUG ADMINISTRATION

SERT (Besitran© Pfizer S.A. Madrid, Spain) was administered orally at a dose of 5.0 mg/kg/day in the animals' drinking water, starting on postnatal day 30 (P30) and continuing until the end of experiments (P90). The SERT solution was prepared using filtered water as a vehicle. Liquid consumption was controlled (with calibrated bottles) and monitored every 2 days, and the dose of the drug was adjusted on the basis of the liquid consumption and animal's weight. Freshly prepared solutions were then provided. Filtered water was given to control animals. During this period, the rats were kept in groups of four animals in polycarbonate boxes (45 cm × 30 cm × 20 cm), with unrestricted access to food. A dose of 5 mg/kg/day of SERT was chosen based on the pharmacokinetic and pharmacodynamic profiles of the drug (Byerley et al., 1987; West and Weiss, 2005; Matar et al., 2006), and to minimize the chronic side effects deriving from its administration (Greenberg et al., 2014). Its elimination half-life (approximately 26 h) makes administration once a day adequate (Doogan and Caillard, 1988; Murdoch and McTavish, 1992) and

oral administration is more akin to clinical reality and provides adequate and maintained plasma levels (Murdoch and McTavish, 1992).

MEASUREMENT OF THE ACOUSTIC STARTLE RESPONSE

At P30, and again at P90, all animals of both sexes were tested for the ASR. Before testing, the rats were habituated to the experimental conditions, especially regarding their introduction into the ASR apparatus. All testing was carried between 9:00 and 12:00 h, using the SR-LAB system (SDI, San Diego, CA, USA), as described by Castellano et al. (2009). The acoustic startle reflexes were measured in six identical startle-response cages (SR-LAB). Acoustic stimulus intensities and response sensitivities were calibrated (using an SR-LAB Startle Calibration System) so that they would be nearly identical in each of the six SR-LAB systems (maximum variability <1% of stimulus range and <5% of response ranges). Each testing session consisted of an acclimatization period of 5 min followed by 64 trials presented pseudo-randomly, with a mean inter-trial interval of 30 s. Sixteen of the trials involved a single-noise pulse (115 dB SPL, 20 ms of burst of white noise, used to determine the ASR), and the remaining trials consisted of 48 trials of a white noise prepulse at each of three intensity levels (65, 70, or 80 dB SPL) lasting 20 ms, followed by the startle stimulus (as above), at 50 ms inter-stimulus intervals. The session had three blocks of pulse and prepulse, with prepulse-to-pulse intervals of 50 ms. The first and last blocks were composed of pulses alone (5 in each block); the second block comprised 6 pulses alone and 9 of each of the prepulse intensities, all administered randomly. Whole body movements corresponding to startling responses were recorded and analyzed by the SR-LAB system, providing ASR latencies and amplitudes. The background noise of 65 dB SPL was generated throughout the entire session in order to avoid interference from external noise and to ensure equal experimental conditions. The percentage of habituation was calculated as the reduction in startle magnitude from block 1 to 3 of five pulses at 115 dB (%HAB = 100 × (first block – last block)/first block).

OPEN FIELD TEST

Spontaneous behavior was studied with the open-field (OF) test. The apparatus consisted of a round, white wooden arena (100 cm diameter, enclosed by a 50-cm-high wall), divided into an inner (7 areas subdivided into a large and a small center of 6 and 1 areas respectively) and an outer zone (12 areas adjacent to the wall). The OF apparatus was illuminated by an 80 W bulb, focused onto the field from a height of 100 cm above the floor. The behavior of each animal was studied for a period of 3 min over 3 consecutive days, and the occurrence of the following types of behavior was recorded: outer (OA) and inner (IA) exploratory activity (number of times the animal crossed into each zone and area) and rearings (Rear) (number of episodes in which the animal reared up on its back legs).

Between the introductions of each animal, the surfaces were cleaned with water and 70% ethanol. To minimize subjectivity, the behavior of the rats was recorded by two experimenters blind to the treatment conditions. All trials were performed between 11:00 and 14:00 h.

LEUKOCYTE COUNTS AND SUBPOPULATIONS

After the behavioral tests had been completed and after 60 days of SERT-treatment blood samples were taken between 09:00 and 11:00 h from a subset of animals from each group ($n = 6$ per group and sex) by cardiac puncture following intraperitoneal anesthesia with a mixture of ketamine (200 mg/kg) and xylazine (10 mg/kg). The blood was immediately transferred to EDTA (K3)-containing tubes and processed on an automatic cell counter (ADVIA 120 cytometer, Bayer, Leverkusen, Germany).

STATISTICS

The variability within litters for all rats on each experimental group (given by standard deviations) was similar than the variability across litters, on all our dependent measures. Also, when data were analyzed using the effect of litter as a covariate, we found no significant effects. Thus, in the final analyses the litter as a variable was not considered and the data from each individual animal were used.

Statistical analysis were performed using the IBM® SPSS® software, version 20 (IBM Corp. and SPSS Inc., Chicago, IL, USA, 2011). The differences between groups were analyzed by ANOVA (one, two and three way), followed by the Fisher-PLSD-test for *post hoc* comparison if appropriate, and ANOVA mixed (or "SPLIT-PLOT") with the Bonferroni-test. Mean differences were subjected pairwise to Student's *t*-test, using the Levene Test for equality of variances. Pearson's coefficient was used to determine correlations. Differences were regarded as statistically significant when $p < 0.05$.

RESULTS

EFFECTS OF PRENATAL STRESS ON BODY WEIGHT GAIN BEFORE ADOLESCENCE

As expected, as a main effect of prenatal stress, differences were observed in the neonates' body weights the day after delivery (P1), but only among the female pups (**Table 1**, $F_{1,30} = 14.04$, $p < 0.01$), since among the males the body weights of the pups were similar.

Body weight gain was analyzed using a three-factor ANOVA (prenatal treatment by sex by age) with repeated measures on the age factor (at this stage, three levels were used: 1, 21 and 28 days of age). Analysis of these data revealed that the prenatal treatment affected body weight gain. As the animals' age advanced, weight by treatment ($F_{2,66} = 3.42$, $p = 0.04$) and weight by treatment by sex ($F_{2,66} = 3.47$, $p = 0.037$) interactions were found, indicating

Table 1 | Animals' body weight (g) before the beginning of pharmacological treatment: at birth (P1), at weaning (P21) and at 4 weeks of age (P28) (n = 18–20 animals per group and sex).

	Females		Males	
	Control	Prenatal stress	Control	Prenatal stress
P1	6.4 ± 1.1	5.9 ± 1.5[aa]	6.7 ± 1.3	6.2 ± 1.6
P21	40.8 ± 2.4	39.8 ± 2.1	39.5 ± 2.2	43.2 ± 2.7
P28	57.8 ± 2.9	64.8 ± 2.7[a]	68.2 ± 2.5	66.2 ± 2.8

[a] $p < 0.05$ and [aa] $p < 0.01$, *indicate a main effect of prenatal stress (Fischer LSD test) (Mean ± standard error).*

differences, in the effect of PS on growth rate, that were sex-dependent.

Post hoc analysis showed that even though in the males no differences were found as an effect of prenatal treatment, but in the females, these differences were present ($F_{2,30} = 6.24$, $p = 0.005$). Female pups from stressed mothers had lower birth weights (P1) than the controls, but these differences disappeared by the time of weaning (P21). However, this process changed at P28, and PS females exhibited a higher body weight than their controls (**Table 1**, $p = 0.03$).

At this early stage of development, sex *per se*, did not influence weight gain, and no sex differences in the neonates' body weights were found at any point.

EFFECTS OF PRENATAL STRESS AND SERTRALINE ON BODY WEIGHT GAIN FROM ADOLESCENCE TO ADULTHOOD

Pharmacological treatment started once adolescence had begun, and the animals' weights were recorded weekly (for statistical analysis, five levels were used, and mean values were determined from P35 to P90). During this period, a significant overall effect of age was observed ($F_{4,73} = 1460$, $p < 0.001$), with group, $F_{16,304} = 1.69$, $p = 0.048$; sex, $F_{4,73} = 98.8$, $p < 0.001$; and group by sex, $F_{16,304} = 1.81$, $p = 0.029$ interactions affecting body weight, indicating the influence of the different treatments on weight gain in each sex. As expected, there was a clear difference between the growth of males and females throughout adolescence in all experimental groups (weight by sex, $F_{4,73} = 70.8$, $p < 0.001$), with males weighting more than females (**Figure 1**). Accordingly, further analyses were performed separately for each sex.

At the beginning of this phase (P35) neither the prenatal manipulation nor the pharmacological treatment affected the animals' body weights. However, at P90 (after 2 months of pharmacological treatment), differences in body weight (g) were found, both in females ($F_{3,30} = 4.15$, $p = 0.014$) and males ($F_{3,30} = 1.8$, $p = 0.025$).

Among the females, there was a main effect of group affecting weight gain with time, $F_{12,87} = 2.12$, $p = 0.023$. As depicted in **Figure 1A**, the animals' growth was faster in the prenatally stressed females than in all the other experimental groups, suggesting a long-term effect of the prenatal treatment that was reversed by SERT-treatment. In fact, SERT treatment affected, at least marginally, weight gain in the females, but this was only observed at this age (P90). In males, a major effect of SERT, affecting body weight gain was observed. After 2 months of treatment it reached significance among the non-stressed males (**Figure 1B**).

Fluid consumption increased with age and was different between the sexes (final average: 30 ml/day for female and 35 ml/day for male rats), but not between treatments.

ASR MEASURES

The startle response was first examined using a three-way mixed-design analysis of variance group by sex by age. A significant increase in startle amplitude was observed from P30 to P90 ($F_{1,77} = 312.3$, $p < 0.001$), with no group interaction, but with a strong sex interaction ($F_{1,77} = 69.6$, $p < 0.001$), marked by the differences between sexes seen at P90 ($F_{1,77} = 58.7$, $p < 0.001$), which were not seen at P30 (**Figure 2**).

On performing further analyses separately for each sex and age, no effects of prenatal stress at P30 were observed, whereas at P90, the greatest difference between groups were found ($F_{3,77} = 2.67$, $p = 0.058$). Although the difference did not reach statistical significance by sex, *post hoc* analysis revealed a marginal effect of prenatal stress, increasing startle amplitude in rats from both sexes regardless of SERT treatment. Additionally, SERT treatment did not induce changes in mean startle amplitude (**Figure 2**).

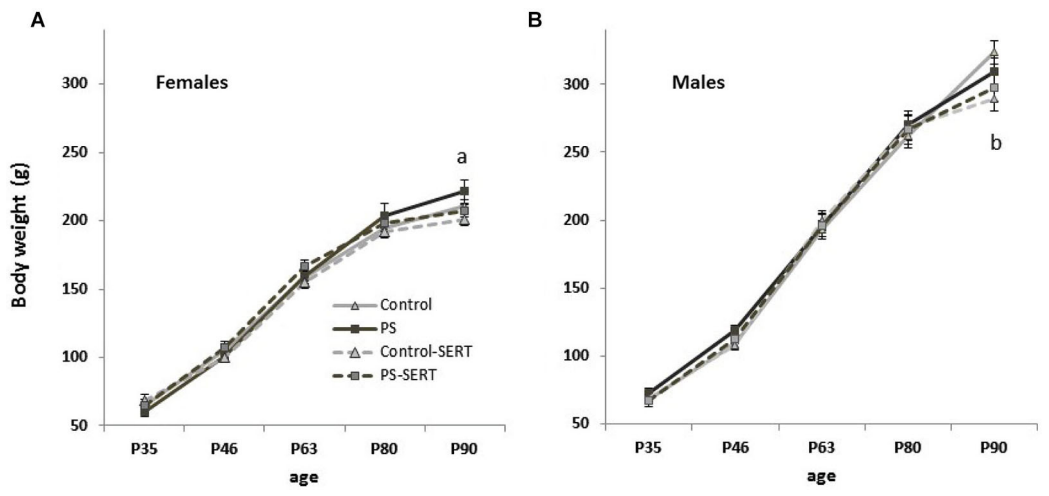

FIGURE 1 | Effects of prenatal stress and SERT treatment (5 mg/kg/day) on body weight gain (g) over time in females (A) and males (B) ($n = 9–11$ animals per group and sex). a, $p < 0.05$ indicates a main effect of prenatal stress; b, $p < 0.05$, indicates a main effect of SERT in Control-SERT vs. Control (Means ± standard error).

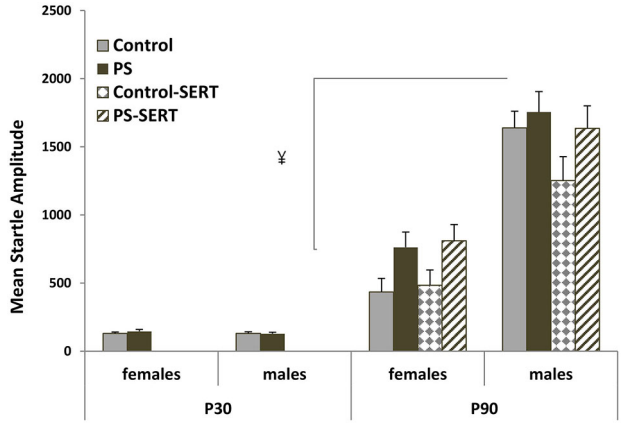

FIGURE 2 | ASR amplitude (in arbitrary units) at P30 and P90 performed in the animals of both sexes subjected or not to prenatal stress (PS and CONTROL), vs. subjected or not to prenatal stress and treated with SERT (Control-SERT and PS-SERT) (5 mg/kg/day). Mean values ± standard error (at P30, n = 18–20 animals per group and sex; at P90, n = 9–11 per group and sex). ¥, $p < 0.05$ indicates a main effect of sex in all experimental groups.

On using a block-to-block analysis to study startle amplitude, differences between the different experimental groups were observed. Startle habituation (difference between the first and the last block, expressed as percentages) was significantly different as effect of group, specifically in females ($F_{3,28}$= 3.3, $p = 0.034$), probably given the deterioration of habituation found in PS females (**Figure 3**). Startle amplitude remained persistently high in PS females and was reversed by SERT. Given the different response to the startle test in the prenatally stressed animals ($F_{1,69} = 3.5$, $p = 0.02$), sex differences were observed specifically in PS animals ($p = 0.03$).

Additionally, there was an overall increase in the latency to startle from P30 to P90 ($F_{1,77} = 284.9$, $p < 0.001$). Whereas at P30 no differences between groups were found; at P90, the latency to startle of PS animals was shorter than in the controls ($F_{3,77}$ = 4.53, $p = 0.006$). SERT has no effect on this parameter (**Figure 4**), and the males showed a higher latency to startle than females ($F_{1,77} = 23.6$, $p < 0.001$).

Interestingly, a strong correlation was seen between ASR amplitude and its latency ($r = 0.74$, $p < 0.001$), such that the animals with higher latency responses were those with greater startle amplitudes.

OPEN-FIELD ACTIVITY

Neither prenatal stress nor treatment with SERT significantly changed the activity of the animals in the OF test, although a strong influence of sex was found.

Upon analyzing the total exploratory activity (total crosses + rearings) (ANOVA with session by group by gender as within factors), no group effect was observed, but a major effect of sex was observed ($F_{1,69} = 11.1$, $p = 0.002$). Females were more active than males in all the sessions, this difference being significant in both the Control (+20.59 ± 9.4, $p = 0.034$) and PS animals (+26.1 ± 9.1, $p < 0.01$). In the case of the SERT-treated animals, the differences between sexes lost significance (Control-SERT: +5.9 ± 9.5, n.s.; PS-SERT: +14.4 ± 9.8 n.s.).

A comparison of the horizontal and vertical activity performed in the sessions did not reveal differences due to stress or SERT treatment in any case (**Figures 5A,C**, **6A**). Again, a major effect of sex ($F_{1,69} = 10.2$, $p = 0.02$; $F_{1,69} = 3.86$, $p = 0.05$; and $F_{1,69} = 4.7$, $p = 0.035$, respectively) was observed, given the overall differences between PS males and females in their willingness to engage in exploratory activity. PS males exhibited significantly less outer-field activity (**Figure 5A**; $F_{1,69} = 4.23$, $p = 0.045$) and inner-field activity (**Figure 5C**; $F_{1,69} = 4.24$, $p = 0.038$) and also fewer rearings than the females from the same group (**Figure 6A**; $F_{1,69} = 7.47$, $p = 0.01$).

With the exception of the Control females, in all experimental groups of both sexes the horizontal activity remained unchanged over the 3 days. In response to repeated exposure to the test, exploration in the OF increased in Control females (**Figures 5B,D**; both outer and inner activity).

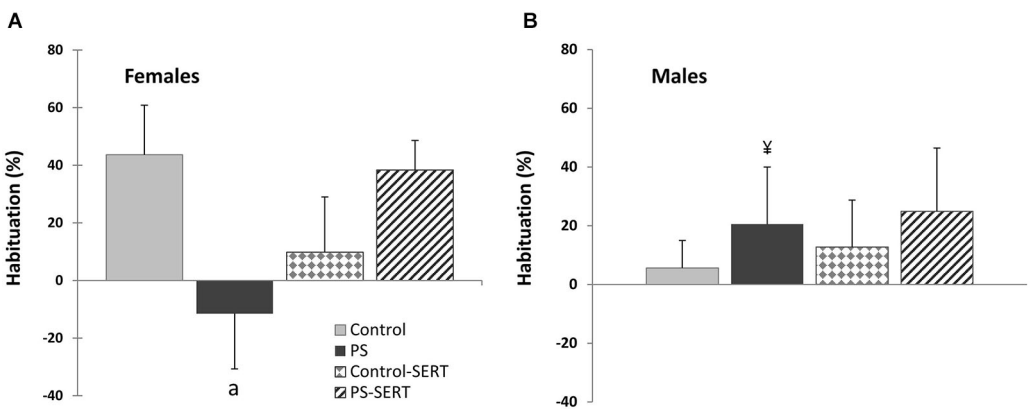

FIGURE 3 | Effects of prenatal stress and SERT treatment (5 mg/kg/day) on habituation (percentage) in females (A) and males (B) at P90. a, $p < 0.05$, indicates a main effect of prenatal stress; ¥, $p < 0.05$, indicates a main effect of sex. Means ± standard error.

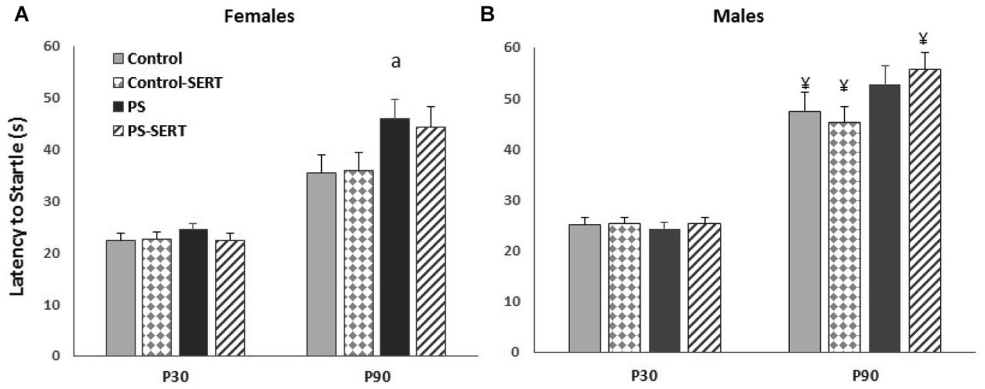

FIGURE 4 | Startle latency (stimuli 115 dB) at P30 and P90 in females (A) and males (B). a, $p < 0.05$ indicates a main effect of prenatal stress in females; ¥, $p < 0.05$, indicates a main effect of sex in all groups except of PS animals (Fischer LSD test). Mean values ± standard error.

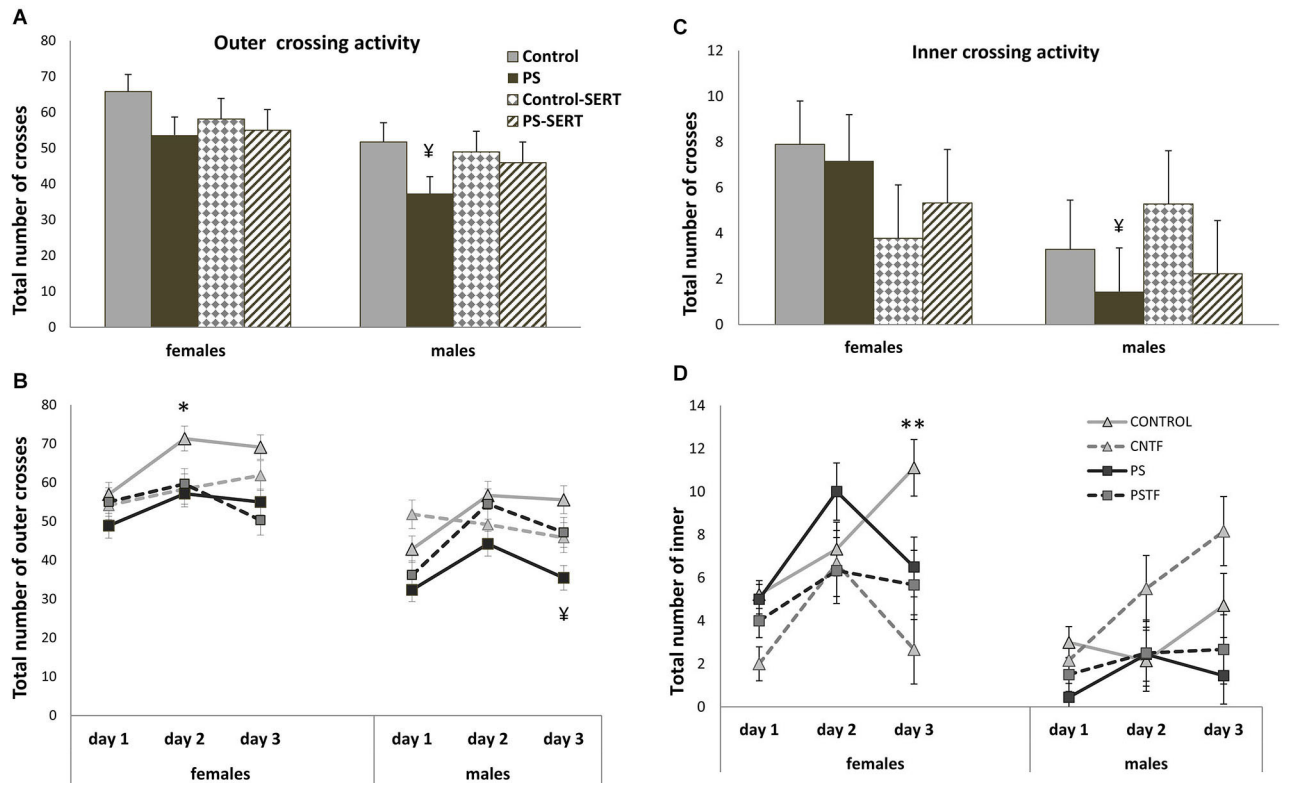

FIGURE 5 | Effects of prenatal stress and SERT treatment on the horizontal activity in the OF test (n = 8–9 per group and sex). Means ± standard error for outer crossing activity (panels **A** and **B**) and inner crossing activity (panels **C** and **D**) observed, during 3 min sessions on 3 consecutive days. No differences as effect of prenatal stress or SERT-treatment were found in outer crossing activity, $F_{3,69} = 2.47$; or inner crossing activity, $F_{3,69} = 0.25$. ¥, $p < 0.05$ indicates a main effect of sex on PS animals. *$p < 0.05$ and ** $p < 0.01$, indicates a significant increase on both the number of outer and inner crosses in Control females.

Furthermore, a different effect of the drug on inner-field activity was observed for each sex; whereas in females SERT slightly reduced inner exploration (**Figures 5C,D**), in males SERT treatment did not affect it, and prenatal stress seemed to be the only factor that affected such activity, and then only to a slight extent.

Moreover, the number of rearings also changed significantly over the 3 days with repeated exposure to the OF ($F_{2,68} = 4.38$, $p = 0.018$), with no sex or group interactions (**Figure 6B**).

As an anxiolytic indicator, the ratio between inner-field and outer-field activity (IA/OA) was further analyzed, and no overall differences were found. However, when the analyses

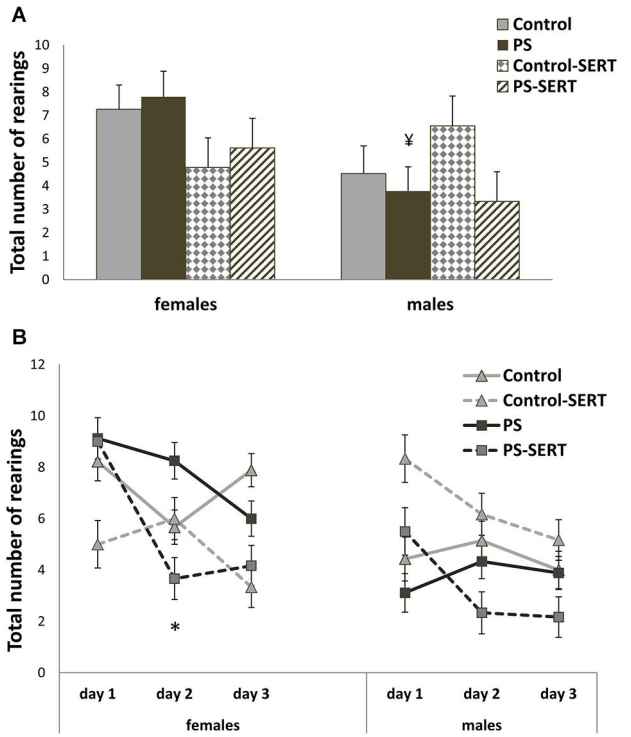

FIGURE 6 | Effects of prenatal stress and SERT treatment on the vertical activity in the OF test. Number of rearings observed during 3 min sessions on 3 consecutive days (Panels **A** and **B**). Mean values ± standard error. No differences as effect of prenatal stress or SERT-treatment were found, $F_{3,69} = 0.58$, $p > 0.05$. ¥, $p < 0.05$ indicates a main effect of sex on PS animals. * $p < 0.05$, indicates a significant decrease from day 1 to day 2 of test, on the number of rearings in PS-SERT females.

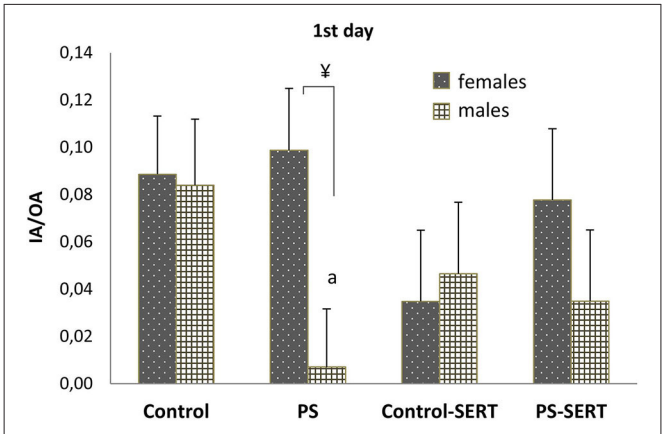

FIGURE 7 | Ratio between inner exploratory activity and outer exploratory activity (IA/OA) on the 1st day of the OF test. a, $p < 0.05$ indicates a main effect of prenatal stress in males; ¥, $p < 0.05$ indicates a main effect of sex.

were split by day and sex, in males, a major effect of group was noted, specifically on the first day of the test ($F_{3,35} = 2.98$, $p = 0.048$), indicating an anxiogenic effect of prenatal stress (**Figure 7**, $p = 0.043$). Sex differences were also found in the PS animals ($p = 0.014$), indicating that PS increases anxiety in males, specifically, and that SERT treatment reversed it.

HEMOGRAM: WHITE BLOOD CELL COUNT

The blood leukocyte formula did not vary as a function of sex. Gestational stress compromised the immune function (**Table 2**). Quantitative analysis of the total of the leukocyte count revealed pronounced leukopenia in previously stressed animals and its formula was disturbed. In control animals, SERT treatment did not affect the immune response and in the stressed animals it returned pre-leukocyte failure values to normal levels. In stressed animals, differential leukocyte counts (in absolute numbers) disclosed lymphocyte levels below the normal range.

DISCUSSION

The main findings of the present study highlight the importance of the exposure during development to environmental challenges affecting the serotonergic system, these effects persisting into adulthood. The sex-specific effects of prenatal stress on later physiological development and anxiety-related behaviors were remarkable. The impact of prenatal stress on immune function, later reversed by SERT administration, was observed. Importantly, it was also noted that, in rats, chronic exposure to a low dose of SERT (5 mg/kg/day) from adolescence until adulthood was safe and effective in reversing the harmful effects of prenatal stress.

Table 2 | Plasma values obtained in the arterial blood in animals of both sexes (at P92).

Group	Leukocytes ($10^3/\mu l$)	Neutrophils ($10^3/\mu l$)	Lymphocytes ($10^3/\mu l$)	Monocytes ($10^3/\mu l$)	Eosinophils ($10^3/\mu l$)	Basophils ($10^3/\mu l$)
Control	5.45 ± 0.6	0.86 ± 0.1	4.34 ± 0.3	0.09 ± 0.01	0.12 ± 0.01	0.01 ± 0.001
Control-SERT	4.78 ± 0.7	1.16 ± 0.2	3.53 ± 0.6	0.07 ± 0.02	0.12 ± 0.03	0.02 ± 0.003
PS	1.85 ± 0.5$^{aa\ bb}$	0.63 ± 0.1	0.93 ± 0.4$^{aa\ b}$	0.06 ± 0.01	0.07 ± 0.02	0.003 ± 0.002^{a}
PS-SERT	4.43 ± 0.6	1.00 ± 0.2	3.22 ± 0.6	0.07 ± 0.02	0.09 ± 0.02	0.01 ± 0.003
	$F = 16.3$, $p < 0.001$	$F = 1.9$, $p = 0.14$	$F = 18.3$, $p < 0.001$	$F = 0.8$, $p = 0.49$	$F = 1.6$, $p = 0.23$	$F = 7.2$, $p < 0.01$

Means ± standard error (n = 6 per group). [a] *$p < 0.05$ and* [aa] *$p < 0.001$ indicates a main effect of prenatal stress;* [b] *$p < 0.05$ and* [bb] *$p < 0.001$ indicates a main effect of SERT.*

BODY WEIGHT GAIN

Perturbations, such as maternal stress, in the uterine environment during development can permanently alter metabolism and body weight in the offspring (Pollard, 1984; Jahn et al., 1993; Lordi et al., 1997; Drago et al., 1999). The latter authors accounted for this in terms of a decrease in growth hormone (GH) and androgen production in prenatally stressed animals, which—having negative influence on growth and food intake—would lead PS animals to gain less weight.

By contrast, our results indicate that PS females increased more in weight during growth. Whereas in males no effects of early stress on weight gain were found, prenatally stressed females weighed less than their controls at birth. This difference rapidly disappeared, and was later counteracted, a trend that persisted into adulthood. Even though, this is in disagreement with earlier studies addressing prenatal stress in which PS delayed development (Pollard, 1984; Lordi et al., 1997; Berger et al., 2002), recently it has been reported in males (Chung et al., 2005; Mueller and Bale, 2006; Abe et al., 2007) and in both sexes (García-Cáceres et al., 2010). Many factors may be involved in these differences, including maternal sensitivity (Mueller and Bale, 2006), the timing of exposure to stress, or the physical properties of the stressors employed (Abe et al., 2007).

The rapid weight gain, or "catch-up" growth, reported here in PS females, that follows low birth weight has already been described in humans (Cottrell and Ozanne, 2008). According to Cottrell and Ozanne (2008) the rapid weight gain following maternal stress has important effects on later health, and so children born with abnormally low weights have been reported as being at increased risk of later obesity and related metabolic issues (Breier et al., 2001; Ozanne and Nicholas Hales, 2005). "Fetal programming" has been suggested as the origin of this (Breier et al., 2001; Tabacchi et al., 2007; Cottrell and Ozanne, 2008).

One of the biological causes of disorders involving the loss of control of the energy balance in humans is dysregulation of the serotonergic system (Kaye et al., 2005; Marston et al., 2011; Avena and Bocarsly, 2012). Brain imaging studies in patients with such disorders have uncovered alterations in 5-HT circuitry (Kaye et al., 2005; Lam et al., 2010), which have also often been described in PS animals. Accordingly, it could be speculated that such changes may at least partly affect the feeding behavior that we observed in PS females, which was reversed by SERT treatment.

SERT administration also changed weight gain and appetite. Whereas at the beginning of treatment SERT did not affect food intake, after 2 months it did. Following SERT treatment the animals exhibited a loss in body weight in comparison with their controls. This is in agreement with findings concerning food seeking behavior reported previously for adult rats, which describe the effects on weight gain of a variety of drugs able to increase the synaptic availability of 5-HT (Lucki et al., 1988; de Magalhães-Nunes et al., 2007; Mandelli et al., 2008). In adolescent male rats, the group headed by de Jong et al. (2006) also found a slight effect of another two SSRIs—fluvoxamine and paroxetine—which reduced growth. As far as we know, the present study is the first to report the long-term effects of SERT administration during development in both sexes over such a long study period (60 days).

A lack of changes in overall liquid intake was found between the groups; i.e., adding SERT at 5 mg/kg/day to the drinking water did not seem to influence the search for water. By contrast, de Magalhães-Nunes et al. (2007) reported that SERT treatment affected water and sodium intake in rats. However, those authors used a 20 mg/kg/day dose.

ANXIETY-LIKE BEHAVIOR

According to the literature, the serotonergic system modulates behavioral states (Dulawa and Geyer, 2000; Siepmann et al., 2003; Quednow et al., 2004; Iñiguez et al., 2010). However, our data show that SERT exerted no changes in mean startle reflex amplitude or in its latency in non-stressed rats.

Prenatal stress in rodents usually results in increased emotionality (Fride et al., 1986; Martí and Armario, 1998). High ASR amplitudes are found in more emotive animals (Kjær et al., 2010) and are thus considered good markers of anxiety disorders (Rasmussen et al., 2008). It is to be expected that the progeny of stressed mothers would have increased startle responses. However, the various research groups investigating the effects of PS reported no, or only marginal, differences in ASR amplitude in prenatally stressed animals (Lehmann et al., 2000; White and Birkle, 2001; Koenig et al., 2005). This is in accordance with our results, where only a slight elevation in startle amplitude was seen as an effect of prenatal stress in both sexes.

When a block-to-block analysis was performed, differences were observed. In the present study, PS males, regardless of the antidepressant treatment, exhibited a marginal elevation in the acoustically elicited startle reflex over the first few trials in comparison with the controls (data not shown). This initial increase reflects the influence of the novel, potentially aversive stimulus on the central nervous system, thus indicating PS males as being more sensitive (Meincke et al., 2004). Also, in rodents, White and Birkle (2001) reported no differences in mean startle amplitude values between PS and control progeny, except when response to novelty was tested.

By contrast, it is expected that with further stimulation, the controls would interpret the stimuli as being less relevant and the amplitude course decays in ensuing trials, revealing a process of habituation (Martí and Armario, 1998). Our data show that whereas PS males showed habituation, this was significantly impaired in PS females, SERT treatment reversing it. Acoustic startle habituation is a central point in the concept of vulnerability to stress, because it reveals the extent to which animals are able to withstand the homeostatic disturbances induced by stress over time (Koch, 1999; Meincke et al., 2004). The persistence in behavioral responses to repeated stimuli reflects difficulties in adapting to subsequent stressors and it is seen in psychotic patients (Meincke et al., 2004), meaning that SERT is an important tool for reversing stress vulnerability in PS females.

In accordance with our results, other researchers studying the physiological responses to postnatal stress in rodents found sex differences, reporting that, when stressed, males showed habituation while females showed sensitization (Chadda and Devaud, 2005; Buynitsky and Mostofsky, 2009). This sex specificity of persistently increased ASR in PS females was first reported by Hougaard et al. (2005).

Also, the latency to startle was only marginally affected by prenatal stress. PS animals from both sexes were slightly slower in becoming startled than their controls, and SERT exerted no effects. As far as we know this is the first time this effect of prenatal stress has been reported.

After the animals had been subjected to the OF test, we found that neither the prenatal stress nor SERT treatment changed locomotor activity in this test, although there was a marginal effect of early stress, anxiety increasing specifically in males. A major effect of sex was found in all parameters measured, females proving to be more active than males.

In the present work, the OF paradigm was used as a simple model to study anxiety-like behavior and locomotor activity (Durand et al., 1999; Prut and Belzung, 2003; Van den Hove et al., 2005; Rayen et al., 2011). We found sex specificity in susceptibility to early stress; the PS males exhibited increased anxiety, although this was hardly significant when compared with the PS females. PS males showed less exploratory activity and less central exploration than the females from the same group. SERT reversed it, even though it did not affect either locomotor behavior or anxiety significantly in animals from both sexes, in agreement with previous reviews about this class of antidepressants (Prut and Belzung, 2003).

The sexual dimorphism found in locomotor activity with the OF test (total crossings + rearings) has already been reported in rodents (Wakshlak and Weinstock, 1990; Pallarés et al., 2007; Duchesne et al., 2009), females being always more active than males in this kind of test. Also, vulnerability to early stress has been seen previously in the OF test (Wigger and Neumann, 1999; Nishio et al., 2001; Zueña et al., 2008). According to these authors, the effects of PS are more pronounced in males, these proving to be more emotional during the OF test. For instance, Nishio et al. (2001) reported a decrease in motor activity in neonate PS males, no effects being observed for PS females.

In the OF test, animals face contradictory motivations—the fear of an open enlightened environment, and the motivation to explore it (novelty preference) (Archer, 1973). An internal conflict underlies the motivation of the animals' behavior (Fride et al., 1986), where high emotivity would inhibit exploration and low emotivity would facilitate it, indicating a better adaptation to the new environment (Gilad and Shiller, 1989; Durand et al., 1999). In our study, the control animals were the only ones that increased their activity with repeated exposure to the OF test.

Nonetheless, the lack of differences caused by PS observed here is in accordance with previous works (Van den Hove et al., 2005). Thus, as additional indices of anxiety-like behaviors in the OF we further determined the animals' IA/EA activity (Archer, 1973; Durand et al., 1999; Prut and Belzung, 2003) and found that, only on the first day, prenatally stressed males exhibited a lower IA/OA ratio than controls. Again, PS males proved to be more sensitive to novelty (White and Birkle, 2001).

Additionally, although most authors defend the notion that SSRIs can normalize anxiety disorders, in rodents they behaved as anxiogenic, or anxiolytic substances, or even had no effects when the animals subjected to treatment were tested in behavioral paradigms (Durand et al., 1999; Prut and Belzung, 2003; Graeff, 2004; de Jong et al., 2006). Many variables can be invoked to account for the heterogeneity of the results, e.g., the animal model, sex, the dose or management of the SSRIs and the inclusion of a rest period. However, the timing of exposure is a determinant factor. In the present study, we tested the effects of a low dose of SERT administered during the developmental period from adolescence to adulthood, anticipating changes in serotonin-related behavior (Byerley et al., 1987; West and Weiss, 2005; Greenberg et al., 2014).

Adolescence in rodents occurs from postnatal day 28 to day 60 (Spear, 2000) and continues to be an important period of the development of the nervous system in which the serotoninergic system is still maturing (de Jong et al., 2006). Being even suggested the adolescence as a sensitive period (Eiland and Romeo, 2013; Holder and Blaustein, 2014).

Besides the changes in 5-HT transporters and their receptor activity, the release of 5-HT from the DRN in adolescent rats is increased in comparison with adult rats (de Jong et al., 2006) and the levels of 5-HT in several brain areas are also increased. As a consequence, the effects of SSRI administration during this period might be different from those elicited in adults. Actually, de Jong et al. (2006) reported that giving SSRI to adolescent rats increased anxiety, and this became apparent when they were tested as adults. In our work, we found no significant differences in anxiety-related behaviors between animals receiving SERT as compared with the controls. However, as expected, SERT played an important role in mediating the deleterious effects of prenatal stress in both sexes.

While it is known that SSRIs act mainly by binding to 5-HT transporters, then blocking 5-HT reuptake and increasing 5-HT availability, it is also known that some SSRIs have other non-specific neuropharmacological effects (Manji et al., 2001), partially acting on the inhibition of other neuroactive monoamine reuptake (Kitaichi et al., 2010) or decreasing corticotropin-releasing hormone (CRH) neuronal activity (Matar et al., 2006). More importantly, they modulate glucocorticoid receptors (GRs) activity in several brain areas (Anacker et al., 2010).

Thus, in addition to the cellular and molecular alterations induced by chronic treatment with SSRIs, reversing depressive states, other beneficial effects exist. Following the antidepressant—due increase in 5-HT concentrations in the median raphe nuclei and hippocampus, the release of neurotrophins (such as BDNF) and hippocampal neurogenesis are stimulated (Anacker et al., 2010; Willner et al., 2013). These neuroprotective and neurotrophic effects of 5-HT are known to block the damaging effects of stress on neurons (Manji et al., 2001; Morley-Fletcher et al., 2003; Hajszan et al., 2009). In fact, a role has even been suggested for antidepressants in the structural plasticity of certain cerebral areas (Pittenger and Duman, 2008). The effects of antidepressants on the hippocampus seem to be partly modulated by modifications in other brain areas that also are the sites of action of SSRIs (Castro et al., 2010). This allows at least part of the system to be restored to an almost normal state (Willner et al., 2013).

IMMUNOMODULATORY EFFECT

SERT has been shown to have an immunomodulatory action once it has reversed the state of leukopenia found in prenatally

stressed animals. As expected, prenatal stress induced alterations in the immune function of the offsprings; this could still be seen when the immune function was determined in adulthood. A decrease was found in both total leukocyte counts in blood and in all subpopulation types, lymphocytic cells being those most affected. This has been extensively described before by authors proposing that gestational stress compromises immune function in the offspring (Kay et al., 1998; Coe and Lubach, 2005; Vanbesien-Mailliot et al., 2007; Merlot et al., 2008), with deleterious effects on leukocyte proliferation (Götz and Stefanski, 2007), and specifically on IgG levels (Sobrian et al., 1992), natural killer activity (Kay et al., 1998) and immune dysregulation by promoting pro-inflammatory and type-2 cytokine responses (Vanbesien-Mailliot et al., 2007). All these authors reported that prenatal stress altered the immune function of progeny with no, or only marginal differences, as regards sex, as was observed in the present study.

The mechanisms underlying the effects of prenatal stress on the immune system of progeny, probably result from the action of maternal stress hormones (Barbazanges et al., 1996). The increased level of glucocorticoids (GCs) reaching the developing fetus are known to affect the development of the neuroendocrine and immune systems (Kay et al., 1998; McEwen, 2008). One consequence is the HPA axis hyperactivity found in prenatally stressed rat pups that is correlated with high hormone levels, caused by an impaired feedback inhibition of GCs (glucocorticoid resistance), and these hormonal changes have been shown to regulate the magnitude and duration of the immune responses (Sobrian et al., 1992). It is known that GC binding to GRs induces their activation (transactivation). However, GRs can instead bind to transcription factors (see Anacker et al., 2010 for review), resulting in the so-called transrepression. The typical target genes of GR-mediated transrepression include inflammatory cytokines, and these latter perform the immunosuppressive action of GC hormones (Dhabhar, 2007; Anacker et al., 2010). Given such a dynamic link between both the immune and neuroendocrine systems, it could be suggested that prenatally stressed animals exhibit some impairment of the HPA axis, which would affect postnatal immune function.

Moreover, gestation is an ontogenic period during which some of the most critical events that allow normal functioning of the immune system are taking place, and, it is reasonable to assume that prenatal exposure to environmental protocols might also cause a temporary or permanent disruption in the genesis or functioning of the immune system itself (Sobrian et al., 1992). Many previous studies have shown that a critical immune organ, the thymus, is extremely sensitive to stress-responsive adrenal corticosteroids during development (Hougaard et al., 2005). Involution of the thymus, as well as lytic and apoptotic death of T cells, occurs even in adults, but is particularly pronounced in the stressed fetus (Coe and Lubach, 2005; Hougaard et al., 2005).

The long-term consequences of this early type of stress on immune function are less well known. Most of these studies reported effects in neonate or juvenile offspring. Sobrian et al. (1992) described for the first time that disruption of the immune system in PS progeny altered the postnatal response of this system to stress. Here we found that leukocyte count impairment persisted in 90-day old aged animals.

Finally, we demonstrate the central role of SERT in restoring leukocyte levels to normality. The mechanisms underlying the effects of SSRIs on the immune system under normal and pathological situations remain to be clarified (Gobin et al., 2014). Taler et al. (2008) and Gobin et al. (2014) suggested that the immunomodulatory effect of SSRIs would be related to their pro-apoptotic activity, and to their action on lymphocyte proliferation and to cytokine secretion. This may have been the case in our work as a consequence of normalizing the altered neuroendocrine function in prenatally stressed animals. Moreover, it is known that SSRIs improve 5-HT levels and can act directly on hippocampal cells, changing GR binding and regulating GRmRNA expression in neuronal cells (Smythe et al., 1994; Anacker et al., 2010).

As far as we know this is the first work to study the impact of SERT in reversing the adverse effects of prenatal stress on immune competence. The low doses used here may have been crucial, since Sacre et al. (2010), for instance, reported that a low dose of another SSRI (fluoxetine) was needed to produce significant changes in autoimmune disease and cancer.

In conclusion, our results contribute to the general knowledge about the beneficial effects of SERT, a drug known to exert antidepressive effects, reversing early stress-associated impairments. Such beneficial effects are therefore of considerable interest in clinical practice. Also, the central role of sex susceptibility to maternal restriction stress is highlighted. The sex-dependent effects reported here could be due to the sex-specific timing of developmental processes during gestation (Roussel et al., 2005). This should be addressed in future studies.

ACKNOWLEDGMENTS

This research was supported by grants from the Spanish Ministry of Science and Innovation (MICINN, #BFU2007-65210 and #BFU2010-17754) to Dr. Dolores E. López.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 May 2014; accepted: 14 July 2014; published online: 06 August 2014.
Citation: Pereira-Figueiredo I, Sancho C, Carro J, Castellano O and López DE (2014) The effects of sertraline administration from adolescence to adulthood on physiological and emotional development in prenatally stressed rats of both sexes. Front. Behav. Neurosci. ***8****:260. doi: 10.3389/fnbeh.2014.00260*
This article was submitted to the journal Frontiers in Behavioral Neuroscience.

ANEXO III

Journal of Behavioral and Brain Science, 2015, 5, 251-265
Published Online July 2015 in SciRes. http://www.scirp.org/journal/jbbs
http://dx.doi.org/10.4236/jbbs.2015.57026

Sex-Dependent Effects of Prenatal Stress on Learned Helplessness and Anxiety-Related Behaviours in Wistar Rats

Inês Pereira-Figueiredo[1], Consuelo Sancho[1,2,3], Juan Carro[1,4], Dolores E. López[1,2,5], Ricardo Gómez-Nieto[1,2,5], Orlando Castellano[1,2,5*]

[1]Neuroscience Institute of Castilla y León, University of Salamanca, Salamanca, Spain
[2]Institute of Biomedical Research of Salamanca, University of Salamanca, Salamanca, Spain
[3]Department of Physiology and Pharmacology, University of Salamanca, Salamanca, Spain
[4]Department of Basic Psychology, Psychobiology and Methodology, University of Salamanca, Salamanca, Spain
[5]Department of Cell Biology and Pathology, University of Salamanca, Salamanca, Spain
Email: *orlandoc@usal.es

Received 10 June 2015; accepted 7 July 2015; published 10 July 2015

Abstract

There has been an increasing importance of studies that link sex to stress coping processes. Recently, we reported that male and female Wistar rats responded differently to prenatal stress (PS) under basal conditions. The aim of the present study was to determine the influence of sex on behaviour and coping strategies, as an effect of gestational adversity in rats that were exposed to an uncontrollable stressor. Once the animals reached adulthood, the offspring from stressed/non-stressed dams were subjected or not to antidepressant treatment with Sertraline. After that, they were exposed to a single inescapable shock (IS) session, in which the rats were further tested for escape behaviour along 10 days, as a model of learned helplessness (LH). In prenatally stressed animals after the IS, behavioural differences appeared in a sex specific manner. Males proved to be more susceptible to the adverse context than females, exhibiting behavioural despair in a large percentage of the cases. Surprisingly, PS did not affect shock escape failure, but did affect learning performance in a sex dependent manner. In females, PS led them to learn to avoid shocks, learning better than controls, and by contrast, PS males did not learn to avoid shocks and displayed some signs of anhedonia. Sertraline did not help animals to avoid shocks, but helped them to escape from it. Our data indicate the existence of sex dependent behavioural differences in PS animals when facing an uncontrollable stress situation, in which the changes induced by PS were not only different, but opposite between sexes.

*Corresponding author.

How to cite this paper: Pereira-Figueiredo, I., Sancho, C., Carro, J., López, D.E., Gómez-Nieto, R. and Castellano, O. (2015) Sex-Dependent Effects of Prenatal Stress on Learned Helplessness and Anxiety-Related Behaviours in Wistar Rats. *Journal of Behavioral and Brain Science*, **5**, 251-265. http://dx.doi.org/10.4236/jbbs.2015.57026

Keywords

Gestational Stress, Gender Differences, Sertraline, Escape Behaviour, Inescapable Footshock

1. Introduction

It is currently established that early adverse experiences cause changes in the nervous system that persist throughout life [1] [2]. Such experiences have a high impact as regards the future predisposition of an individual's reactivity and propensity to suffer from psychological illnesses [3] [4]. In pregnant females, prenatal stress (PS) is an adverse experience known to enhance developmental disorders and to elicit several psychopathologies associated with anxiety or depression in newborns [5]-[8]. The majority of the studies using prenatal stress animal models were carried out in males without any further investigations into sex-related behavioural differences [3] [9] [10]. Even though early reports on the sex variation in response to early stressors were inconsistent [11], more recent studies addressing prenatal stress in several species have confirmed the existence of sex-dependent factors [2] [12]-[14] that are responsible for the divergent epigenetic changes in males and females. This suggests the possibility that early stressors might elicit sex-specific effects on later vulnerability to stress in the offspring.

Overall, animals that are exposed to inescapable or uncontrollable stress frequently develop learned helplessness (LH) [15]. This condition reflects the despair they possibly feel when they have no control over their circumstances, and it has been proposed as a model of the "stress and coping" paradigm [15] [16]. It is considered that certain forms of human depression are shared with LH animals and it has been suggested that the underlying mechanism is similar [17] [18]. Both, humans and animals show an apparent lack of interest in their environment as they feel their actions are meaningless, and that there is a lack of congruence between actions and consequences, which might have key medical implications. Moreover, this phenomenon is sensitive to antidepressant administration [19] [20]. Thus, in the present study, our aim is to determine what happens to animals previously exposed to inescapable shocks (IS) when they are re-exposed to the same box. Our study attempts to determine whether these animals will exhibit contextual fear and escape deficits and whether chronic treatment with sertraline (SERT), a selective serotonin reuptake inhibitor (SSRI) known to reverse depressive symptoms [20], will counteract its effects. Recently, we have reported that the administration of SERT along adolescence is safe in rodents, and reverses some of the prenatal stress effects. Thus, in the present study this drug was administered during the same period to rats. In this sense, we analyzed prenatal stress-induced changes in the later behaviour of the offspring of stressed mothers, focusing on the potential differences between sexes both, as regards stress vulnerability and the impact of chronic treatment with SERT, as a modulating factor.

2. Materials and Methods

2.1. Animals

Virgin female Wistar rats CLS: WI (HAN) (n = 12) weighing 250 g were obtained from highly out bred rats from the animal facility at the University of Salamanca. Vaginal smears were collected daily for 8 days before mating to determine the stage of the oestrus cycle and the day of conception. On the day of proestrus, sexually experienced male Wistar rats were introduced for mating. The day on which spermatozoa were found in the smear was designated as day 1 of pregnancy. For all experiments, the animals were allowed access to food and water *ad libitum*, and were maintained on a regular light-dark cycle (lights on: 07:00 to 19:00 h) in a temperature- and humidity-controlled environment. The animals were handled and cared for according to the guidelines of the European Community's Council Directive (2010/63/EU), current Spanish legislation (RD 1201/05), and those established by the Institutional Bioethics Committee for the care and use of laboratory animals.

2.2. Prenatal Stress Exposure

Pregnant female rats were randomly assigned to the stress or control groups (n = 6 per group) and housed individually in plastic breeding cages. Stress consisted of placing females in the third trimester (days 15 - 21 of gestation) in transparent cylindrical restrainers (7 cm diameter, 19 cm long); under a bright light (50 W bulb) di-

rected onto the surface of the restrainer for 45 min three times a day (at 09:00, 12:00 and 16:00 h). Control mothers were left undisturbed, only being handled for routine activities (cleaning, etc.). Only offspring from litters containing 9 - 12 pups were used in the present experiments. Offspring were weaned at 21 days of age and were separated into group cages housing four animals of the same sex and treatment as previously described [13]. No differences in litter sizes, in the male-to-female ratio of the offspring or inpre-weaning-mortality were found.

2.3. Drug Administration

Starting at postnatal day 30 (P30) and continuing until the end of experiments (P115), pups from each condition—Control or PS depending on the previous treatment—were subdivided to receive either chronic treatment with SERT (Control-SERT and PS-SERT) or saline (Control, PS) (n = 8 per sex and group). Based on previous reports [13], we decided to administer SERT orally (Besitran© Pfizer S. A. Madrid, Spain) at a dose of 5.0 mg/kg/day. Liquid consumption was controlled by using calibrated bottles, and was monitored every two days, and the dose of the drug was adjusted on the basis of the liquid consumed. During this period, the rats were kept in groups of 4 animals in polycarbonate boxes (45 × 30 × 20 cm), with unrestricted access to food.

2.4. Inescapable Shocks

Over 2 days before testing, the animals were habituated to the experimental conditions by being placed in the apparatus and left undisturbed for 3 minutes. The shuttle box used to administer the footshocks had two equal chambers (50 × 25 × 25 cm) separated by a black Plexiglas partition with a gate that could be opened or closed (Letica Scientific Instruments, Spain). In this test, footshocks were administered in the left chamber with the gate closed. The floor consisted of a stainless-steel bars set 2 cm apart, connected in series to a main control module (Letica, LI-2900, Spain) through which the electric shocks were administered. The rats were placed individually in the experimental chamber and after 1 min of acclimatization, a sequence of 3 electric footshocks (0.35 mA, 5 s, with 20 s between each one) were given, with no warning light signal either before or during each shock. After another minute of rest, the rats were returned to their home cages. The sensitivity to shocks was assessed by the occurrence of jump reactions and audible vocalizations, both as measures of physical integrity [21] [22]. The value of each shock vocalization response was classified as no response, when no audible sound was detected (corresponding to a value of 0); a mild response, the emitted sound lasted less than a second (corresponding to a value of 0.5); and a strong response, when the emitted sound last more than one second (corresponding to a value of 1). The total time spent freezing, as seen by a period of at least 4 consecutive seconds with no visible movements, including the vibrissae, was also assessed along the sessions. All observations were carried out in a single-blind assessment by two different investigators.

2.5. Escapable Shocks

Four days after the IS, all animals were re-exposed to the same shuttle box used previously to test their escape/avoidance performance. During this test, the gate was opened to allow free movement between the compartments. Thus, the animals were able to learn that they could escape the shocks. Both compartments of the box were equipped with a light source (15 W)—located on the upper lateral wall and connected to an electrifiable bar-floor—that was used as the conditioned stimulus (CS). The delivery of footshocks, unconditioned stimulus (US), in both compartments was independent; the floor was hinged to operate a switch when depressed, allowing the responses to be controlled automatically. Rats were tested in 10 consecutive daily sessions, each session with 10 trials. The animals were placed in the left chamber of the shuttle box and allowed to explore for 30 s, before the first trial began. Each sequence of 10 trials consisted of a 5 s warning signal with the light on, followed by 3 s of 0.25 mA electric shock and 30s of inter-trial time. Crossing into the opposite chamber while the light signal was on was counted as avoidance, and crossing the chamber while receiving the electric shock was counted as escape. The shock and light signal were terminated immediately once the rat had moved to the other chamber. A non-crossing response during shock delivery was considered escape failure [23] and the rat received a shock lasting 3 s. The criterion for conditioning was the observation of 2 consecutive sessions with more than 70% avoidance responses, and an extreme behavioural response was considered when the animals failed to escape in 3 consecutive sessions (with 100% of non-crossing responses). The movements through the gate during

the 30 s prior to each session were counted as a crossing during adaptation and the movements during the session with no stimulation, were counted as inter-trial crossings [24]. Both were used as indicators of locomotor activity under the experimental condition. The number of faecal boluses, and the reactions to CS (light) and US (shocks) were also assessed throughout the sessions.

The oestrous cycle was not determined in order to prevent differential manipulation of the male and female rats. In light of the previous studies [17] [25] the despair behaviour or the escape latency upon using LH tests is not influenced by the different phases of oestrus.

2.6. Statistical Analysis

Statistical analyses were performed using the SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA). The differences between groups were analyzed by analysis of variance (one, two, and three way ANOVA), followed by the Fisher-PLSD-test for post-hoc comparison if appropriate, and ANOVA mixed ("SPLIT-PLOT") with the Bonferroni test. Differences between groups were regarded as statistically significant when $p < 0.05$. Pearson's coefficient was used to determine correlations. All values are expressed as mean values ± standard error (S.E.M.)

3. Results

3.1. Inescapable Footshocks

The sensitivity to footshock presentation was assessed by the responses of jumps and vocalizations (**Figure 1**). An effect of PS was revealed in the number and intensity of vocalizations ($F_{3.56} = 7.38$, $p < 0.001$). Prenatally stressed animals of both sexes vocalized less in their reaction to shocks than controls (**Figure 1**). In both sexes, SERT acted by reversing the effect of early stress on decreasing the response to shocks. Regarding the jump reaction, differences between sexes were found to affect the animals' response to footshocks ($F_{1.56} = 9.8$, $p = 0.003$). Within non-treated animals, the females responded more intensely than males ($p < 0.01$). In females, a main effect group ($F_{3.28} = 4.9$, $p = 0.008$) was found, once SERT decreased the jump reaction to shocks (**Figure 1**). In males, again, early stress was the only factor affecting this measure; PS males responded less than the controls, although the difference was not significant ($p = 0.47$).

Moreover, the analysis of the period of time animals spent freezing during the footshock session revealed a main effect of group ($F_{3.56} = 5.7$, $p = 0.002$), with a sex vs. group interaction ($F_{3.56} = 6.12$, $p = 0.001$). An overall

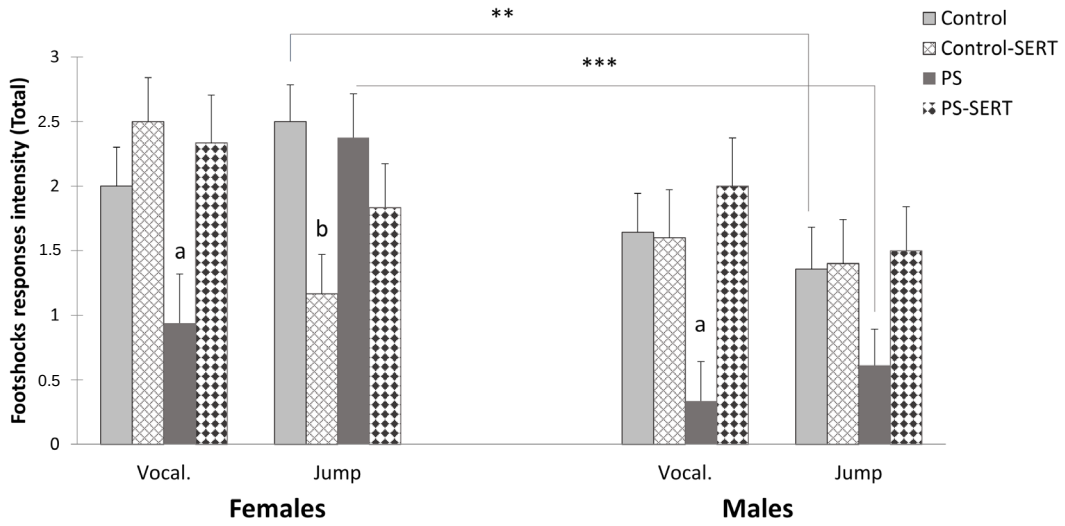


Figure 1. Effects of prenatal stress and SERT treatment on footshocks reaction. It is shown the effects of prenatal stress and SERT treatment (5 mg/kg/day) on footshocks reaction in the animals of both sexes. a: $p < 0.05$, indicates a main effect of prenatal stress; b: $p < 0.05$, indicates a main effect of SERT; $^{**}p < 0.01$; $^{***}p < 0.001$, indicate a main effect of sex within non treated animals. Each bar represents the sum of the 3 footshocks presentation intensity, expressed as means ± S.E.M.

effect of SERT in decreasing the freezing response to shocks was found in both sexes; but also differences according to sex are shown, as an effect of the early stress ($p < 0.001$) (**Figure 2**). Post-hoc reveals that PS females freeze more than controls ($p = 0.06$); otherwise, PS males displayed significantly less time freezing than their control counterparts ($p < 0.001$) (**Figure 2**).

3.2. Escapable Footshocks

3.2.1. Avoidance Behaviour

Differences were found depending on group ($F_{3.49} = 5.7$, $p = 0.002$), and sex ($F_{1.49} = 8.6$, $p = 0.005$), and a sex group ($F_{3.49} = 3.18$, $p = 0.032$) interaction was observed.

A mixed-factor ANOVA for sex vs. treatment vs. sessions revealed an overall increase in avoidance behaviour along the daily sessions ($F_{9.441} = 5.7$, $p < 0.001$) (**Figure 3(A)** and **Figure 3(B)**). When each group was compared with its pair-matched control, post-hoc analysis revealed sex differences for the prenatally stressed and Control-SERT animals; the females exhibited a higher number of avoidance responses than the males ($p < 0.001$ in both groups). Among the females, post-hoc analysis revealed that the PS females developed the greatest ability in learning to avoid adverse stimuli (PS vs. Control + 15.2% ± 3.9, $p = 0.001$). In males, prenatal stress did not affect the acquisition of avoidance behaviour (**Figure 3(A)** and **Figure 3(B)**). SERT administration affected the learning avoidance of the shocks differently in controls and PS animals (**Figure 3(C)**). In control females, SERT administration helped the learning avoidance, an effect that, from the 6th session reached significance ($p = 0.018$) (**Figure 3(A)**). Paradoxically, in prenatally stressed females, an effect of the drug disrupting such behaviour was found (**Figure 3(A)**). Also, the males receiving SERT showed the poorest avoidance performance (**Figure 3(B)**). On comparing the percentage of animals in each group that reached the conditioning criterion, differences were also found: the prenatally stressed females obtained the highest success rate of avoidance, 87.5% of PS females fulfilled the proposed criterion vs. 44.4% of Controls; 66.6% of Control-SERT; and 16% of PS-SERT. In males, neither prenatal stress (22 % males PS vs. 14% of Control males) nor SERT treatment (16% of PS-SERT vs. 16% of males Control-SERT males) affected the percentage of animals reaching the conditioning criterion (data not shown).

3.2.2. Escape Behaviour

The analysis of escape behaviour (sex vs. group vs. sessions) revealed a significant change ($F_{9.441} = 8.43$, $p < 0.001$), with a group interaction ($F_{27.441} = 2.26$, $p < 0.001$), probably due to the effect of SERT on this parameter in both sexes ($F_{3.49} = 23.2$, $p < 0.001$). The animals receiving SERT displayed significantly more escape responses than their control counterparts (**Figure 4**). Among the males (**Figure 4(B)**), the differences reached significance in both groups of SERT-taking animals, regardless of the previous stress treatment. In females (**Figure 4(A)**), the differences reached significance in previously stressed animals. Although in the first three sessions, Control-SERT females also exhibited significantly more escape responses than controls, there was a moment as from which this response decreased in the Control-SERT females (**Figure 4(A)**).

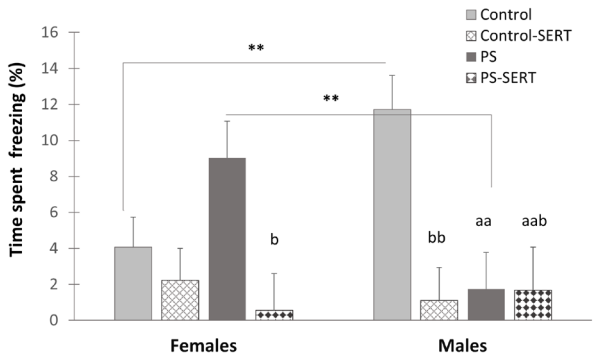
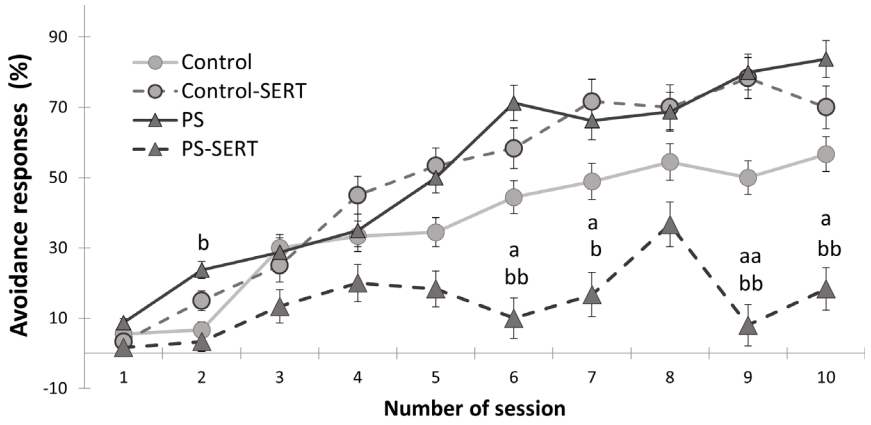
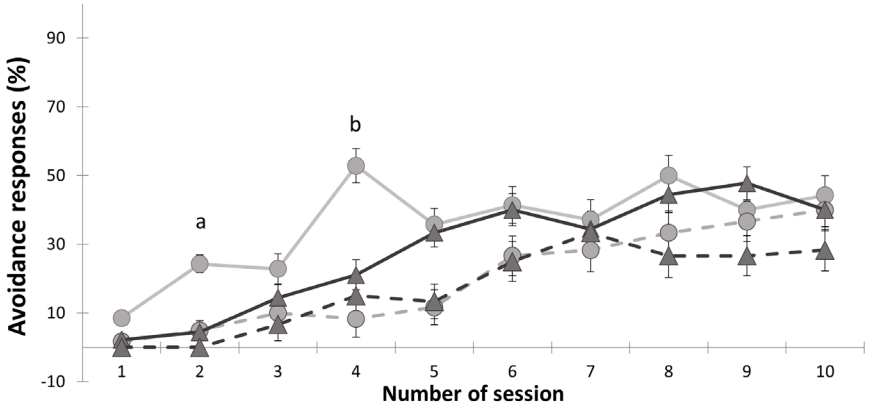


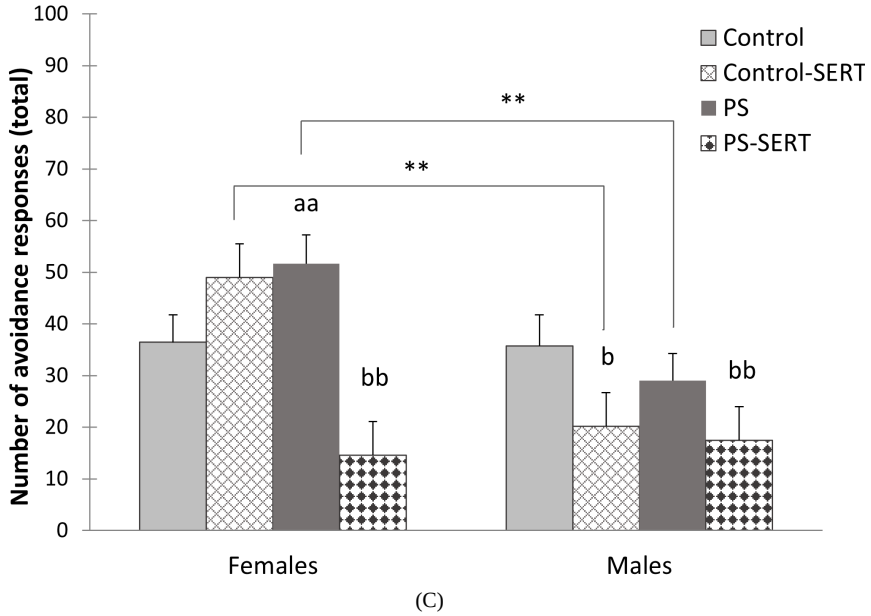
Figure 2. Effects of prenatal stress and SERT treatment on the time rats spent freezing throughout the footshocks session. You may see the effects produced by prenatal stress and SERT treatment (5 mg/kg/day) on the time the rats spent freezing-expressed in percentage-throughout the footshocks session. aa: $p < 0.01$, indicates a main effect of prenatal stress in males; bb: $p < 0.01$; b: $p < 0.05$, indicate a main effect of SERT; aab: $p < 0.01$, indicate differences between PS-SERT and Controls. $^{**}p < 0.01$; indicate a main effect of sex within non treated animals (mean values ± S.E.M.).



(A)



(B)



(C)

Figure 3. Acquisition of the avoidance responses. Avoidance responses all over the daily sessions in females (A) and males (B), in each experimental group; and (C) comparison between males and females in each of the experimental groups (each bar represents the sum of avoidance responses in the ten daily sessions). n = 7 - 9 per group and sex. a: $p < 0.05$ and aa: $p < 0.01$ indicate a main effect of prenatal stress; b: $p < 0.05$ and bb: $p < 0.01$, indicate a main effect of SERT. $^{**}p < 0.01$; indicate a main effect of sex (mean values ± S.E.M.).

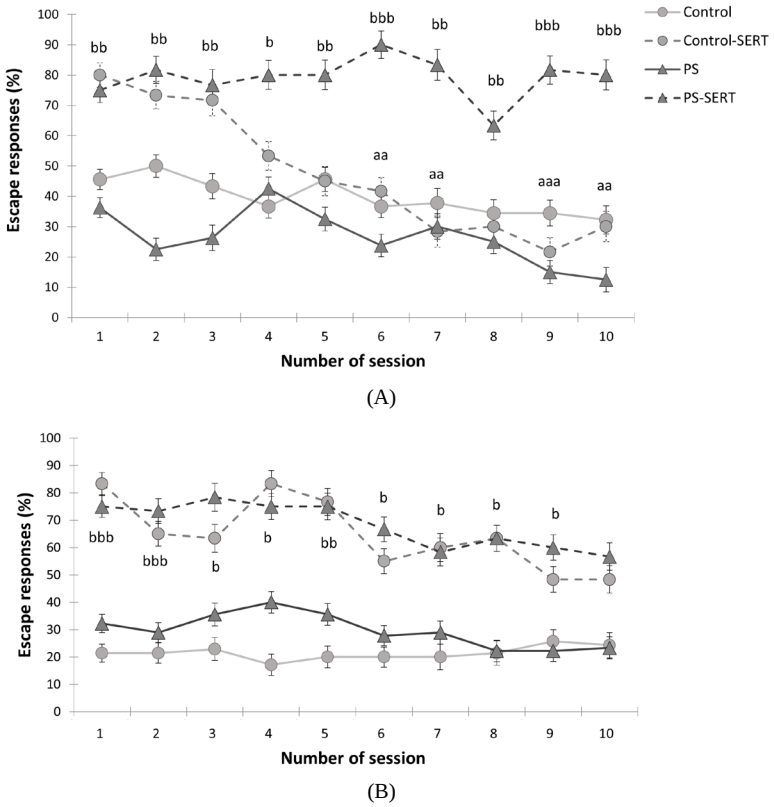


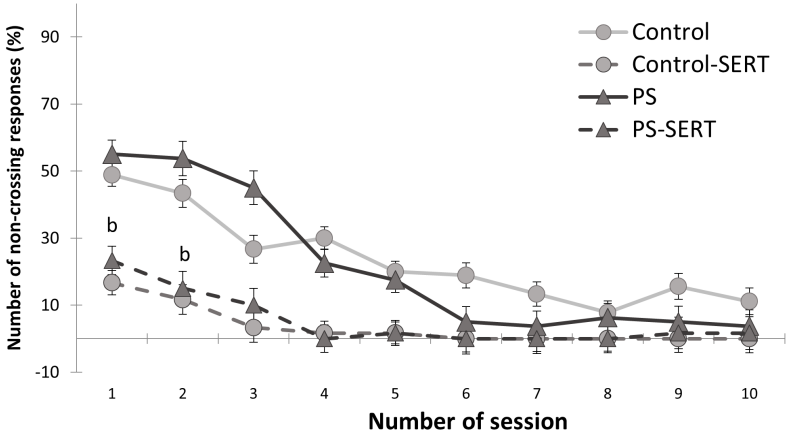
Figure 4. Acquisition of the escape responses all over the daily sessions. Escape responses in females (A) and males (B), in each experimental group. n = 7 - 9 per group and sex. aa: $p < 0.01$ and aaa: $p < 0.001$ indicate a main effect of prenatal stress; b: $p < 0.05$, bb: $p < 0.01$ and bbb: $p < 0.001$, indicate a main effect of SERT (mean values ± S.E.M.).

3.2.3. Escape Failure

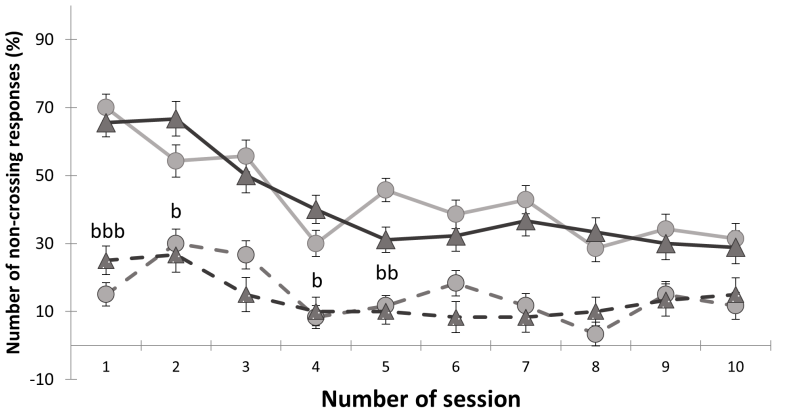
An overall decrease in escape failure along the training sessions was found ($F_{9.441} = 17.23$, $p < 0.001$), with a group interaction ($F_{9.441} = 1.6$, $p = 0.03$), probably due to the effect of SERT treatment ($F_{3.49} = 14.2$, $p < 0.001$), on prenatally stressed or Control animals in both sexes. SERT acted, by helping the animals to escape from shocks (**Figures 5(A)-(C)**). The SERT-treated animals of both sexes exhibited significantly fewer non-crossing responses than the untreated rats (**Figure 5(C)**), regardless of previous stress. Within the untreated animals, all of them began by failing to escape the shocks, but in the following sessions—during which the females began to avoid orescape from the shocks (**Figure 5(A)**)—the males, regardless of stress, exhibited escape failure and failed to respond at all (**Figure 5(B)**). Thus, differences between males and females were observed, the males displaying significantly more non-crossing responses than females ($p < 0.05$) (**Figure 5(C)**).

3.2.4. Inter-Trial Activity

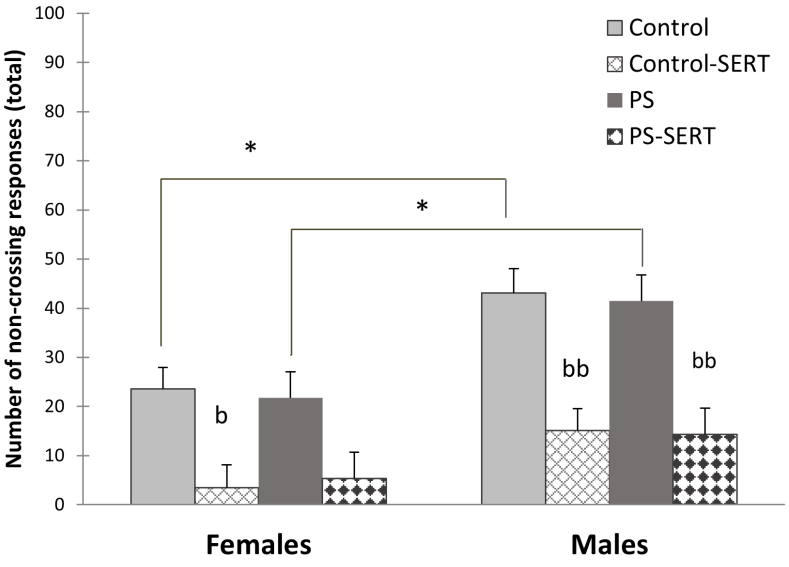
No significant differences between the experimental groups or sexes were found upon changing the activity before the beginning of the session (crossing in adaptation). By contrast, the number of inter-trial crossings was affected by group ($F_{3.49} = 4.3$, $p = 0.009$), with a group vs. sex interaction ($F_{3.49} = 3.83$, $p = 0.015$). In both sexes, previously stressed animals receiving SERT administration showed significantly fewer crossings than the animals from the other experimental groups (**Figure 6**). Also, in prenatally stressed animals a main effect of sex, $F_{1.49} = 6.4$, $p = 0.015$, was observed. Whereas the PS males did not show any motivation to cross the gate, PS females displayed more crossings than the controls ($p = 0.04$). The increase in inter-trial activity was positively correlated with the avoidance responses ($r = 0.91^{*}$), meaning that the more active they were during the test, by passing from one compartment to the other by trial and error, the more the rats learned to associate the stimuli. By contrast, this parameter was negatively related to the non-response level ($r = -0.6^{*}$).



(A)



(B)



(C)

Figure 5. Effects of SERT on prenatally stressed or control animals in the escape failure. Escape failures all over the daily sessions, in females (A) and males (B) in each experimental group; and (C) comparison between males and females in each of the experimental groups (each bar represents the sum of escape failures in the ten daily sessions). n = 7 - 9 per group and sex. b: $p < 0.05$ and bb: $p < 0.01$, indicate a main effect of SERT. $^{*}p < 0.05$ indicates a main effect of sex within non treated animals (mean values ± S.E.M.).

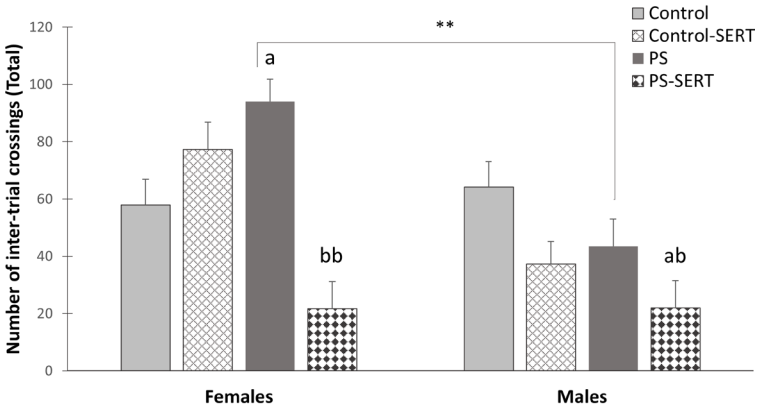


Figure 6. Inter-trial activity performed by the animals. Inter-trial activity exhibited by both sexes in each experimental group. a: $p < 0.05$ indicates a main effect of prenatal stress; ab: $p < 0.05$ indicates differences between the PSTF and Control males; bb: $p < 0.01$, indicates a main effect of SERT; $^{**}p < 0.01$; indicate a main effect of sex in prenatally stressed animals. Each bar represents the sum of inter-trial crosses all over the ten escapable footshocks sessions (mean values ± S.E.M.).

3.2.5. Emotionality Measures

Throughout testing, differences between groups in the number of faecal boluses were revealed ($F_{3.49} = 20.9$, $p < 0.001$). When exposed to the footshocks' box the rats of both sexes receiving SERT exhibited an increase of the faecal boluses (**Figure 7(A)**); however, no effects of PS nor differences between the sexes were found. The responses to the stimulus were also counted (the footshocks as the US; and the light source as the CS). Throughout the escapable shock sessions, neither treatment nor sex affected the response to the footshocks (the vocalizations and jump reflex number). However, looking towards to the light source was dependent on the treatment ($F_{27.441} = 2.67$, $p < 0.001$) (**Figure 7(B)**). Once again, in both sexes the animals receiving SERT exhibited more responses to stimuli than the untreated animals.

4. Discussion

According to the literature, individuals' responses to stressors or their propensity to suffer from affective disorders is partly based on their experiences early in life [1] [4] [5] [8]; sex-dependent differences remain to be elucidated. The main findings of the present study indicate that the restraint stress applied to rat mothers during the last week of their pregnancies acts differently in males and females as regards to their ability to respond under a stressful condition. After being exposed to uncontrollable shocks, all animals exhibited learning deficit when re-exposed to the fear context; the exceptions were PS females. These results extend recent findings reporting that rather than being unfavourable, mild early-life stress may help animals to adapt better to a stressful context later on in life [10] [26]. However, the differences according to sex must be taken into account. Furthermore, despite no particular differences in escape failure were found as an effect of PS in both sexes, the PS males exhibited signs of anhedonia, responding less to footshock delivery. SERT has shown to be effective in reversing the LH in both sexes, but increased anxiety in each exposure to the fear context.

4.1. Inescapable Shock Reaction

With footshock administration, in the present study, all animals responded with vocalizations or jumps and freezing. These types of reaction are considered important defensive reflexes that may help to protect the animal from injury [21] [27] [28]. Our data show that prenatal stress affected the footshocks response, especially in males. Prenatally stressed males jumped, vocalized and froze less when exposed to the shocks, in comparison with their controls. In both sexes, SERT also induced a decrease in the time spent freezing. It is well documented that there are fundamental dimorphic sex differences at the level of the brain structures and in neurotransmitter systems [29]. In the present study, when subjected to footshocks, the females were consistently more reactive than males (jumped significantly more), while the males froze more often, in complete agreement with the literature [22] [27]. Furthermore, it has been suggested that the nociceptive system of offspring may be

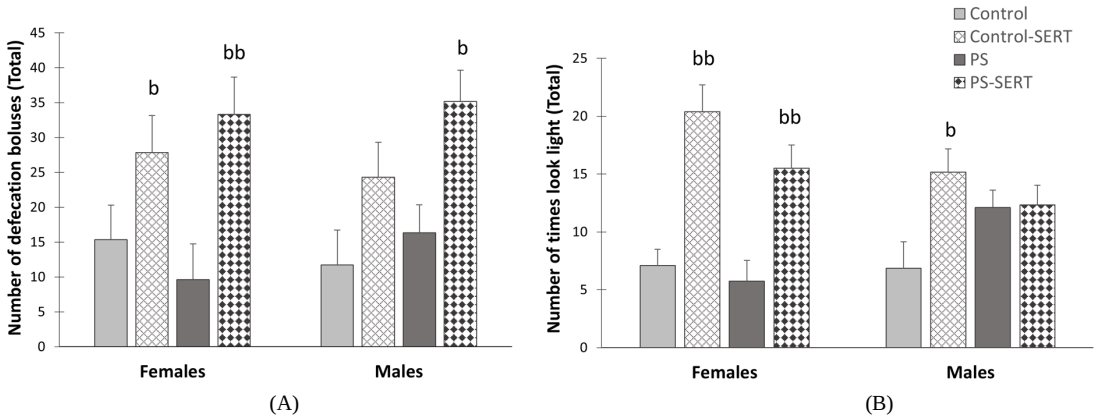


Figure 7. Effects of prenatal stress and SERT treatment on emotionality measures. Prenatal stress and SERT treatment (5 mg/kg/day)effects in the animals of both sexes when repeatedly exposed to the shuttle box previously paired with Inescapable footshocks. bb: $p < 0.01$ and b: $p < 0.05$, indicate a main effect of SERT. Each bar represents the sum of boluses (A) and the light orientation responses (B) in the ten daily sessions (mean values ± S.E.M.).

altered by exposure to early stressors. The endogenous opioid changes, induced by a stressful pregnancy, can significantly affect the mediation of the nociceptive systems in the offspring that may be different in each sex [30] [31]. In fact, it could be proposed that prenatally stressed males exhibit a lower sensitivity to shocks, which SERT reversed. However, when the shocks reaction were examined later (throughout the escapable shock procedure), no differences were found. Thus, the reduced reactivity to painful shocks we found, in prenatally stressed males, was not due to a lower sensitivity to shocks, but probably it was governed by other patterns of emotional behaviour, which could reflect differences in the activation threshold of some brain structures [32] [33]. On the other hand, SERT was effective in reversing these effects of the PS, possibly, by regulating the autonomic functions [34].

4.2. Sex-Differences in Learning Performance and Escape Failure

The exposure to IS prior to escapable shocks affected the learning performance and the shocks avoidance in a sex-dependent manner. According to the literature, subjecting rats to an uncontrollable traumatic event, such as the IS, disturbs their later escape performance [4] [17] [20] [35] [36]. It was reported that more than 95% of male rats develop an escape deficit after they had been subjected to an inescapable stressful situation 24 hours earlier [35]. Normally, the LH paradigm uses a large number of high-intensity footshocks [20] or is applied over several consecutive days [17]. In the present study, the single mild footshock session we used was apparently sufficient to trigger the same deleterious effects in all the animals, except the prenatally stressed females. The PS females exhibited the greatest ability to learn to avoid the adverse stimuli, and did not display escape deficit.

During the first trials, when the rats were in the charged compartment, they did not enter the opposite chamber readily, as expected in this type of bi-directional training, even when is not preceded by IS [37]. In ensuing sessions, rats can either learn to avoid shocks-they learn by association that after light comes the shock, and that this can be avoided by crossing the gate-or they fail to learn, and receive repeated footshocks. In a previous experiment carried out at our laboratory, the avoidance behaviour was tested and 90% of the naïve male rats managed to achieve scores of 60% - 80% in conditioning [38]. The data obtained in the present work reveal differences in the effects of PS on the learning performance and escape failure according to sex. Our results show that after being exposed to IS, whereas the males born from stressed mothers did not reach to learn, the PS females learned to escape from the shocks, as they were not affected by the previous IS. It could thus be suggested that the model of gestational stress we tested, helps females, at least regarding the ability to learn in a fear context. In males this was not the case. By contrast, the present data show that males subjected to PS were the most susceptible in developing the escape deficit, apart from being the only experimental group in which the animals exhibited an extreme behavioural response. Most PS males failed to respond to the adverse situation remaining still (while receiving the shocks), and they exhibited a significantly reduced interest in their environment; these symptoms can be attributed to the animal model of LH [15] [33] [35] [36] [39]. Importantly, and in contrast to males, most of the females did not exhibit this depressive-like state, regardless of previous stress. This agrees

with the literature, whereas women are more susceptible than men to suffer from depression or *post-traumatic stress disorder* [40] [41], female rats rarely develop LH [17] [18].

Also, it was suggested that when tested on a learning or memory task context the better performance of females could be related to differences in their activity, in which motor function is required [32] [36] [42]. In the present study, the exposure to IS confirmed the sex-differences in escape behaviour and in the activity levels, but only if the females were subjected to PS. In the animals raised in standard conditions (Controls) no sex differences were found.

4.3. Sex-Dependent Effects of Prenatal Stress on Learned Helplessness

Helplessness behaviour and learning deficits in prenatally stressed animals have been reported previously [5] [8] [43]. With the PS paradigm, most authors have reported a greater immobility in the helplessness test in stressed animals and reduced memory retention on the passive avoidance tasks; and some differences according to sex have been noted. It has been described that female offspring appear to be relatively more resistant to stress exposure than males during development, the effects of PS affecting memory and learning being less evident in females [6] [44] [45].

Several studies reported that the differences between sexes, in the response to a fear context, arise from changes in the gonadal hormone levels during foetal development [6] [17] [46], persisting in gonadectomized adult animals [17]. Testosterone and estrogens could exert an active role, exacerbating or protecting the effects of the manipulation during pregnancy [47]. It is known that gestational stress reduces the level of testosterone in adults [45] and that low testosterone levels are associated with memory loss in men. This is consistent with our results showing disturbed behaviour in PS males that could be related to inadequate levels of testosterone, originally caused by the gestational stress. Considering that there are sex-dependent differences in the sensitivity to foetal programming [16], it is possible that during such critical brain development period, sex hormones give rise to sex differences with regards to learned helplessness-eliciting behaviours. Moreover, although the cause remains to be totally established, other authors also reported that PS may alter the ability of estrogens to alleviate some depressive behaviour in female but not male rats [47].

4.4. The Effects of Sertraline

In addition to the disturbance of serotoninergic metabolism by PS [48], subjecting rats to inescapable stressors also induces a decrease in the levels of 5-HT in some brain areas [20], and it is expected that chronic treatment with SERT, which increases the level of this neurotransmitter, would reverse such an effect. Our data show that the LH phenomenon was sensitive to the treatment with SERT. SERT was effective in reducing escape failure and in preventing males' hypoactivity.

Moreover, among non-stressed animals our data show that SERT was effective in reversing IS-induced learning deficits, but only in control females; in males, this was not the case. Although the existence of sex differences in responses to drugs is not remarkable, since these have been reported in both humans and rodents [32], and specifically in the response to SERT [49] [50], the effects of PS affecting drug effectiveness is more difficult to interpret. The animals escaped from the shocks, but did not avoid them. Many of these animals, which were previously stressed and were receiving SERT, though not despaired, did not manage to avoid the shocks. It was expected that the rats treated with SERT would improve their shocks avoidance. In a stressful situation, 5-HT should help to establish behavioural alterations in the most appropriate direction [51] [52]. Nevertheless, the present results strengthen past reports in the sense that learning is affected by drug treatment with serotonin precursors (e.g. 1-tryptophan and 5-HT) [16]. In such study the deficit in the acquisition of escape behaviour was only induced by the combination of exposing the animals to IS and the increase in 5-HT levels [16]. It was suggested that the animals would be slower due to alterations at the level of motor activity. It is known that in addition to its role in the regulation of emotions, 5-HT can also influence other functions such as sensory perception or motor activity [53]. However, we found no differences in the locomotor activity as effect of SERT administration. This supports previous results [13] [51] [54] in which fluoxetine or SERT reduced the stress-induced immobility, without influencing other activities.

Moreover, our data show an increase in most emotionality parameters in the SERT-taking animals. In response to an unexpected stress the dorsal area of the raphe nuclei (DRN) is activated, with the consequent release of 5-HT in the limbic area and subcortical structures [51]. Apparently, the activation of the DRN seroton-

ergic neurons facilitates anxiety-like behavioural responses [55]. Thus, by increasing serotonin turnover in this circuitry, SERT treatment modulates depressive behaviours, but may have increased anxiety during each exposure to the stressful context. Despite this, it could be suggested that the improved anxiety in SERT-receiving animals might also be a consequence of not escaping from shocks. In contrast to the protocol of previous experiments [23], in the present work, the animals that did not acquire avoidance responses were exposed persistently to the shocks.

In light of the results, we surmise that the learning deficit exhibited by stressed animals receiving SERT would not be due to a deficit in perception, memory, pain or motor impairments. In fact, we did not find stimulus perception to be affected, and the rats did not find difficulties in associating the CS with the US. The rats looked at the light when this was on, and jumped and escaped when they were subjected to shocks, but for some reason they did not avoid them. Indeed, we observed that some animals from this experimental group exhibited a preparatory behavioural posture (data not shown), which would allow them to rapidly pass from one compartment to the other-they stand at the door-but even though they could escape they did not, waiting for the shock to pass. This behavioural posture had already been observed [37].

5. Conclusion

Our data agree with and extend previous reports based on the idea that exposure to PS affects subsequent behaviour and the response to stress, later in life, in a sex-dependent way. In this study, the males were more likely to develop behavioural despair than the females. It could be speculated that the functional connection between the forebrain and brainstem regions has been affected, as an effect of the early stress, in males but not in females, leading to a dysregulation of the stress response. When exposed to a fear context, oestrogen levels could have been modulating the behavioural and neurobiological responses, and somehow, protecting the effects of early stress in females. Further research is required to identify the neuronal mechanisms that mediate the differing responses, between males and females, to dramatic events and their recovery with antidepressants.

Acknowledgements

This research was supported by grants from the Spanish Ministry of Science and Innovation (MICINN, #BFU2007-65210 and #BFU2010-17754) to Dr. Dolores E. López and by the University of Salamanca Research Support Grant 2015 to Dr. Dolores E. López.

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ANEXO IV

Title: The higher susceptibility of prenatally stressed rats to develop post-traumatic stress disorder-like symptoms is reversed by Sertraline in a sex-dependent manner

Article Type: Research Reports

Keywords: fear conditioning, footshocks, behavior, physiologic, re-stress

Corresponding Author: Dr. Consuelo Sancho, Ph.D

Corresponding Author's Institution: Institute for Neuroscience of Castilla y León

First Author: Inês Pereira-Figueiredo

Order of Authors: Inês Pereira-Figueiredo; Orlando Castellano; Juan Carro; Dolores E. López, Consuelo Sancho, Ph.D

Abstract

The present study attempted to evaluate the role of prenatal stress affecting the development of post-traumatic stress disorder (PTSD)-like symptoms and it reversion by Sertraline, a front-line medication for PTSD in humans. To achieve this purpose, when adults, prenatally stressed (PS) or unstressed (Controls) offspring rats of both sexes, following chronic Sertraline treatment or not, were studied, using the animal PTSD model, the contextual fear conditioning paradigm. Before it, anxiety-like behavior during the prepulse inhibition test, a modulation of the startle reflex, was examined in all animals. Subsequently, the animals were subjected to inescapable footshocks (IS) in a shuttle box that was then followed by four days of situational reminders in the aversive context. Finally, the physiological response to re-stress was compared with a group of undisturbed controls. Whereas prior to the IS, no effects of PS nor Sertraline were found - the PPI and the habituation to the shuttle box did not change; posteriorly, PS lead animals to exhibit behavioral and physiological disturbs. When compared to controls, PS animals of both sexes exhibited less activity in the aversive environment, and the males displayed extinction issues. The PS females also exhibited lower ACTH levels in response to re-stress. PS heightened the IS-induced response to re-stress and caused leukopenia, however both effects were reversed by Sertraline. Nevertheless, Sertraline lessened the behavioral impact of PS only in females. Current results extend previous data, from our laboratory, showing that PS strengthened PTSD-like symptoms and Sertraline act differently in males and females.

The higher susceptibility of prenatally stressed rats to develop post-traumatic stress disorder-like symptoms is reversed by Sertraline in a sex-dependent manner

Inês Pereira-Figueiredo[a], Orlando Castellanos[a,c], Juan Carro[a,b], Dolores E. López[a,c] Consuelo Sancho[a]

[a]Neuroscience Institute of Castilla y León/IBSAL. University of Salamanca, Salamanca, Spain.

[b]Department of Basic Psychology, Psychobiology and Methodology. University of Salamanca, Salamanca, Spain.

[c]Department of Cell Biology and Pathology. University of Salamanca, Salamanca, Spain.

***Correspondence:** Neuroscience Institute of Castilla y León, Consuelo Sancho Sánchez
University of Salamanca, Medicine Faculty
C/ Pintor Fernando Gallego 1, 37007 Salamanca, Spain. email: sanchoc@usal.es

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Abstract

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1. Introduction

Stress is described as a fundamental adaptation for an organism to cope with emergencies (McEwen, 2000). When the stress response exceeds certain limits of intensity, the potentially beneficial alterations earlier mentioned can cause pathological states, or exacerbate latent or pre-existing morbid states (Wakizono et al., 2007; McEwen, 2008; Maier and Watkins, 2010). In humans, the inability to adequately respond to a stressful situation may lead to psychopathological disorders, such as the post-traumatic stress disorder (PTSD) (Cohen et al., 2003; Yehuda et al., 2006; Wilson et al., 2014), exhibiting an exaggerated sensitization to fear, hypervigilance, exaggerated startle response, and sensitization of the physiological responses to stress (Yehuda, 2001; De Kloet et al., 2006; Cohen et al., 2006; Lehrner et al., 2014). However, not all individuals that experience the same stressful trauma develop PTSD (Cohen et al., 2003). Despite that the definitive susceptibility factors triggering such a disorder have not been identified, risk factors may include individual neurobiology as well as past experiences (Diehl et al., 2007).

According to the literature, early adverse experiences, including prenatal stress, have profound and long-lasting effects on the development of neurobiological systems (Kapoor et al., 2006; Diehl et al., 2007; Clinton et al., 2008; Louvart et al., 2009; Green et al., 2011; Pivina et al., 2014) that may "program" later vulnerability to stress events (Harris and Seckl, 2011; Xiong and Zhang, 2013; George et al., 2013). In humans, it has been reported that adverse early experiences are critical factors in the later development of PTSD and other anxiety disorders (Gunnar and Quevedo, 2007; Cirulli et al., 2009). Both in humans and rodents, early life stress induces low birth weight (Kajantie and Räikkönenc, 2010) and low baseline corticoid hormone levels in the fetuses or children (Oitzl et al., 2010; Lehrner et al., 2014), and have been described as vulnerability factors for PTSD (Cohen et al., 2006; Morris et al., 2012). With this in mind, in the present study, our main goal was to determine how prenatal stress may affect later vulnerability to PTSD in the animals of both sexes.

The offspring of stressed female rats were initially tested, upon reaching adulthood, in a condition of pre-trauma by using the prepulse inhibition test (PPI); a diagnostic tool believed to index essential mechanisms in the neural control of behavior (Koch et al., 1999). In fact, although deficits in PPI were originally identified in schizophrenic patients, reflecting a poor somatosensory integration, this condition has also observed in several disorders related to anxiety (Geyer, 2006; Li et al., 2009).

Considering that PTSD emergency is totally dependent on confrontation with a traumatic event (Liberzon et al., 1997; Louvart et al., 2009; Zoladz et al., 2012), the animals were then subjected to a session of mild inescapable shocks (IS) in a shuttle box that was followed by situational reminders, in the absence of shocks, in the same adverse environment (the contextual fear conditioning paradigm). By inducing a stressful situation from which the animal cannot escape, this paradigm probes the acquisition of conditioned emotional responses and their extinction, and is often used as a model of PTSD (Vermetten and Bremner, 2002; Wakizono et al., 2007; Blundell et al., 2011).

In recent studies using animal models for PTSD, putative candidates for determining the development of such a disorder are being unraveled, and have been shown to include abnormalities in the neuroendocrine, sympathoadrenal modulatory systems, the immune system and in the levels of neutransmitters (Olff et al., 2006; Yehuda et al., 2006; Lehner et al., 2006; Wilson et al., 2014; Pivina et al., 2014) where most of it have a close relationship with the PS phenotype. The common features include the inability to cope with stressful situations that has associated with deficits in the activity of brain systems controlling the hypothalamic-pituitary-adrenal (HPA) axis (Gunnar and Quevedo, 2007) and the sympathoadrenal medullary system (Xiong and Zhang, 2013; Hoffman et al., 2014). So, after the behavioral tests were conducted, the assessment of physiological parameters during the recovery from a re-stress condition (by immobilization stress) were examined, in all animals and compared with a group of undisturbed controls.

Considering the role of the serotonergic system on stress responsiveness and the modulatory effects of serotonin in the PTSD (Vermetten and Bremner, 2002; Wilson et al., 2014) a pharmacological treatment with Sertraline (SERT) was used. SERT is an antidepressant, widely used in our laboratory, which is effective in reversing the impact of stress (Pereira-Figueiredo et al., 2014), and the behavioral despair in prenatally stressed rats (Pereira-Figueiredo et al., 2015a). This type of antidepressant is also the front-line medication for treating PTSD in humans (Davidson, 2006; Hien et al., 2015). However, its effectiveness remains controversial when used in PTSD models in rodents (Matar et al., 2006; Wilson et al., 2014). According to our results, we suggest that the prolonged administration of SERT is needed to achieve clinical improvement (Pereira-Figueiredo et al., 2015b), and therefore we decided to administer SERT for 3 months, beginning at the first month age.

Moreover, considering that in human patients sex differences in disease prevalence, symptoms and clinical outcome have been established (Sloan and Kornstein, 2003; Olff et al., 2006; Kajantie and Räikkönenc, 2010; Simpson et al., 2012), we anticipate that the inclusion of both male and female rats in our study may enhance the validity of the SERT treatment by reversing stress-induced changes.

2. Experimental procedures

2.1. Animals

The present report is part of a long-term survey on the effects of prenatal stress on the adult progeny of Wistar rats (see also Pereira-Figueiredo et al., 2014). Virgin female Wistar rats (n=14) weighing 250 g were obtained from highly outbred rats from our own animal facility at the University of Salamanca. Vaginal smears were collected daily for 8 days before mating to determine the stage of the estrus cycle and the day of conception. On the day of proestrus, sexually experienced male Wistar rats were introduced for mating. The day spermatozoa were found in the vaginal smear was designated as day 1 of pregnancy. For all experiments, animals were allowed *ad libitum* access to food and water, and maintained on a regular light-dark cycle (lights on: 07:00 am-19:00 pm) with a constant temperature of 21º C. The animals were handled and cared for according to the guidelines of the European Communities Council Directive (2010/63/CE) for the care and use of laboratory animals.

2. 2. Prenatal stress exposure

As previously described (Pereira-Figueiredo et al., 2014), pregnant female rats were randomly assigned to either the stress or unstressed groups (n=7 per group) and individually housed in plastic breeding cages. Stress consisted of placing the females, in the third week of gestation (days 15-21), into transparent cylinder restrainers (7 cm diameter, 19 cm long), under a bright light (50 W bulb) directed onto the surface of the restrainer for 45 min three times a day (at 09:00, 12:00 and 16:00 h). Control mothers were left undisturbed, being handled only during routine activities (cleaning, etc.). All stress and control mothers gave birth naturally, and no differences in litter sizes, in the male-to-female ratio of the offspring or in pre-weaning-mortality were found. Only offspring from litters containing 9 - 12 pups were used in the presented experiments. Offspring were weaned at 21 days of age and were separated into group cages housing four animals of the same sex and treatment, with the criterion that groups included no more than two pups from the same litter.

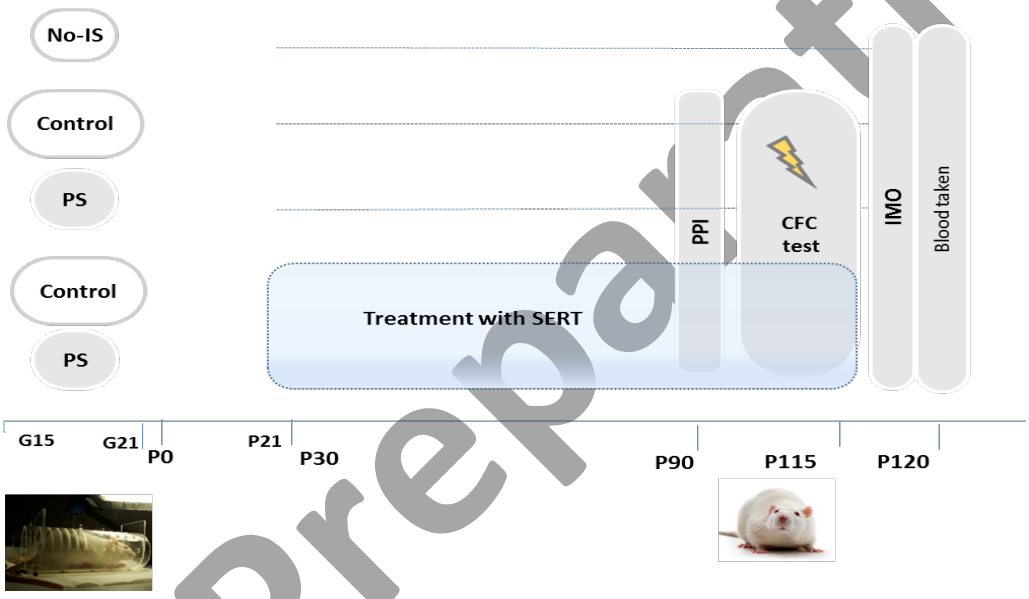

FIGURE 1. Experimental design and timeline. The Control or PS offspring treated with SERT or not, were exposed to the PPI test (P90), Contextual fear conditioning paradigm (CFC) and later to IMO stress (N= 8 on each group and sex, respectively). A set of animals (No-IS, N =7 on each group and sex, respectively) has been added, and exposed to IMO only, to serve as controls of the experimental conditions.

2.3. Drug Administration

Starting at postnatal day 30 (P30) and continuing until the end of experiments (P115), pups from each condition—Control or PS depending on the previous treatment—were subdivided to receive either chronic treatment with SERT (Control-SERT and PS-SERT) or saline (Control, PS) (N = 8 per sex and

group). Based on previous reports (Pereira-Figueiredo et al., 2014), we decided to administer SERT orally (Besitran© Pfizer S. A. Madrid, Spain) at a dose of 5.0mg/kg/day. Liquid consumption was controlled by using calibrated bottles, which was monitored every two days, and the dose of the drug was adjusted on

the basis of the liquid consumed. During this period, the rats were kept in groups of 4 animals in polycarbonate boxes (45 × 30 × 20 cm), with unrestricted access to food.

2.4. Prepulse inhibition (PPI)

At P90, all animals of both sexes were tested for PPI, in a simple paradigm routinely implemented in our laboratory (Castellano et al., 2009) using the SR-LAB system (SDI, San Diego, CA, USA). Previous to testing, the rats were habituated to the experimental conditions, especially regarding their placement into the apparatus. The acoustic startle reflexes (ASR) were measured in six identical startle chambers simultaneously. Acoustic stimulus intensities and response sensitivities were calibrated (using an SR-LAB Startle Calibration System) to be nearly identical in each of the six SR-LAB systems (maximum variability <1% of stimulus range and <5% of response ranges). The background noise of 65 dB SPL was generated throughout the entire session. A session consisted of an acclimatization period of 5 min and 64 trials presented pseudo-randomly. Sixteen of the trials were a single noise pulse (115 dB, 20 ms of bursts) that were used to determine the ASR. The remaining 48 trials were a white noise prepulse presented at different intensity levels (65, 70, or 80 dB SPL) with a duration of 20 ms, followed by a startling stimulus with an interstimulus interval of 50 ms. Whole-body movements corresponding to the startling responses were recorded with a piezoelectric accelerometer and converted into analogical signals that provided the latencies and amplitudes of the ASR. The percentage of the magnitude of PPI was calculated for each respective prepulse intensity according to the following formula: % prepulse inhibition = 100-(100x startle amplitude in prepulse followed by pulse trial)/(startle amplitude on pulse trial alone). The PPI latency was the time taken between each prepulse intensity stimulus and the corresponding response, expressed in ms (Castellano et al., 2009). This test was performed between 10:00 and 14:00 in a separate room away from the other behavioral tests.

2.5. Contextual fear conditioning test

One week after the PPI procedure, all animals were subjected to the Contextual fear conditioning paradigm (CFC) Hoffman et al., 2014). The shuttle box was used as the Conditioned stimulus (CS) and the footshocks as the unconditioned stimulus (US). The box had two equal chambers (with a 25 cm depth x 29 cm height x 25 cm width, each) separated by a black Plexiglas partition with a gate that could be opened or closed (Letica Scientific Instruments, Spain); in the present study, the rats were tested in the left chamber with the gate closed. The anterior wall was transparent to be able to observe the actions of the animal. This test consisted on 7 daily consecutive sessions lasting for 3 min. The first two days allowed for the individuals' habituation to the experimental conditions - the animals were introduced into the apparatus and let undisturbed. On the third day, after a minute of acclimatization, a sequence of 3 shocks (5 sec, 0.35 mA, with 20 sec between each one) were administered as a current, equally distributed through a metal grid floor; the rats were allowed to stand for another minute and were then transported back to their home colony. Twenty-four hours later, and during four more daily sessions, the animals were re-exposed to the testing apparatus. These sessions also lasted for 3 min, occurred without shocks, and served to introduce a situational reminder of the shocks to the animal (Armario et

al., 2008). Behavior was videotaped using a camera (Sony HDR-CX 220) mounted on the ceiling and by using a videocassette recorder. Footage and audio material was later used by observers blind to the experimental conditions for scoring. The acquisition of fear was given by the percentage of time the animals spent motionless when re-exposed to the context: *freezing* for at least 3 sec with no detectable movements (only *vibrissae)*, accompanied by flection of the body with a shrug and flexion of the neck (as previously described by Greenwood et al., 2010); or *crouching (*similar to *freezing,* but with detectable movements of the neck). Both postures were analyzed at the same time and were defined as *defensive* (DEF) (Yang et al., 2004). The extinction of fear was acquired when less than 10% of the time was DEF after the fourth shock session. Also, the number of *rearings*, and the number of *groomings* were recorded over the entire procedure.

2.6. Immobilization stress

Fifteen days after the last behavioral test ended (at P120 days), all animals were subjected to a single session of immobilization stress (IMO) (see Pereira-Figueiredo et al., 2015_b). Each rat was placed in a transparent cylinder restrainer under a bright light for 30 min, afterwards they were removed and allowed to stand for 10 min in a new cage (Viau et al. 1993). Two types of restrainers were used so that both female and male rats would be confined to a similar degree (70 vs. 80 mm diameter for females and males, respectively). The length of the cylinder was adjusted to the body size of the rat by the use of an end plate that was 15 to 19 cm long. After recovery time ended, each animal was anesthetized and trunk blood was taken by cardiac puncture (Maccari et al., 2003). We carried out this procedure simultaneously for three animals, from 10:00 h to 13:00h. The body weight was also registered.

2.6.1. Plasma Hormone Radioimmunoassay (RIA) ACTH.

Trunk blood samples were processed using EDTA (K3)-containing tubes that were freshly centrifuged (1800 x g for 15 min at 4º) to obtain the plasma. Plasma was stored at - 80° C until processing in the laboratory of Physiology of the Faculty of Veterinary Medicine at the University of Lisbon. ACTH concentrations were measured in unextracted plasma, in duplicate, using a commercial radioimmunoassay (RIA) kit according to the instructions of the manufacturer (Immu Chemm Double Antibody^{125} hACTH I RIA kit, MP Biomedicals). The minimal detectable level was 3.5 pg/ml with an intraassay and interassay coefficient of variation of 6.9% and 9.6%, respectively.

2.6.2. Hematological analyses

For hematological analyses, the trunk blood was also collected into EDTA (K3)-containing tubes that were freshly processed on an automatic cell counter (ADVIA 120 cytometer, Bayer, Leverkusen, Germany). The obtained hematological parameters were number of leukocytes, erythrocytes, hemoglobin concentration, mean corpuscular hemoglobin (MCH), hematocrit and mean platelet volume (MPV).

2.7. Statistics

Statistical analyses were performed using IBM® SPSS® software, version 20 (IBM Crp. and SPSS Inc., Chicago, IL, USA, 2011). Differences between groups were analyzed by ANOVA (one, two and three way), followed by the Fisher-PLSD-test for post hoc comparison if appropriate, and ANOVA mixed (or "SPLIT-PLOT") with the Bonferroni-test. Mean differences were subjected pairwise to Student's t-test, using the Levene Test for equality of variances. Pearson's coefficient was used to determine correlations. When appropriate the chi square analyses were used. Differences were regarded as statistically significant when $p < 0.05$.

3. Results

3.1 PPI measures

To evaluate the behavioral effects of PS and SERT administration, the analyses were performed using the highest prepulse intensity (80 dB SPL), as the PPI highest inhibitions were obtained with the highest intensities, as previously reported (Castellano et al., 2009). No differences in relation to the treatments were revealed, the 2-way ANOVA (with PS and SERT treatment vs. sex as factors) showed no significant effects in the PPI levels, ($F_{3,71}$ = 0.91, n.s), although, a main effect linked to the sex of the animal was found ($F_{1,71}$ = 14.2, p < 0.001). As shown in Figure 2 A and B, males exhibited higher PPI values and longer latency than females; the differences reaching significance only among animals that were not taking SERT (p<0.001), regardless of the previous stress applied.

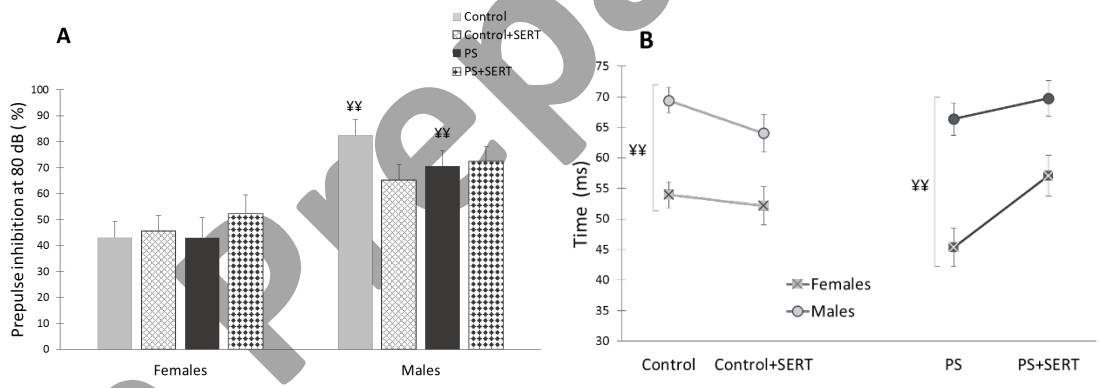

Figure 2. PPI values (A) and PPI latency (B), performed in the animals of both sexes at the age of P90 days, previously submitted or not to prenatal stress (PS and Control) and treated with SERT (5 mg/kg/day) (PS-SERT and Control-SERT). N= 8 animals per group and sex (mean values ± S.E.M.). ¥¥, p < 0.01, indicate a main effect of sex within non treated animals.

3.2 Contextual fear conditioning test

3.2.1 Habituation to context

Prior to the footshocks (IS), during the two habituation sessions, the animals of all groups displayed little defensive behavior, and the percentage of DEF time was comparable for all of the animals (mixed three-way ANOVA with group, sex and sessions as factors, $F_{7,49}$= 1.92, n.s.). Within PS animals, there were differences between the sexes (P=0.03), PS males spent more time *freezing* or *crouching* than females (Figure 3).

3.2.2. Context-shocks pairing

During the IS session, the fear related to the box increased significantly ($F_{3,49}$= 35.13, p<0.001), and the ANOVA revealed a group effect ($F_{3,49}$=7,8, p=0.002). *Post-hoc* analyses showed the effect of SERT, decreasing the defensive response to footshocks, in both sexes (Figure 3). The animals taking SERT spent less time *crouching* or *freezing* than their control counterparts (p<0.001). Again, differences between sexes were revealed in prenatally stressed animals, with females displaying more time showing DEF responses than males (Figure 3).

3.2.3. Fear Conditioning

In general, the exposure to IS induced a significant increase in the time spent defensive when the animals were re-exposed to the box, in the absence of shocks($F_{1,49}$=65.6, p<0.001).

To explore the effects of the drug and prenatal stress in the acquisition of conditioned fear, sessions were repeated daily and the analyses were performed separately from day 1 to day 2 post-shocks. Contrasting the 1st session in the absence of shocks, the 3-way ANOVA (group*sex*session as factors) revealed differences according to group ($F_{6,98}$= 12.13, p<0.001) and sex ($F_{1,98}$= 4.76, p=0.034) and a group*sex interaction ($F_{6,98}$= 4.48, p=0.007). In both sexes, SERT treatment significantly increased fear towards the box, regardless of the previous stress condition ($F_{3,49}$= 15,4, p<0.001). In males, Post-hoc analyses showed significant differences (p<0.001) in the time the animals spent exhibiting DEF behaviors, when taking SERT, as compared to the non-treated males (Figure 3).

In the 2nd session after shock, the effects of the treatments regarding the fear demonstration depended on subjects' sex ($F_{1,49}$= 7.5, p = 0.008). *Post-hoc* shows sex differences in the response to SERT treatment, with no influence of previous stress (Figure 3), as the females taking SERT began to display less DEF behaviors. Within non-treated animals of both sexes, DEF behaviors did not change, or remained increasing.

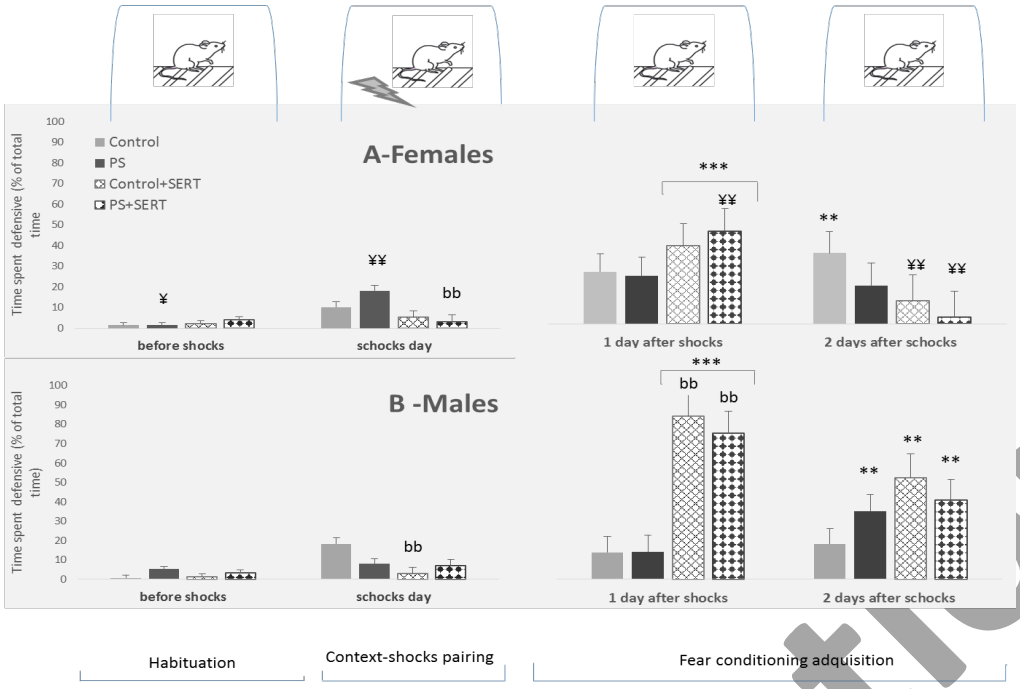

Figure 3. Effects of prenatal stress and SERT treatment (5 mg/kg/day) in the time rats spent defensive (expressed in percentage) all over the contextual fear conditioning paradigm test. bb, $p < 0.01$, indicates the significant differences as effect of SERT; ¥, $p<0.05$ and ¥¥, $p<0.01$, indicate the differences between sexes; **, $p<0.01$, indicates the significant change when related to the session of the footshocks (Mean values ± S.E.M.).

3.2.4. Fear extinction

In the 4th day after the shocks, the 2-way ANOVA revealed no longer differences between groups in DEF behaviors ($F_{3,49}$= 1.2, n.s. in females and $F_{3,49}$= 1.5, n.s., in males). However, when compared on each experimental group, the number of animals that managed to extinguish the fear from the context (less than 10% of time was *defensive)*, differences were found (Figure 4). In overall, SERT helped the animals in the extinction of fear (X^2 = 7.99, df= 3, p=0.046), with the only exception of the PS males, in whose SERT had no effect. In males, the early stress affected the fear extinction, and differences are revealed when PS males are compared to controls (X^2 =7.9, df= 3, p= 0.046), an effect that SERT did not reverse. On the other hand, in females, SERT was the only factor affecting the number of animals that managed to extinguish fear (X^2 = 5.98, df=3, p=0.014), meaning SERT was quite effective, regardless of previous stress.

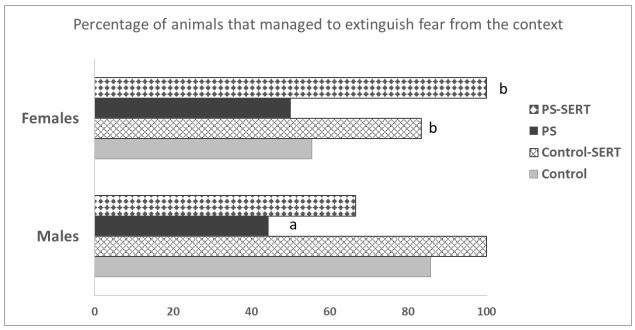

Figure 4. Percentage of animals that managed to extinguish fear from the box. Each bar represents the percentage of animals that exhibited less than 10% of time defensive in the 4th session after footshock in each experimental group. a, $p < 0.05$, indicates the differences as effect of prenatal stress; b, $p < 0.05$, indicates the significant differences as effect of SERT.

3.2.5. Rearings

Before the IS, no differences as effect of the treatments were revealed in the number of rearings (Figure 5). The footshocks exposure induced an overall decrease in the number of rearings in all animals ($F_{3,49}$= 55.1, p<0.001), with sex influence ($F_{1,,49}$= 17.41, $p < 0.001$).

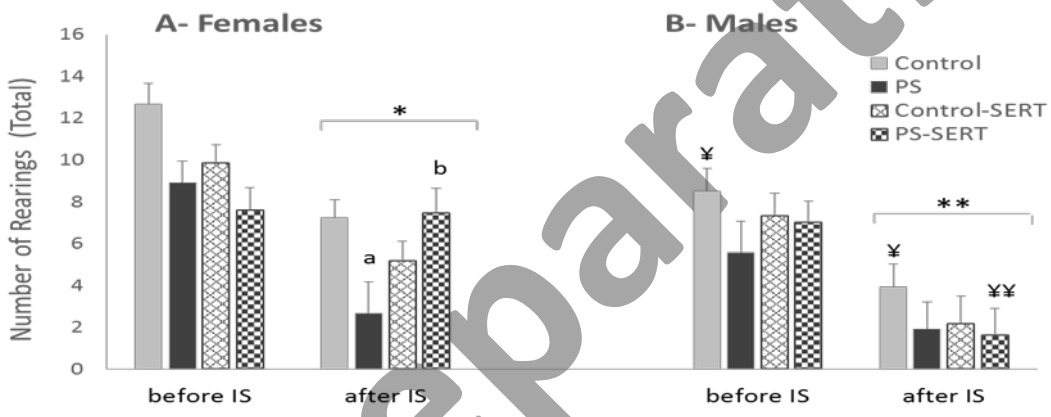

FIGURE 5. Effects of the inescapable shocks (IS) in the number of Rearings on the shuttle box (mean ± S.E.M.). Comparison between females (A) and males (B) from the different experimental groups. Before IS: each bar represents the mean values+ S.E in the two sessions before the inescapable shocks (IS); afer IS: each bar represents the mean values+ S.E. in the three sessions after the inescapable shocks. a, $p < 0.05$, indicates the differences as effect of prenatal stress; b, $p < 0.05$, indicates the differences as effect of SERT in females; ¥, p<0.05, and ¥¥, p<0.01, indicates the differences between sexes; **, p<0.01, indicates the significant change between the sessions before vs. after IS.

The days after the IS, most animals recovered the exploratory will, while the fear of the box decreased (r= -0.41**), however in PS subjects it did not happen (Figure 6 A and B). As a consequence, after the animals have been exposed to the IS, the differences between groups became evidenced, in both sexes ($F_{3,49}$= 3.8, p=0.016; $F_{3,49}$= 3.2, p=0.031 in females and males, respectivelly). Post-hoc analysis shows the effect of prenatal stress affecting the number of rearings, the PS animals of both sexes displayed significantly less rearings than Controls (p<0.05). In females, the SERT treatment reversed the effects of the shocks in prenatally stressed subjects, in PS males it did not happen (Figure 5 and 6).

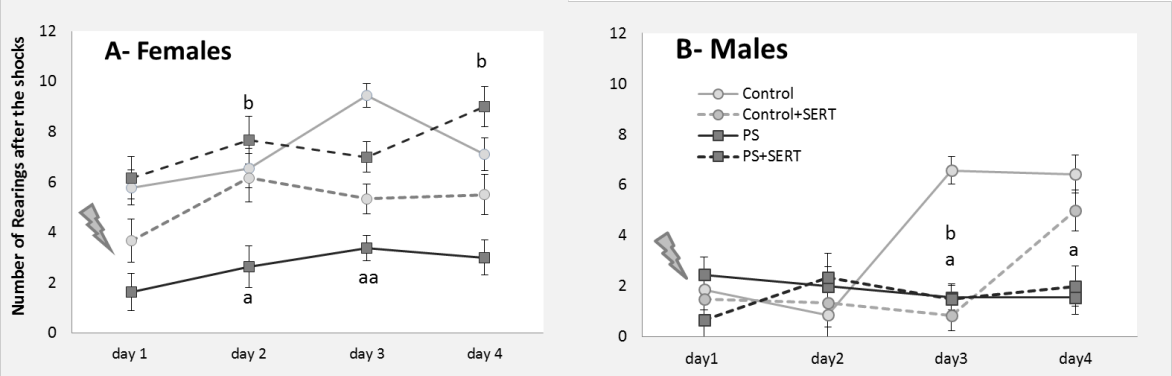

FIGURE 6. Effects of the inescapable shocks (IS) in the number of *r*earings in the shuttle box, all over the sessions after the inescapable shocks (mean ± S.E.M.). Comparison between females (A) and males (B) from the different experimental groups. aa, $p < 0.01$, and a, $p < 0.05$, indicate the differences as effect of prenatal stress; b, $p < 0.05$, indicates the differences as effect of SERT.

3.2.6 Groomings

The number of groomings was affected by the footshock session, ($F_{1.49}$ = 5.45, p = 0.024). When comparing the effects of the footshocks in the different animal groups, post-hoc shows that the reduction in the grooming's behavior reached significance only in PS males (p = 0.012) (data not shown).

3.3. Physiological measures

3.3.1 Changes in the body weight

On the day postnatal 120 (P120) differences in the body weight are revealed (UNIANOVA) in both sexes, due to the treatments the animals experienced ($F_{4,73}$= 4.25, p=0.006). Whereas no differences are revealed among the animals exposed to IS, post-hoc shows, in males, that SERT-taking induced a body weight' loss when compared to the undisturbed controls (No-IS) (p<0.05) (Table 1).

	Females	Males
Experimental Groups		
No-IS	**244.8± 4.5**	**390.2 ± 10.6**
Control	227.1± 4.4	358.2 ± 11.5
Control+SERT	226.3± 4.8	340.6± 10.2 c
PS	**242.9± 4.4**	357.5± 9.3
PS+SERT	224.1± 5.2	335.5± 9.6 cc

Table 1. Body weight at P120 in the animals of both sexes of all the experimental groups when compared with a group of undisturbed animals (NO-IS). Mean values ± S.E. (N = 7-8 per group and sex). cc, p<0.01, and c, p<0.05, indicate the main effect of IS in the males taking SERT (different from No-IS).

3.3.2 ACTH plasmatic values

The ANOVA (with group and sex as factors) revealed that the ACTH levels, after exposure to IMO stress, have been affected by the treatments ($F_{4,62}$= 6.112, p=0.001). In overall, the animals of both sexes, previously submitted to foot-shock exhibited an attenuated plasma ACTH response to re-stress (Figure 7). In females, post-hoc reveals that early stress heightened the effect of the IS, and in both groups of prenatally stressed subjects (PS and PS-SERT), plasma ACTH secretion were significantly lower in response to IMO-stress exposure when compared to the females' group exposed to IMO only (No-IS), an effect that SERT did not reverse (p<0.05).

In males, SERT was the only factor affecting the ACTH response to IMO stress, the males taking SERT exhibited the lowest ACTH levels (regardless of stress) that reached significance when compared to NO-IS males (p<0.05).

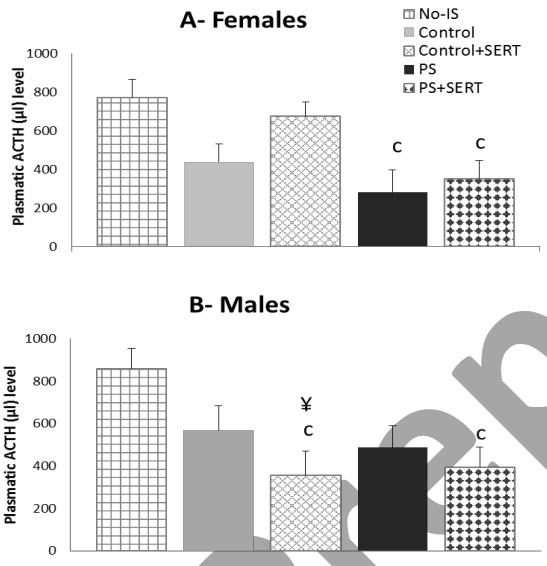

FIGURE 7. Plasma ACTH levels (pg/µl) obtained in the arterial blood in the females (A) and males (B), 10 min after the end of a 30-min exposure to IMO stress. Mean values ± S.E. in the different experimental groups (N = 7 per group and sex). c, p<0.05, indicates the significant differences as effect of the IS; ¥, p < 0.05, indicates the significant differences between males and females.

3.3.3 Hematological Analyses

Experimental groups		Leukocytes (10^3 /µ l)	Erythrocytes (10^6 /µ l)	Hemoglobin (g/dl)	Hematocrit (%)	MCH (pg)	MPV fL(µm^3)
Basal values		*3.3 – 8.7*	*5.5 -9.3*	*10.6 – 15.6*	*32.7 – 44.8*	*15.8-19.9*	*5.4 -9.2*
Females	No-IS	4.9 ± 0.6	7.9 ± 0.2	16.0 ± 0.3	44.9 ± 1.0	19.8 ± 0.2	10.6± 0.2
	Control	3.4 ± 0.6	8.1 ± 0.3	14.6 ± 0.5	41.5± 1.7	18.3 ± 0.7 **c**	8.8 ± 0.4 **c**
	PS	1.4 ± 0.5 **aa**	7.8 ± 0.2	14.3 ± 0.3	44.0± 1.1	18.5 ± 0.4 **cc**	7.5 ± 0.3 **ccc**
	Control+ SERT	5.3 ± 0.3	7.9 ± 0.2	16.0 ± 0.5	43.9 ± 1.7	20.2 ± 0.4 **bb**	11.3 ± 0.4 **bb**
	PS+ SERT	4.1 ± 0.5 **bb**	8.0 ± 0.5	16.1 ± 0.5 **b**	45.2 ± 1.7	20.2 ± 0.4 **b**	10.6 ± 0.5 **bbb**
		F=8.2, p<0.001	**F=0.1, ns**	**F=4.2, p=0.006**	**F=0.95, ns**	**F=7.9, p<0.001**	**F=22.5, p<0.001**
Males	No-IS	5.8 ± 0.3	8.5 ± 0.2 ¥	17.1 ± 0.3 ¥	47.8 ± 1.0 ¥	19.9 ± 0.2	11.0 ± 0.3
	Control	3.1 ± 0.3 **c**	8.1 ± 0.1	15.1 ± 0.5 **c**	42.5 ± 1.7	18.1 ± 0.4 **cc**	7.4 ± 0.4 ¥ **ccc**
	PS	1.9 ± 0.5 **a**	8.6 ± 0.3 ¥	15.3 ± 0.3 ¥ **cc**	44.5 ± 1.0	17.9 ± 0.2 **ccc**	8.2 ± 0.3 **ccc**
	Control+ SERT	4.2 ± 0.3 **b**	8.3 ± 0.3	16.6 ± 0.5	46.7 ± 1.7	20.0 ± 0.4 **b**	10.6 ± 0.4 **bbb**
	PS+ SERT	4.7 ± 0.5 **bb**	8.5 ± 0.3	16.4 ± 0.4	44.4 ± 1.5	19.7 ± 0.4 **bb**	10.7 ± 0.4 **bbb**
		F=7.8, p<0.001	**F=0.5, ns**	**F=6.3, p<0.001**	**F=2.3, p=0.04**	**F=12.5, p<0.001**	**F=22.9, p<0.001**

Table 2. Hematological data: Plasma values obtained in the arterial blood in the animals of both sexes 10 min after the end of a 30-min exposure to IMO stress. Mean values ± S.E. in the different experimental groups (N = 7 per group and sex). Abbreviations: MCH, mean corpuscular hemoglobin; MPV, mean platelet volume. aa, p<0.01 and a, p<0.05, indicate the effect of prenatal stress (different from Controls); bb, p < 0.01 and b, p<0.05, indicate the effect of SERT (different from their control counterparts); ccc, p<0.001; cc, p<0.01; c, p<0.05, indicate a significant difference as effect of the IS (different from No-IS); ¥, p < 0.05, indicates significant differences between males and females.

10 min after the exposure to IMO stress, we found the hematological values of the NO-IS animals were above the basal condition values (table 2). In the animals exposed to a previous stress condition (PS or Controls), the plasma levels of hemoglobin, mean corpuscular hemoglobin (MCH) and clotting factors (represented by the mean platelet volume (MPV)), were significantly lower when compared to the animals not exposed to shocks (NO-IS) (Table 2). Moreover and importantly, the treatment with SERT reversed the stress-induced changes on the hematological parameters, and the animals treated with the drug exhibited hematological values comparable with the No-IS animals. Also, a strong leukopenia was found in prenatally stressed animals from both sexes (table 2), an effect reversed by SERT.

4. Discussion

In the last decades, it was shown that early life stressors, as prenatal stress, can alter the threshold of vulnerability of the brain systems, determining a sort of sensitization to subsequent stressors (Chung et al., 2005; Abe et al., 2007; Cirulli et al., 2009; Green et al., 2011), but some behaviors, even if changed by the PS procedure, are not manifested under low anxiety conditions (Kjær et al., 2010; Pereira-Figueiredo et al., 2014). The main findings of the present study reinforce this idea- whereas previously to footshock exposure, no differences as effect of early stress were observed- after footshock exposure, the PS animals exhibited clear signs of disturb, despite some differences according to sex were evidenced.

The exposure to a session of inescapable shocks (IS), by itself, induced an overall disturb in most of the behavioral and physiological parameters studied, that prenatal stress heightened. Then, after the IS, the PS animals of both sexes exhibited difficulties in getting used to the aversive context and less exploratory motivation; extinction issues in males, and attenuated ACTH stress-induced response in females. These results are in line with what happens in humans with PTSD, where the symptoms of the disease are exacerbated or just revealed in the presence of stressful stimuli (Cohen et al., 2003; Wakizono et al., 2007). However, whether prenatal stress would determine the susceptibility to PTSD-like symptoms, depending on individual's sex, was not still clear.

Our data show that after the exposure to footshock, differences according to sex were also found in SERT effectiveness. SERT taking was quite less effective in males than in females, reversing the effects of PS (restored the fear extinction and the rearings activity). Also, some disturbing signs were evidenced in males due to SERT administration (weight gain and the ACTH levels).

4.1. Previously to IS neither prenatal stress nor SERT induced PPI changes

Neither prenatal stress nor SERT changed the PPI of the startle response**.** Only sex differences in PPI and latency values were found, as expected and previously described (Kumari et al., 2003).

It is assumed that maternal stress lead to increased levels of corticotrophin-releasing hormone reaching the brain of the fetus, which may disturb the development of several systems, such as the dopaminergic mesolimbic system, which is closely related to PPI modulation (Koch, 1999). Nevertheless, in the present study, we did not observe a clear relationship between prenatal stress and PPI levels. Using the same paradigm, we have recently reported that under basal conditions, neither prenatal stress nor SERT treatment induced remarkable alterations in behavior and had no effects on mean startle reflex values (Pereira-Figueiredo et al., 2014). Other authors have also failed to demonstrate changes on PPI modulation when using others SSRIs in non-stressed animals (Jensen et al., 2007); and some researchers have reported inconsistent results in the effects of this type of early stress (Koenig et al., 2005; Kjær et al., 2010.

4.2. The fear-conditioning acquisition

When the animals were re-exposed to the shuttle box previously paired with the electric shocks, they displayed emotional and defensive responses. The experimental box elicited a fear response in all animals, and an overall suppression of the activity, as expected (Cordero et al., 2003; Santos et al., 2006; Baran et al., 2009; Blundell et al., 2011). The animals learned, by contextual conditioning, the box was a hostile environment and adopted the *crouching* or *freezing*, postures requiring the animal to be motionless but alert, as normal defensive behaviors that rodent animals take to be prepared for a possible coup or attack (Blanchard et al., 1991; Hashimoto et al., 1999; Blanchard et al, 2001; Yang et al., 2004; Popa et al., 2008).

For many years, the *freezing* as a conditioned fear response was considered an index of anxiety (Hashimoto et al., 1999). In our work, the context, the shuttle box where animals received the

footshock, did not provide information about the possibility of ending the negative event, and this remained as an eminent threat, thus the *crouching* was the response the most frequently adopted, as previously reported (Yang et al. 2004; Popa et al., 2008). I.e., rats with a mild level of fear associated with a given context may not freeze, but they could limit its locomotor activity and adopt the *crouching* (Blanchard et al., 1991). Such assessment is very informative because of its sensitivity to lower levels of fear, undetected by the response of *freezing*.

Whichever the case, the conditioned fear is a form of learning that depends on hippocampal receptors (Cordero et al., 2003; Sanders et al., 2003) and the amygdala (Swaab et al., 2005), thus, we expected that prenatally stressed animals would have some disturb on fear conditioning acquisition (Griffin et al., 2003). However, in early stressed animals, the fear demonstration was milder and delayed when compared to SERT-taking animals, but did not differ from controls. Also, Abe et al. (2007), found that, despite PS enhanced emotionality, the prenatally stressed male rats showed intact ability to acquire context conditioning. In the present study, SERT was the only factor acting in the fear conditioning. In these animals of both sexes, the demonstration of defensive behaviors has been quicker and more intense than in non-treated animals (regardless of previous stress). This result partly agrees with Santos et al., (2006), whose found that the contextual fear conditioning was sensitive to the subchronic treatment with fluoxetine, another SSRI. Consistent with other authors differences according to sex were also found (Padilla et al., 2009), with males displaying more time *crouching* or *freezing*, except that such differences were mostly found after the footshock and in the animals taking SERT, as consequence to a different response to the drug after the traumatic event. Also, Wilson et al., (2014) reported in male rodents that, despite attenuating PTSD signs, SERT-taking did not diminish anxiety and even increased the noradrenergic response.

4.3. The effects of Prenatal Stress in the number of *rearings* and *groomings*

After the footshock, accompanying the increase of defensive behaviors, a reduction in the exploratory activity and in the number of *groomings* took place. The retraction in these activities, due to the orientation to a higher demand, was expected (i.e. surveillance vs hygiene) (Sanders et al., 2003).

Our data show differences in the number of *rearings* due to early stress. The decrease in the exploratory activity found in PS animals, corresponds to that previously reported with early stressors (Louvart et al. 2005; Abe et al., 2007). On each re-exposition to the box previously paired with the IS, we found that whereas Control animals gradually re-explored the experimental box, the PS animals of both sexes did not retake this activity, as they were unable to get used to the context that was no longer aversive. Our results agree with Louvart et al. (2005), they observed that after exposing female rats to footshocks, PS females exhibited a decrease in the exploratory activity, and lack of motivation. Moreover, our data show that SERT was able to reverse the effect of PS, only in females, in males did not act.

Some differences according to sex were also found, but only in Control subjects. Control males explored less than females (displayed fewer *rearings*). It has already been suggested that males show a strong

tendency towards passiveness, in comparison with females, and are more affected by the IS exposure than females (Kosten et al., 2005; Padilla et al., 2009; Ter Horst et al, 2009). Quite recently we found that males are also more affected by the early stress than females when exposed to an uncontrollable stress (Pereira-Figueiredo et al., 2015), however in the present study no sexual differences are reported in the PS animals.

Together our data show that after the footshock, the PS animals, adopted relatively to the environment a passive strategy, displaying a lack of responses to the environment, not exploring nor taking a defensive behavior, clear signs of lack of motivation. An effect that SERT reversed only in females; disagreeing with previous reports using Fluoxetine in males (Leventopoulos et al., 2009).

4.4. The effects of prenatal stress and SERT on Fear Extinction

On each re-exposition to the shuttle box (the CS) in the absence of the shocks (the US), the ability of this to induce fear gradually becomes diminished, thus any expression of emotionality should to be replaced by the behaviors adopted before the shocks, and the fear is extinguished (Burghardt et al., 2013). In the present study we found the early stress affected the fear extinction, specifically in males. The number of prenatally stressed males that managed to extinguish fear was significantly lower than their control counterparts. Consistent with the present finding, other authors have already reported the difficulty of prenatally stressed animals, of both sexes, on extinguishing fear: in males (Green et al., 2011) and females (Louvart et al, 2005).

The feature of the fear extinction has an extreme importance. Being alert is a protective feature of all organisms, but it support is detrimental to the organism (Cordero et al., 2003; McEwen, 2008). Yet, the fear extinction only occurs if the animals learn that the context previously paired with the aversive event has no longer a threatening value. If the defensive mechanisms are disturbed, they fail to adapt and the fear does not extinguish (Armario et al., 2008; Grissom and Bhatnagar, 2009). Also, in humans, the patients with PTSD exhibit a strong memory of an aversive event that resists extinction (Cohen et al., 2003; Ishii et al., 2010).

In the present study we suggest that SERT helped the animals to become faster accustomed to stress conditions. Our data show that SERT administration helped the fear extinction and reversed the apparent lack of motivation stress-induced, that we found in most animals, except the PS males. According to previous works using this drug (Davidson et al., 2001; Matar et al., 2006), and other SSRIs (Hashimoto et al., 1999; Leventopoulos et al., 2009), it would be expected the SERT to be able to lessen the impact of a traumatic event in rodents. Recently we demonstrated that giving SERT for two months to young rats we could reverse the learned helplessness previously induced by footshocks (Pereira-

Figueiredo et al., 2015), but it did not affect learning and also increased anxiety. However, as far as we know, few studies have examined the effects of antidepressants specifically in the extinction of the aversive memories; and in these studies, differences were reported in the effects of the antidepressants (Melo et al., 2012; Burghardt et al., 2013) and specifically of SERT (Wilson et al., 2014).

4.5. Physiological changes induced by prenatal stress and the inescapable shocks

4.5.1 Weight loss

Previously we reported that from birth, the animals' growth was affected in females by early stress (Pereira-Figueiredo et al., 2014). Several studies show that indicators such as birth weight or length of gestation are associated with alterations in blood pressure, autonomic nervous system and hypothalamic-pituitary-adrenal axis (HPAA) response (Kajantie and Räikkönenc, 2010). In the present study, we found no correlation between these parameters and, at the age of four months, no significant differences in the offspring's body weight, as effect of the prenatal stress were found. SERT was the only factor affecting the body weight, decreasing it significantly, only in males.

4.5.2. HPA changes induced by prenatal stress

The revision of the literature suggests there exist a relationship between early life adverse events and changes in the neuroendocrine stress response (Maccari et al., 2003; Jaferi and Bhatnagar, 2006; Kapoor et al., 2006). The classical phenomenon described is the prolongation of the Glucocorticoids (GCs) secretion. Juvenile rats submitted to PS, have higher levels of CORT and ACTH, and exhibit a potentiated and prolonged release of these hormones in response to aversive experiences when compared to controls (Grissom and Bhatnagar, 2009).

In the present study, our data suggest that exposure to stress during the fetal development affected the HPA function in females. Instead, the PS females previously submitted to IS, displayed the lowest ACTH levels after being exposed to re-stress, possibly leading to an increased feedback of the HPA axis (Viau et al. 1993). Such reversal of the prolongation in the ACTH secretion has already been reported- with some surprise, recently some authors ascertained this same effect in PS animals- in females (Burton et al., 2007; Louvart et al. 2009) and in males (Chung et al., 2005), that were exposed later in life to some trauma. The authors also reported there are sex-differences in the susceptibility of the HPA axis during this stage of early development (Szuran et al., 2000). In fact, a close interaction between the HPA-axis and the hypothalamic-pituitary-gonadal (HPG)-axis exists. Being suggested that such mechanisms may be a basis for the higher prevalence of mood disorders in women as compared to men (Swaab et al., 2005)

In our work, the association of the early stress and the traumatic event (the IS) has been necessary to make such effect to reach significance in females, once among the animals exposed to IS the differences in the ACTH levels were not significant.

Previously Liberzon et al. (1997), and Girotti et al. (2006) using the *stress-restress* paradigm (with different stressors), also found that aversive experiences had effects on the later reactivity of the HPA response to stress in young rats, apparently decreasing it. Contrary to what they supposed, these authors observed that the *stress-restress* paradigm induced structural and functional adaptations in the HPA axis, decreasing the neuroendocrine response or activating the negative feedback. However the same authors suggested that the effects of this *stress-restress* kind was little lasting, disappearing in last few days (Girotti et al., 2006). In the present study, our results reinforce the importance of the

persistence on the effects of prenatal stress- that were still at 120 days old, and this is a key consideration determining the effects of early environment.

Such a change in the ACTH levels in PS females that were later re-exposed to stress, may not reflect a better ability to deal with stress, but yet to be a sign of PTSD (Swaab et al., 2005; Yehuda et al, 2006; Morris et al., 2012; Zoladz et al., 2012). In studies with PTSD patients, other authors (Yehuda et al., 2006; Gunnar and Quevedo, 2007) found low cortisol levels and a faster decline of plasma ACTH levels in response to exogenous GCs, relatively to healthy individuals. According to the literature, such reduction in the levels of GCs and ACTH following a traumatic event should be the result of changes in the adaptation to stress (Jaferi and Bhatnagar, 2006; Louvart et al, 2009; George et al. 2013).

Moreover, our data also show that in males, specifically, the SERT treatment also changed the neuroendocrine response, the males that were taking SERT exhibited the lowest ACTH levels after exposure to re-stress. According to the literature, the antidepressants administration should normalize the activity of the HPA axis (Broadbear et al., 2004), and control the sympathetic drive (Wilson et al., 2014). After been exposed to a stressfull condition, the 5-HT released from serotonergic neurons in the paraventricular nuclei of the hypothalamus (PVN) terminals, will interact with CRH releasing neurons, that once stimulated activate the HPA response. Also Broadbear et al. (2004) reported that the acute administration of 5 mg/kg of SERT activated the HPA axis response, and the effects differed between males and females. These sex differences in response to the treatment with SERT may reflect differences in the HPA serotoninergic activation.

In overall, our data shows evident differences in the SERT effectiveness between sexes, and so in the prenatally stressed males, exposed to footshock, SERT did not act. Apparently, the sex is a great factor acting in the response to psychomotor stimulant drugs (Simpson, 2012) and SSRIs (Leuner et al. 2004), females being often more responsive than males. According to Sloan and Kornstein (2003), in humans, this antidepressant specifically, was more efficient in women than men, and accordingly we reported that in rodents, SERT was again more effective in females than males (Pereira-Figueiredo et al., 2015 $_{b}$), confirming that there are sex- specific differences in the behavioural responses to stress and to antidepressants. The present study suggest these differences are heightened when the animals suffer from some traumatic experience.

4.6. The hematologic response to stress has been affected by the IS

In a previous work (Pereira-Figueiredo et al., 2015$_{a}$) we reported that after being exposed to IMO stress the young rats of our study exhibited a significant increase in hematocrit, hemoglobin and platelet numbers. These physiological changes are of utmost importance to the animal's survival when facing an acute stress condition (Jaferi and Bhatnagar, 2006; Armario et al., 2008).

In the present work, our results show that in those animals that previously to IMO stress, were exposed to footshock, the changes in hematological values were rather moderate. Such effect was heightened in the prenatally stressed animals of both sexes and was fully reversed by SERT treatment.

In humans, PTSD is typically accompanied by acute and chronic alterations in the stress response (de Kloet et al., 2006). The psychiatric research has documented that changes in stress responsiveness, that originates in the LC and ends in the basolateral nucleus of the amygdale, could be the major factor in the pathophysiology of most stress-induced fear-circuitry disorders and specifically in PTSD (Wilson et al., 2014). When facing a potentially threatening stimuli, the LC is activated. In the present study the change in responsiveness to re-stress in the PS animals previously submitted to footshock could be the consequence of a more intense and prolonged discharge of the LC neurons increasing the noradrenergic levels in their brain circuitry. Such a catecholamine increase would over activate by it hand, the sympathetic division of the autonomic nervous system (Swaab et al., 2005; Girotti et al., 2006; Wilson et al., 2014) being able to change the hemodynamic factors in response to the IMO stress (Kopp et al, 2004).

Our data show the chronic treatment with SERT has been able to reverse such a re-stress response impairment. Despite complex, the relationship between the serotoninergic function and the sympathetic adaptation under conditions of stress has already been described (Von Kanel et al., 2004; Zoladz et al. 2012; Zoladz et al. 2013; Pereira-Figueiredo et al., 2015a).

It could be speculated that early stress affecting the foetal development, might have disturbed the functionality of the circuitry between the prefrontal cortex, amygdala and the brainstem nuclei, leading animals to not be able to adapt as well to re-stress conditions. However the sexual differences in such cerebral structures vulnerability to developmental challenges and the treatments to prevent their outcome, must be taken account in further studies.

5. Conclusions

The present study supports the hypothesis that events in the womb are putative biological risk factors for PTSD. The present data seem to indicate that most of the effects of early stress are manifested only if the animals are confronted with adversities in adulthood. Our data evidenced the behavioral differences between males and females in early life susceptibility and in the response to the antidepressant. We found that with the chronic treatment with SERT we were able to reverse the deleterious effects stress-induced in most behavioral and physiological parameters, more efficiently in females than in males.

We hope the present study to help in elucidating the mechanisms and treatments to be followed in the conditions of uncontrolled fear or PTSD in humans, highlighting the differences between sexes.

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