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Facultad de Ciencias

HDAC5 Y SIRT2, DOS DIANAS FARMACOLÓGICAS IMPLICADAS EN EL FENOTIPO DEPRESIVO Y EN LA ACCIÓN ANTIDEPRESIVA

MERCEDES M. ERBURU CALVO

Universidad de Navarra

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Memoria presentada por Dª Mercedes M. Erburu Calvo para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Farmacología y Toxicología y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, octubre de 2016

Dra. Rosa María Tordera Baviera

Dra. Elena Puerta Ruiz de Azúa

Recordarás algo de lo que leas, bastante de lo que oigas,
mucho de lo que veas y todo lo que hagas.

Harrison

A mis padres y hermanos

A ti, Koldo. Y a nuestros peques

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Cuando bebas agua, recuerda la fuente. Proverbio chino

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Por tanto, GRACIAS es lo único que puedo decir.

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Abreviaturas más empleadas

5-HIAA	Ácido 5-hidroxi-indolacético
5-HT	5-Hidroxitriptamina, Serotonina
AcH	Acetylated histone, histona acetilada
ADN	Ácido desoxiribonucleico
AEMPS	Agencia Española de Medicamentos y Productos Sanitarios
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionato
ARN	Ácido ribonucleico
BDNF	Brain-Derived Neurotrophic Factor, Factor neurotrófico derivado del cerebro
CMS	Chronic Mild Stress, estrés crónico suave
CT	Cycle Threshold, umbral de ciclo
CREB	cAMP Response Element-Binding, proteína de unión al elemento de respuesta de AMP cíclico
CSDS	Chronic Social Defeat Stress, estrés crónico social por derrota
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition Manual diagnóstico y estadístico de desordenes mentales, 5ª edición
EPM	Elevated Plus Maze, laberinto elevado
GABA	Gamma-aminobutyric acid, ácido gamma-amino butírico
HAT	Histone acetiltransferase, histona acetiltransferasa
HDAC	Histone deacetylase, histona desacetilasa
HPA	Hipotálamo-Pituitario-Adrenal
IMAO	Inhibidor de la monoaminoaoxidasa
ISRS	Inhibidor selectivo de la recaptación de serotonina
IPA	Ingenuity Pathway Analysis
LIMMA	Linear Model for Microarray Data, modelo lineal para datos de microarray
LTD	Long term depression, depression a largo plazo
LTP	long term potentiation, potenciación a largo plazo
MAO	Monoaminoxidasa

MT	Melatonina
NAD	Nicotinamida Adenina Dinucleótido
NMDA	N-methyl-D-aspartato
PFC	Prefrontal cortex, corteza prefrontal
PMI	*Post mortem* interval, intervalo *postmortem*
NA	Noradrenalina
NAc	Núcleo accumbens
NMDA	N-metil-D-aspartato
OMS	Organización Mundial de la Salud
RT-PCR	Real Time PCR, PCR a tiempo real
SIRT	Sirtuin, sirtuina
SNC	Sistema Nervioso Central
TLDA	Taqman Low Density Array
VGLUT	Vesicular Glutamate Transporter, transportador vesicular de glutamato

CAPÍTULO I

CAPÍTULO 1

Introducción

1. LA DEPRESIÓN

1.1. Definición y epidemiología

Los trastornos afectivos, es decir, aquellos trastornos mentales en los que el humor o la afectividad se ven profundamente alterados, son una de las principales enfermedades neuropsiquiátricas. En concreto, la depresión es el trastorno afectivo más común y una de las causas principales de discapacidad a nivel mundial. Actualmente se estima que afecta a 350 millones de personas y se espera que este número aumente en los próximos años. Esta tendencia se refleja en los últimos estudios epidemiológicos realizados en España; la Encuesta Nacional de Salud del Instituto Nacional de Estadística mostraba, en 2006, que un 11,5% de la población española sufría ansiedad o depresión, proporción que en la encuesta de 2012 ascendió al 14,6%. En la última encuesta publicada, perteneciente al año 2014, se revela que casi 5 millones de personas presentan sintomatología depresiva leve o moderada y otro millón moderadamente grave o grave. En total, más del 15% de la población residente en España presenta sintomatología depresiva de distinta gravedad. Este trastorno es prácticamente el doble de frecuente en mujeres (20,36%) que en hombres (10,63%) en todos sus grados de severidad.

La depresión es un desorden mental crónico caracterizado, principalmente, por una triada de síntomas como son el humor depresivo, la pérdida de interés por estímulos placenteros, también llamada anhedonia, y fatiga o baja energía. Frecuentemente se encuentra asociado a trastornos del sueño, baja autoestima, sentimientos de culpa y tendencias suicidas (Wong y Licinio, 2001).

Según el Manual Diagnóstico y Estadístico de los Trastornos Mentales de la Asociación Psiquiátrica Americana (DSM-5), en los episodios depresivos típicos, el enfermo debe mostrar, al menos, uno de los síntomas centrales de la depresión. Además, otras manifestaciones que, muy frecuentemente, pueden acompañar a los episodios depresivos son:

1. Pérdida o ganancia de peso significativa (p.ej. cambio superior al 5% del peso corporal en un mes)
2. Trastornos del sueño (insomnio o hipersomnia)
3. Agitación o enlentecimiento psicomotor
4. Fatiga o pérdida de energía
5. Sentimiento de inutilidad o culpabilidad excesiva o inapropiada
6. Disminución de la capacidad para pensar, concentrarse o tomar decisiones
7. Pensamientos recurrentes sobre la muerte o ideación suicida

Estos síntomas producen, además del malestar clínico, un deterioro a nivel social, laboral o de otras áreas importantes de funcionamiento.

Para ser diagnosticado de un episodio depresivo, en general, los síntomas deben mantenerse al menos durante dos semanas, aunque períodos más cortos podrían ser aceptados si los síntomas son excepcionalmente graves.

En cuanto a los tipos de depresión, se suelen clasificar según diferentes criterios. Según criterios etiológicos, la depresión puede ser endógena, cuando no se asocia a factores estresantes externos, o reactiva, cuando se conoce el agente desencadenante. Según la sintomatología, puede dividirse en psicótica o neurótica, diferenciadas por la presencia o ausencia de delirios y/o alucinaciones. Basándose en la aparición de la enfermedad, puede ser primaria, si aparece de forma independiente a un cuadro clínico afectivo, o secundaria, cuando se desarrolla a raíz de una alteración médica o psicológica. Teniendo en cuenta la evolución de la enfermedad, puede ser unipolar, cuando únicamente existen episodios depresivos, o bipolar, si se combinan con estados de excitación o manía. Según la duración e intensidad, se clasifica en trastorno depresivo mayor (cumple los criterios diagnósticos del DMS-5), trastorno depresivo recurrente (episodios depresivos, de unos tres meses de duración, alternos con periodos libres de síntomas de al menos un año), trastorno afectivo estacional (episodios depresivos que aparecen en los meses con menos horas de sol), trastorno distímico (síntomas depresivos de intensidad leve que se prolongan durante periodos superiores a dos años) y por último los trastornos adaptativos (síntomas ansiosos y depresivos de carácter leve en respuesta a factores psicosociales identificables).

Como ocurre con otros trastornos neuropsiquiátricos, la depresión tiene una etiología variada; de hecho, se considera que es el resultado de interacciones entre factores genéticos, psicosociales y ambientales. Existen evidencias genéticas que relacionan la depresión con la herencia familiar (Duman y col., 2016). En efecto, hijos de padres con un cuadro depresivo tienen una probabilidad tres veces mayor de desarrollar la enfermedad respecto a aquellos que carecen de estos antecedentes (Wurtman, 2005). También se asocia con alteraciones neuroendocrinas (hipercortisolismo o hipotiroidismo), alteraciones de neurotransmisores, cáncer (como el adenocarcinoma pancreático o tumores de pecho, por ejemplo) o con el consumo de ciertos fármacos (isotretinoina o beta-bloqueantes, entre otros) y drogas de abuso (Nestler y col., 2002; Henry y col., 2008). Sin embargo, es el estrés uno de los principales desencadenantes de la depresión, de manera que se ha llegado a la conclusión de que las personas que padecen episodios de depresión mayor son las que han estado más expuestas a agentes estresantes (Mazure y col., 2000). Por tanto, a pesar de que un trastorno depresivo es diferente a las variaciones del estado de ánimo que surgen

durante la vida (problemas económicos, familiares, sociales), quienes se enfrentan a estas circunstancias estresantes tienen una probabilidad mayor de sufrir depresión. Además, existe una asociación directa entre la severidad y el número de eventos negativos sufridos y la probabilidad de inicio de una depresión (Kendler y col., 2001).

La Organización Mundial de la Salud (OMS) declara que la depresión es una de las principales causas de discapacidad. Esto se debe a que su inicio temprano, su impacto funcional y su tendencia a la cronicidad y a la recurrencia dificultan notablemente la capacidad para afrontar la vida diaria y deterioran tanto el área social como laboral de los enfermos. Es previsible que en el año 2030, la depresión pase a convertirse en la primera causa más común de discapacidad a nivel mundial.

Debido a su alta prevalencia, al coste que origina su tratamiento, a su papel como uno de los principales factores de riesgo de suicidio (unas cincuenta y ocho mil personas se suicidan cada año en la Unión Europea, cifra que supera la de muertes anuales por accidentes de tráfico, homicidios o VIH/SIDA) y a su impacto en la productividad de las personas, la depresión juega un enorme papel económico no sólo en el sistema sanitario sino también en la sociedad.

1.2. Neurobiología de la depresión

Debido al gran impacto de la depresión en la salud y en la sociedad, se han tratado de investigar los factores biológicos que contribuyen a su desarrollo, sin embargo, los estudios no han encontrado una causa que explique el origen del trastorno. Esta falta de información puede ser debida a la complejidad del diagnóstico de la depresión (Schoevers y col., 2008), a la dificultad para observar cambios patológicos en el cerebro y a las limitaciones de las técnicas de estudio *post mortem* y de neuroimagen (Frewen y col., 2008).

Las primeras investigaciones hipotetizaron que un desequilibrio en las monoaminas cerebrales (noradrenalina, serotonina y dopamina) podría contribuir al desarrollo de la enfermedad y que la acción antidepresiva podría deberse a un aumento en la neurotransmisión monoaminérgica (Duman y col., 1997; Manji y col., 2001; Nestler y col., 2002). Los avances en las técnicas moleculares y en neuroimagen han intentado esclarecer los mecanismos relacionados con la depresión. Actualmente se piensa que son varios los aspectos fisiológicos implicados: cambios en estructuras cerebrales y en su funcionamiento; participación de las monoaminas (metabolismo, el papel de sus trasportadores y sus polimorfismos); implicación de neurotransmisores como el glutamato y el ácido amino-γ-butírico (GABA); el papel de la neurogénesis; la interacción entre la vulnerabilidad genética y el ambiente y el papel de la inflamación, entre otros.

Aunque algunos de estos mecanismos se encuentran interrelacionados, por el momento no existe una explicación que logre unificarlos, por lo que parece que la depresión mayor es un trastorno heterogéneo. Se describen, a continuación, las teorías más relevantes acerca de la neurobiología de la depresión.

1.2.1. Hipótesis monoaminérgica

La hipótesis de las monoaminas, propuesta por Schildkraut en 1965, sugiere que los trastornos depresivos son el resultado de deficiencias en las funciones monoaminérgicas en áreas límbicas del cerebro (Schildkraut, 1965). Esta teoría se desarrolló a partir de asociaciones observadas entre los efectos clínicos de varios fármacos que alivian los síntomas depresivos y los efectos neuroquímicos sobre la transmisión cerebral monoaminérgica (aumento de las monoaminas en la hendidura sináptica).

No obstante, diversos hallazgos sugieren que no es posible explicar el desarrollo de la depresión mediante, exclusivamente, la deficiencia de aminas biógenas. En primer lugar, los antidepresivos tradicionales no son efectivos en aproximadamente un 30-40% de los pacientes con depresión mayor (Schatzberg, 2000) y su efecto terapéutico no comienza hasta al cabo de varias semanas de tratamiento, a pesar del aumento inmediato de los niveles de monoaminas. En segundo lugar, la depleción experimental de monoaminas produce un empeoramiento moderado del humor en pacientes deprimidos no tratados, pero no afecta en absoluto a los controles sanos (Charney, 1998). En tercer lugar, se ha visto que drogas que aumentan los niveles de monoaminas como la cocaína o las anfetaminas no mejoran los síntomas depresivos.

Teniendo en cuenta que son necesarias de dos a tres semanas de tratamiento antidepresivo para obtener el efecto terapéutico, la investigación experimental actual se centra en el estudio de los cambios bioquímicos que ocurren en el cerebro después del tratamiento crónico con el antidepresivo. De aquí nace la hipótesis de que, como consecuencia de una activación persistente de los mismos por el aumento de los niveles de serotonina y noradrenalina en la hendidura sináptica, es necesaria una adaptación de los receptores a largo plazo. Por ejemplo, se ha observado que el tratamiento crónico con antidepresivos, desensibiliza los receptores 5-HT_{1A}, 5-HT_2 y α_2-adrenérgicos, fenómeno coincidente en el tiempo con el inicio del efecto terapéutico del antidepresivo.

A pesar de que la hipótesis monoaminérgica no proporciona una explicación suficiente de la depresión, actualmente, la estrategia terapéutica de primera elección del tratamiento antidepresivo sigue siendo el aumento de la transmisión noradrenérgica y serotonérgica.

1.2.2. Hipótesis glutamatérgica/GABAérgica

En los últimos años han surgido teorías que sugieren que alteraciones en los neurotransmisores del sistema nervioso central, glutamato y GABA, pueden contribuir al desarrollo de la depresión (Brambilla y col., 2003; Chiapponi y col., 2016; Ren y col., 2016; Rubio-Casillas y Fernández-Guasti, 2016). GABA es el principal neurotransmisor inhibitorio del cerebro, mientras que glutamato es el neurotransmisor excitatorio. El balance entre los niveles de glutamato y GABA, así como entre el número de sus contactos sinápticos es esencial para un funcionamiento normal del cerebro.

La deficiencia de GABA se ha propuesto como un modelo de ansiedad y depresión. Se ha observado que los niveles de GABA en plasma y en fluido cerebroespinal son inferiores en personas con depresión (Roy y col., 1991; Petty, 1994; Brambilla y col., 2003; Sanacora y Saricicek, 2007). Además, en pacientes deprimidos se ha observado un déficit cortical de GABA, que conllevaría un desequilibrio excitatorio-inhibitorio (Sanacora y col., 2004; Bhagwagar y col., 2007).

Otros estudios *post mortem* muestran una pérdida de interneuronas GABAérgicas en la corteza prefrontal dorsal lateral de pacientes con depresión (Maciag y col., 2010) y una disminución de la ácido glutámico descarboxilasa (GAD67) (Karolewicz y col., 2010).

Como ocurre con GABA, hay algunos estudios que sugieren que el sistema glutamatérgico, especialmente alteraciones en los receptores N-methyl-D-aspartato (NMDA), contribuye a la fisiopatología de la depresión (Petrie y col., 2000; Pfleiderer y col., 2003; Serafini y col., 2013). En este sentido, se ha sugerido que la modulación de receptores glutamatérgicos puede facilitar la neurogénesis y la liberación de neurotransmisores asociados con la respuesta al tratamiento (Rubio-Casillas y Fernández-Guasti, 2016). En línea con esta hipótesis, antagonistas de receptores NMDA han demostrado actividad antidepresiva en estudios preclínicos (Skolnick y col., 2009). En concreto, la ketamina induce una respuesta antidepresiva rápida, efecto que parece ser mediado por la activación de diversas proteínas intracelulares como mTOR (Li y col., 2010), BDNF (Autry y col., 2011) y los receptores glutamatérgicos AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionato) (Koike y col., 2011; Choi y col., 2016).

1.2.3. Alteraciones de la neuroplasticidad

La neuroplasticidad abarca diversos procesos de vital importancia mediante los que el cerebro percibe, se adapta y responde a una gran variedad de estímulos internos y externos. La OMS, en 1982, definió el término neuroplasticidad como la capacidad de las células del

sistema nervioso para regenerarse, anatómica y funcionalmente, después de estar sujetas a influencias patológicas ambientales o del desarrollo, comprendiendo traumatismos y enfermedades.

Las manifestaciones de neuroplasticidad en el sistema nervioso central (SNC) adulto incluyen alteraciones en la función dendrítica, remodelación sináptica, procesos implicados en aprendizaje y memoria como la potenciación sináptica a largo plazo (LTP; long term potentation) y la depresión sináptica a largo plazo (LTD; long term depression), ramificación axonal, extensión neuronal, sinaptogénesis y neurogénesis (Mesulam y col., 1999). La disfunción de estos procesos fundamentales podría contribuir a la fisiopatología de los desórdenes del humor, y el restablecimiento de los mismos podría relacionarse con la recuperación de una plasticidad adecuada. Se postula que la depresión podría ser el resultado de una incapacidad para adaptarse a factores ambientales adversos, consecuencia de una disfunción de los mecanismos anormales de neuroplasticidad (Duman y col., 1999; 2016; Gerhard y col., 2016).

Las alteraciones de la neuroplasticidad se pueden diferenciar en tres tipos: alteraciones estructurales, hipótesis neurogénica e hipótesis neurotrófica, que se describen a continuación.

Respecto a las **alteraciones estructurales**, numerosos estudios de neuroimagen e histopatológicos de varias regiones cerebrales de individuos con trastornos del estado de ánimo sugieren que la depresión se acompaña de cambios morfológicos tanto a nivel histológico como macroanatómico.

La depresión mayor se asocia con una disminución de células gliales (Cotter y col., 2001; Bowley y col., 2002; Rajkowska y Miguel-Hidalgo, 2007), una reducción en el volumen de materia gris y blanca en la corteza prefrontal (Soares y Mann, 1997) y una disminución del tamaño del hipocampo (Sheline y col., 1996) y de la amígdala (Sheline y col., 1998).

Diversos estudios experimentales han observado cómo modelos de depresión basados en estrés crónico suave producen una gran variedad de cambios estructurales en corteza prefrontal, hipocampo y amígdala. En la corteza, una exposición repetida a estrés causa retracción sináptica, pérdida de espinas en las neuronas piramidales y un aumento de la apoptosis (Michelsen y col., 2007; Bachis y col., 2008). En cuanto al hipocampo, el estrés disminuye su volumen (Czeh y Lucassen, 2007) y la remodelación de dendritas (Bessa y col., 2009) y aumenta la apoptosis celular (Czeh y Lucassen, 2007). En la amígdala se produce una hipertrofia dendrítica (Vyas y col., 2004). Estos cambios en las estructuras cerebrales indican que se está produciendo una reorganización de las redes neuronales.

Teniendo en cuenta que el citoesqueleto tiene un papel primordial en la plasticidad sináptica, numerosas evidencias sugieren que esta estructura celular podría ser un blanco terapéutico en el tratamiento de la depresión, para restablecer las dendritas y los axones perdidos y la conectividad sináptica. Estas alteraciones anatómicas se han establecido a partir de estudios histológicos *post mortem* de pacientes que habían padecido depresión. En éstos se observó una disminución del tamaño de las neuronas en la corteza prefrontal, en particular una disminución en la longitud de las dendritas y en el número de las espinas dendríticas (Rosoklija y col., 2000; Chana y col., 2003). Además, recientemente, estudios de imagen y de tejido cerebral humano *post mortem* han revelado alteraciones en la sustancia blanca y en los oligodendrocitos de los pacientes con depresión (Tham y col., 2011). Curiosamente, roedores sometidos a estrés también presentan una disminución en el número de oligodendrocitos a nivel cortical y de la amígdala (Banasr y col., 2007; Czeh y col., 2007), lo que sugiere que existe una relación entre alteraciones en la mielinización y los trastornos del estado del ánimo (Miyata y col., 2016).

La **hipótesis neurogénica** de la depresión propone que una disminución de la neurogénesis podría tener un papel relevante en la patogénesis de la enfermedad. Así, existen numerosos estudios que muestran una reducción de la neurogénesis hipocampal en distintos modelos animales de depresión y una estimulación de la misma tras el tratamiento con diversos tipos de antidepresivos (Malberg y col., 2000; Kempermann, 2002; Kempermann y Kronenberg, 2003; Alonso y col., 2004; Jayatissa y col, 2006; Kronenberg y col., 2009). La exposición a distintos agentes estresantes también disminuye la proliferación celular y la neurogénesis (Gould y col., 1997; Dranovsky y Hen, 2006; Mineur y col., 2007), lo cual lleva a proponer que el estrés crónico podría precipitar episodios depresivos mediante la alteración de la neurogénesis hipocampal.

No obstante, la implicación de la neurogénesis en la precipitación del comportamiento depresivo, está siendo últimamente cuestionada. En los últimos años se ha postulado que la disminución de este proceso no es esencial para que se produzca la sintomatología depresiva (Vollmayr y col., 2003; Sapolsky, 2004; Jayatissa y col., 2009). Por un lado, los estudios clínicos llevados a cabo con pacientes deprimidos no muestran cambios en la proliferación celular (Reif y col., 2006). En roedores, la inhibición de la neurogénesis mediante rayos X (Surget y col., 2008) no parece afectar al fenotipo conductual depresivo y, de la misma manera, la conducta depresiva puede inducirse sin necesidad de una disminución en la proliferación celular (Vollmayr y col., 2003). Además, cada vez hay un mayor número de trabajos que consideran que el aumento de la neurogénesis no es imprescindible para que se produzca la mejoría de la sintomatología depresiva (Holick y col., 2008; Huang y col., 2008; Bessa y col., 2009; David y col., 2009). A su vez, hay que

considerar que para que el incremento de la neurogénesis sea beneficioso, las nuevas neuronas generadas en el hipocampo deben de integrarse adecuadamente en las redes neuronales existentes con una apropiada diferenciación y migración (Scharfman y Hen, 2007; Pechnick y Chesnokova, 2009).

Por último, la **hipótesis neurotrófica** de la depresión se basa en numerosos estudios que han correlacionado descensos del factor neurotrófico derivado de cerebro (BDNF), en el hipocampo y en la corteza prefrontal, con comportamientos depresivos (Karege y col., 2005). Asimismo, el tratamiento antidepresivo aumenta la expresión de este factor neurotrófico (Duman y Monteggia, 2006; Castrén y Rantamäki, 2010).

Los pacientes con depresión mayor tienen niveles disminuidos de BDNF en cerebro (Altar y col., 2009) y suero (Cunha y col., 2006; Sen y col., 2008). Es más, se ha observado una correlación entre la severidad del estado depresivo y los niveles en plasma de BDNF (Duncan y col., 2009). Se ha sugerido que el retraso del efecto antidepresivo se debe al tiempo necesario para producir mecanismos neuroadaptativos que puedan mejorar la plasticidad neuronal (Kozisek y col., 2008; Pittenger y Duman, 2008). En línea con esta hipótesis, varias investigaciones han demostrado que el BDNF podría mediar la acción terapéutica de los antidepresivos (Berton y Nestler, 2006; Groves, 2007; Martinowich y col., 2008). Diversos estudios documentan que los tratamientos crónicos antidepresivos, incluyendo los inhibidores selectivos de la recaptación de serotonina y el choque electroconvulsivo, aumentan la expresión de BDNF en el hipocampo y en la corteza en modelos animales (Nibuya y col., 1995; Russo-Neustadt y col., 2003; Castren y col., 2007). En línea con esta hipótesis, mediante la utilización de ratones deficientes de BDNF, como modelo de depresión, se ha observado que la infusión directa de BNDF en el cerebro produce un efecto antidepresivo y potencia la eficacia del tratamiento antidepresivo, mientras que el bloqueo de la señalización de BDNF impide el efecto de los fármacos antidepresivos (Castrén y col., 2010; Lindholm y Castrén, 2014).

Asimismo, en modelos animales, el estrés desciende los niveles de BDNF en estructuras límbicas, esenciales para el control del humor, y el tratamiento antidepresivo revierte o bloquea estos efectos (Nestler y col., 2002; Duman y Monteggia, 2006),

Dada la estrecha relación entre BDNF y la proteína de unión al elemento de respuesta de AMP cíclico (cAMP response element-binding, CREB), que actúa como factor de transcripción, parece que éste también está implicado tanto en el mecanismo de acción de los antidepresivos, como en la propia enfermedad (Banasr y col., 2004; Warner-Schmidt y Duman, 2006; Im y Kenny, 2012). Estudios *post mortem* en la corteza temporal de

pacientes con depresión mayor tratados con antidepresivos mostraron un aumento de los niveles de CREB, mientras que los no tratados presentaban una disminución de éstos (Dowlatshahi y col., 1998; Yamada y col., 2003).

Estudios en animales muestran que los roedores que sobreexpresan CREB en el giro dentado del hipocampo tienen, en el test de natación forzada, una conducta equiparable a ratones tratados con antidepresivos (Chen y col., 2001). Asímismo, el efecto antidepresivo de algunos compuestos, como el resveratrol, se ha asociado a aumentos en los niveles de CREB en la corteza frontal (Ge y col., 2015; Xu y col., 2016). Curiosamente, la función de CREB no es igual en todas las áreas cerebrales, ya que la sobreexpresión de CREB en la amígdala basolateral o en el núcleo accumbens produjeron respuestas tipo pro-depresivas en los modelos de indefensión aprendida (Pliakas y col., 2001) y de natación forzada (Wallace y col., 2004).

1.2.4. Otras hipótesis

En los últimos años se han propuesto nuevas teorías para explicar la depresión y su biología. Entre ellas se encuentran la hipótesis neuroendocrina, la hipótesis inflamatoria y el papel de la microbiota intestinal.

Respecto a la **hipótesis neuroendocrina**, existen numerosas investigaciones que sugieren que la depresión mayor se asocia a una inadecuada respuesta al estrés, debido a una disfunción del eje hipotálamo-pituitario-adrenal (HPA) (Watson y col, 2002; Ströhle y Holsboer, 2003; Holsen y col., 2013).

En los trastornos depresivos se produce una hipersecreción del factor liberador de corticotropina (Raison y Miller, 2003), un aumento de la respuesta adrenal a la hormona corticotropina (ACTH) (Parker y col., 2003) y, en consecuencia, una hipercortisolemia derivada del deterioro en la retroalimentación negativa mediada por receptores de glucocorticoides (Brown y col., 2004).

Los niveles elevados de glucocorticoides interfieren en la neurogénesis hipocampal contribuyendo al desarrollo de la enfermedad. Esto concuerda con estudios que muestran que la kinasa 1 inducida por suero y glucocorticoides (del inglés, *serum- and glucocorticoid-inducible-kinase 1* (SGK1)), que inhibe la neurogénesis hipocampal, está aumentada en pacientes deprimidos, así como en modelos animales de depresión (Anacker y col., 2013). Además, hay evidencias que sugieren que los corticoesteroides modifican la función de BDNF, lo cual indicaría su posible implicación en la patogénesis de la depresión (Kumamaru, 2008).

La **hipótesis inflamatoria** sugiere que existe una implicación del sistema inmunitario en el trastorno depresivo (Raison y col., 2006; Maes, 2011; Makhija y Karunakaran, 2013). Los pacientes deprimidos tienen niveles aumentados de glucocorticoides, potentes antiinflamatorios, sin embargo, se observa que presentan niveles aumentados de citoquinas proinflamatorias, incluyendo interleucina-1, interleucina-6, factor de necrosis tumoral alfa y algunos receptores del interleucina soluble (O´Brien y col., 2004; Dowlati y col., 2010; Liu y col., 2012). Las citoquinas proinflamatorias no sólo participan en la respuesta inmune innata y la inflamación sino que también tienen importantes efectos metabólicos y endocrinos, incluyendo el metabolismo de neurotransmisores, función neuroendocrina y neuroplasticidad neuronal.

Actualmente, están surgiendo estudios que indican que funciones cerebrales superiores, como el humor o la memoria, y el aparato digestivo están muy relacionados (Hoban y col., 2016). De acuerdo con esta teoría, la **microbiota intestinal** podría tener una influencia a nivel cerebral y, por tanto, en el comportamiento. En investigaciones con animales se han encontrado diferencias en la composición de la microbiota intestinal entre animales deprimidos y sanos (Dinan y Cryan, 2013). Péptidos producidos a nivel gastrointestinal como la leptina, ghrelina y colecistoquinina (Becker y col., 2008; Kluge y col., 2011; Häfner y col., 2012), entre otros, tienen una influencia directa en el sistema nervioso central, incluyendo la neurogénesis, que podría estar implicada en el trastorno depresivo (Villanueva, 2013).

A pesar de los numerosos estudios que apoyan la validez de cada una de las hipótesis recién planteadas, en la actualidad aún no hay una explicación común que las aúne a todas y reconcilie las distintas teorías. Esto pone de manifiesto la necesidad de seguir profundizando en los mecanismos moleculares implicados en el desarrollo del trastorno depresivo. En este sentido, la ausencia de claros efectos genéticos en la depresión, junto con el hecho de que numerosos factores ambientales pueden modificar de manera más o menos estable la expresión génica a través de modificaciones epigenéticas, sin necesidad de alterar la secuencia del ADN, apoyan el concepto de que los **factores de riesgo biológicos de la depresión** sean, en su base, **epigenéticos** (Oh y col., 2008). Además, dichas modificaciones epigenéticas podrían ser la base para la enorme variabilidad interindividual hacia la adversidad o la respuesta al tratamiento (Wilkinson y col., 2009; Duman y col., 2016).

En línea con las hipótesis anteriormente propuestas, evidencias crecientes sugieren que los mecanismos epigenéticos juegan un papel importante en la neuroplasticidad y median cambios funcionales estables en el cerebro en respuesta a estímulos ambientales

(Dulac, 2010). Específicamente, cambios epigenéticos inducidos por estrés en regiones límbicas del cerebro podrían persistir durante mucho tiempo y contribuir a la patogénesis de la depresión y de otras enfermedades como la ansiedad generalizada o el estrés postraumático (Golden y col., 2013). Igualmente, la terapia antidepresiva podría ser alcanzada en parte por mecanismos epigenéticos (Tsankova y col., 2006).

1.3. Epigenética, depresión y tratamiento antidepresivo

Históricamente, la palabra "Epigenética" se ha utilizado para describir aquellos acontecimientos que no podían ser explicados por los principios de la Genética. En 1942, Conrad Waddington acuñó el término Epigenética como "la rama de la Biología que estudia las interacciones causales entre los genes y sus productos, las cuales dan lugar al fenotipo". Una definición más actual define la Epigenética como el estudio de las modificaciones estables y heredables de la cromatina que ocurren sin cambios en la secuencia de ADN y que ayudan a determinar los rasgos fenotípicos de las células durante el desarrollo (Riccio, 2010).

Actualmente, se conocen varios mecanismos epigenéticos, de los cuales la metilación del ADN, la modificación de las histonas y la acción de ARN no codificante, son los más estudiados. **La metilación del ADN** es el mecanismo epigenético más estudiado. Consiste en la adición de un grupo metilo en el carbono 5 de aquellas moléculas de citosina que están seguidas de guanina, es decir, en los dinucleótidos CpG. La principal consecuencia de la metilación del ADN es que se produce una represión de la transcripción, dando lugar a una menor expresión o silenciamiento del gen (Phillips, 2008). A su vez, una menor metilación suele asociarse a una mayor activación en la transcripción del gen (Prokhortchouk y Defossez, 2008). En cuanto a la **modificación de histonas**, éstas tienen un papel clave en la regulación de la expresión génica. Las histonas que forman parte del nucleosoma están sujetas a modificaciones postranscripcionales, en particular en su cola N-terminal: acetilación, metilación, fosforilación, ubiquitinación, sumoilación y ADP-ribosilación, entre otros (Karlic y col., 2010); y estos cambios son tan importantes que pueden ser utilizados como predictores de la expresión génica (Figura 1).

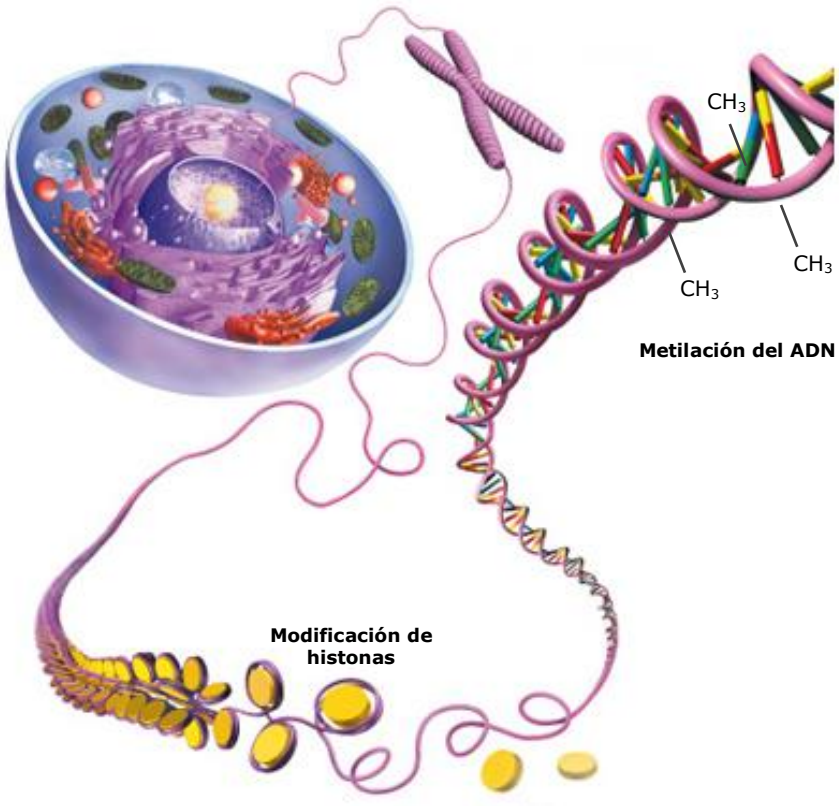

Figura 1. Representación gráfica de dos mecanismos epigenéticos que modifican la expresión génica: la metilación del ADN y la modificación de histonas. (Adaptado de Chowdhury y Dutta. Academic Publishers)

En base a estudios recientes (Covington y col., 2009; 2015; Nestler y col., 2015), de todos los mecanismos epigenéticos mencionados, el presente trabajo se ha centrado en estudiar la implicación de la modificación de histonas, en concreto su acetilación, en la depresión, por lo que es este aspecto el que se va a desarrollar con más profundidad:

La cromatina es el complejo de ADN, formado por histonas y otras proteínas, que se encuentra en el núcleo celular. La remodelación de la cromatina es un proceso dinámico que modula la expresión génica. La unidad fundamental de la cromatina es el nucleosoma, que contiene, aproximadamente, 147 pares de bases de ADN enrollados alrededor de un octámero de histonas. Este octámero está compuesto de dos copias de las histonas H2A, H2B, H3 y H4 (Kouzarides, 2007).

Los mecanismos de remodelación de la cromatina permiten que el ADN permanezca accesible, o no, a la maquinaria transcripcional, facilitando o inhibiendo la transcripción génica. Es decir, la cromatina existe en un estado condensado inactivado, heterocromatina, que impide la transcripción génica, y en un estado activo abierto, eucromatina, que permite que los genes sean transcritos. Esto tiene lugar a través de las modificaciones epigenéticas

que ocurren en cada una de las colas N-terminal que se extienden más allá del nucleosoma, principalmente en los N-terminales de H3 y H4. Estas modificaciones incluyen la acetilación, metilación, fosforilación, ubiquitinación, sumoilación y ADP-ribosilación (Karlic y col., 2010). La modificación mejor estudiada es la acetilación de residuos de lisina, proceso llevado a cabo por las histona acetiltransferasas (Hat) y las histona desacetilasas (Hdac).

Las Hats son enzimas que acetilan los residuos de lisina de las histonas a través de una transferencia de un grupo acetilo desde una molécula de acetil-CoA, formando ε-N-acetil lisina, lo cual desenrolla la conformación ADN-histonas, permitiendo que los factores de transcripción interactúen con el ADN, facilitando la expresión génica. En cambio, las Hdacs tienen como función la eliminación de grupos acetilo de los residuos de arginina/lisina de la cola N-terminal de las histonas y otras proteínas (Strahl y Allis, 2000). Esta desacetilación da lugar, en general, a un estado de la cromatina más compacto y, por tanto, a un silenciamiento de genes (Figura 2).

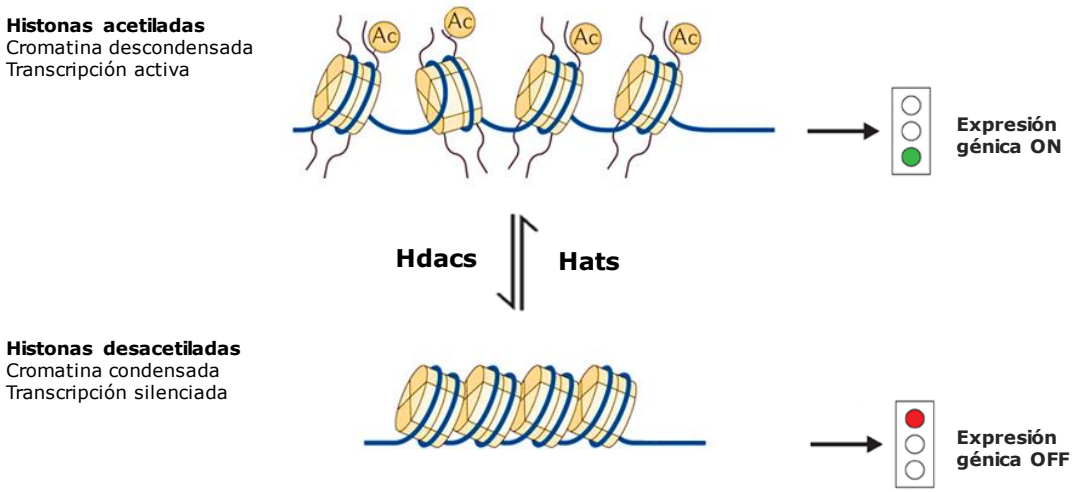

Figura 2. Imagen ilustrativa del mecanismo de acción las histona acetiltransferasas (Hat) y las histona desacetilasas (Hdac) para activar o inhibir la transcripción génica (Adaptado de Verdin y col., 2014).

En comparación con las Hats, las Hdacs tienen una diversidad estructural y funcional más rica, lo cual hace que su estudio sea una interesante herramienta en la investigación de nuevos fármacos y terapias (Abel y Zukin, 2008).

Las Hdacs pertenecen a una familia, conservada evolutivamente, que se divide en cuatro clases (Haberland y col., 2009). Las clases I, II y IV son similares en cuanto a que requieren Zn^{2+} como cofactor (de Ruijter y col. 2003), mientras que la clase III necesita nicotinamida adenina dinucleótido (NAD^{+}) (Sauve y col., 2006).

La clase I (HDACs 1, 2, 3 y 8) se encuentra casi exclusivamente en el núcleo de la célula y, con excepción de HDAC8 que es específica del músculo, el resto se expresa ampliamente en el cerebro (Waltregny y col., 2004; Kazantsev y Thompson, 2008). La clase II se divide, basándonos en parámetros estructurales, en dos subclases: clase IIa (HDACs 4, 5, 7 y 9) y clase IIb (HDACs 6 y 10). HDAC6 se encuentra, predominantemente, en el núcleo, mientras que el resto de HDACs se mueven entre el núcleo y el citoplasma, a través de un mecanismo regulado por fosforilación vía kinasa, dependiente de calcio-calmodulina (Gregoretti y col., 2004). Los miembros de ambas subclases muestran una expresión a nivel celular y tisular específica, pero todos ellos se expresan en el cerebro (Guedes-Dias y Oliveira, 2013). Las Hdacs de clase III, también llamadas sirtuinas, están formadas por siete sirtuinas (SIRT1-SIRT7) y se encuentran ampliamente presentes en todos los compartimentos de la célula y en el organismo (Michán y Sinclair, 2007). Todas ellas tienen localización en el cerebro (Frye, 2000). SIRT 1, 2, 6, y 7 se encuentran en el citoplasma y el núcleo, mientras que SIRT 3, 4 y 5 tienen localización mitocondrial (Michishita y col., 2005; Ramadori y col., 2008). La clase IV consta, únicamente, de HDAC11. Se encuentra, principalmente, en el núcleo. No se conoce mucho acerca de ella excepto que se expresa durante el desarrollo del SNC y, posiblemente, tenga un papel en la inflamación, a través de su efecto inhibitorio sobre la expresión de interleucina 10 (Liu y col., 2008; Villagra y col., 2009).

Por tanto, la mayor parte de las histonas desacetilasas se expresan a nivel del sistema nervioso, y tienen un papel esencial en su desarrollo. La correcta remodelación de la cromatina durante la embriogénesis es crucial para el cerebro y cualquier desregulación de estos mecanismos epigenéticos tiene consecuencias dramáticas, como se ha demostrado en varios estudios que relacionan diferentes enfermedades del desarrollo con una remodelación de la cromatina anormal (Ausio y col., 2003; Levenson y Sweatt, 2005). Aparte de su papel en el desarrollo neurológico, los mecanismos epigenéticos son esenciales para el cerebro adulto, con funciones relacionadas con la homeostasis, neurogénesis, diferenciación, neuroprotección o plasticidad neuronal, entre otros. De acuerdo con esto, las alteraciones en la función de diferentes factores epigenéticos se ha relacionado con alteraciones neurobiológicas observadas en algunas enfermedades psiquiátricas y neurodegenerativas (Tsankova y col., 2006)

Concretamente, la acetilación de histonas se ha relacionado con trastornos afectivos como depresión o ansiedad. Se ha observado que el estrés puede ser un factor ambiental que produce cambios en la acetilación de histonas mediada por Hdacs, de manera que estas enzimas pueden relacionarse con la patogénesis de la depresión (Golden y col., 2013). Por ejemplo, estudios llevados a cabo con ratas estresadas crónicamente muestran cambios en

la acetilación y metilación de la histona 3 y 4, y estudios recientes indican que los efectos terapéuticos de la imipramina podrían estar causados, al menos en parte, por la inhibición de HDAC5, una histona desacetilasa que reprime, entre otras, la expresión de la neurotrofina BDNF (Tsankova y col., 2006).

Respecto a las Hdac de clase III, estudios recientes han sugerido que las sirtuinas tienen un papel importante en la fisiopatología y el tratamiento de la depresión. Varios estudios genéticos sugieren que el gen *SIRT1* está asociado con los trastornos depresivos y de ansiedad (Kishi y col., 2010; Libert y col., 2011; CONVERGE Consortium, 2015; Kim y col., 2016) y se ha observado una disminución del ARNm de *SIRT1*, *SIRT2*, y *SIRT6* en pacientes con depresión y trastorno bipolar (Abe y col., 2011). Sin embargo, estudios en animales han dado lugar a resultados contradictorios: por un lado, ratones knock-out para SIRT1 específicamente en el núcleo accumbens mostraron una reducción de la ansiedad y un fenotipo resistente al estrés (Kim y col., 2016), mientras que los ratones que sobreexpresaban SIRT1 en todo el organismo presentaban un aumento de la ansiedad y mayor vulnerabilidad al estrés (Libert y col., 2011). Por el contrario, otros estudios muestran que la sobreexpresión de SIRT1 exclusivamente en el cerebro no presenta alteraciones conductuales significativas (Watanabe y col., 2014). Además, aunque la exposición al estrés crónico aumenta la actividad de SIRT1 en roedores (Ferland y Schrader, 2011; Ferland y col., 2013; Kim y col., 2016), el efecto antidepresivo del resveratrol, un activador de dicha enzima, ha sido demostrado por varios estudios recientes (Ge y col., 2013; Hurley y col., 2014; Liu y col., 2014). Estos resultados sugieren que la implicación de las sirtuinas en los trastornos depresivos depende del tipo celular, las características genéticas, y la región del cerebro analizada, por lo que son necesarios más estudios para determinar el papel específico y la responsabilidad de las diferentes sirtuinas en la etiología de la depresión y de la ansiedad (Abe-Higuchi y col., 2016).

1.4. Tratamiento farmacológico de la depresión

Una vez diagnosticada la depresión, se debe iniciar el tratamiento, siendo, en la mayoría de los casos, una combinación de terapia psicológica y farmacológica.

Respecto al tratamiento farmacológico, a pesar de que, como se ha explicado anteriormente, la hipótesis monoaminérgica no proporciona una explicación suficiente de la depresión, en la actualidad, la estrategia terapéutica de primera elección sigue siendo el restablecimiento de los niveles de los neurotransmisores serotonina y/o noradrenalina.

Los primeros antidepresivos utilizados en terapéutica fueron los antidepresivos tricíclicos (p. ej. imipramina, amitriptilina, clomipramina), que bloquean en mayor o menor grado la recaptación de las aminas biógenas, noradrenalina y serotonina, y los inhibidores de la monoaminooxidasa (IMAOs) (p. ej. iproniazida), que inhiben la monoaminooxidasa, enzima encargada de la degradación de las aminas. No obstante, la frecuencia de efectos adversos, cardiotoxicidad asociada al empleo de tricíclicos o las crisis hipertensivas producidas por los IMAOs en interacción con alimentos ricos en tiramina, así como su ineficacia en un porcentaje importante de pacientes, originó el desarrollo de nuevos fármacos.

De esta manera, en la década de los ochenta, surgieron los inhibidores selectivos de la recaptación de serotonina (ISRS) (p. ej. fluoxetina, paroxetina, citalopram, sertralina y fluvoxamina), que adquirieron gran relevancia ya que carecen de los efectos adversos anticolinérgicos presentes en los antidepresivos tricíclicos y son fármacos más seguros.

Otras estrategias farmacológicas más recientes han ido introduciendo nuevos fármacos que van ocupando su propio espacio. Entre ellos se encuentran los inhibidores de la recaptación de serotonina y noradrenalina, como la venlafaxina o duloxetina, o con acción selectiva noradrenérgica, como la reboxetina. También se ha confirmado la eficacia clínica de fármacos con una acción combinada, inhibidora de la recaptación de aminas y moduladora de distintos receptores, como la mirtazapina (antagonista de receptores 5-HT_{2A} y α_2-adrenérgicos) o la trazodona (antagonista de receptores 5-HT_{2A}). Por último, los inhibidores de la recaptación de dopamina y noradrenalina, como el bupropion, también han mostrado poseer una amplia eficacia antidepresiva. Sin embargo, en conjunto, estos compuestos no han logrado mejorar la eficacia clínica de los antidepresivos clásicos de manera sustancial, ni tampoco disminuir el tiempo de latencia desde el inicio del tratamiento hasta la aparición del efecto terapéutico esperado (Blier, 2003). En línea con esta estrategia farmacológica, otro fármaco que se ha aprobado recientemente para el tratamiento de la depresión es la vortioxetina. Este medicamento es un inhibidor selectivo de la recaptación de serotonina y modula la actividad de los receptores serotonérgicos. Su administración parece mejorar el funcionamiento cognitivo de los pacientes deprimidos, además de mejorar los síntomas depresivos (Colle y Corruble, 2016).

Otro fármaco que representa una innovación para el tratamiento farmacológico de la depresión es la agomelatina. Este fármaco es un agonista melatoninérgico que activa los receptores de melatonina (MT) MT_1 y MT_2, resincronizando los ritmos circadianos, alterados durante el curso de la depresión. Adicionalmente, antagoniza los receptores 5-HT_{2C}; esta acción antagonista promueve la transmisión adrenérgica y dopaminérgica, especialmente en

corteza prefrontal, sin alterar los niveles extracelulares de serotonina. La eficacia, tolerabilidad y seguridad de este compuesto se ha determinado en varios estudios doble-ciego (Kennedy y col., 2008; Hickie y Rogers, 2011; Srinivasan y col., 2012).

Por otra parte, estudios recientes han puesto de relieve la importancia del glutamato como un objetivo para el desarrollo de nuevos fármacos antidepresivos, particularmente para aquellos casos de depresión resistente al tratamiento (Diazgranados y col., 2010; Ibrahim y col., 2011; Murrough y col., 2013). La ketamina es un antagonista de los receptores NMDA. Actualmente está aprobado por la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) como agente anestésico y, además, es utilizado como medicamento fuera de ficha técnica (off-label) en el manejo del dolor crónico (Mathew y col., 2012). Sin embargo, el uso de la ketamina tiene algunos inconvenientes, como son los síntomas disociativos y psicotomiméticos, así como su potencial abuso y neurotoxicidad después de un tratamiento crónico (Chaki y Fukumoto, 2015; Choi y col., 2015).

En la tabla 1 se recogen los principales fármacos aprobados en la actualidad para el tratamiento de la depresión de acuerdo con la AEMPS así como moléculas de interés con diversos mecanismos de acción que están siendo objeto de estudio en la actualidad por su potencial efecto antidepresivo.

Tabla 1. Fármacos con actividad antidepresiva

Fármaco	Mecanismo	Tiempo respuesta	Uso clínico
Primera generación: antidepresivos tricíclicos e inhibidores monoamino-oxidasa			
Amitriptilina	IR NA y 5-HT	3-4 semanas	Aprobado AEMPS
Imipramina	IR NA y 5-HT	3-4 semanas	Aprobado AEMPS
Doxepina	IR NA y antagonista H1	3-4 semanas	Aprobado AEMPS
Isocarboxacida	IMAO	3-4 semanas	Aprobado AEMPS
Maprotilina	IR NA y antagonista H1	3-4 semanas	Aprobado AEMPS
Trimipramina	IR NA y 5-HT	3-4 semanas	Aprobado AEMPS
Tranilcipromina	IMAO	3-4 semanas	Aprobado AEMPS
Segunda generación de fármacos antidepresivos			
Bupropión	IR NA y DA	3-4 semanas	Aprobado AEMPS
Citalopram	ISRS	3-4 semanas	Aprobado AEMPS
Fluoxetina	ISRS	3-4 semanas	Aprobado AEMPS
Levomepromazina	Antagonista DA	3-4 semanas	Aprobado AEMPS
Mianserina	IR NA/Antagonista RA α-2	3-4 semanas	Aprobado AEMPS
Mirtazapina	Antagonista /5-HT2A	3-4 semanas	Aprobado AEMPS
Moclobemida	IMAO-A	3-4 semanas	Aprobado AEMPS
Paroxetina	ISRS	3-4 semanas	Aprobado AEMPS
Reboxetina	ISRNA	3-4 semanas	Aprobado AEMPS
Sertralina	ISRS	3-4 semanas	Aprobado AEMPS
Tianeptina	Restaura balance glutamato	3-4 semanas	Aprobado AEMPS
Trazodona	IR 5-HT y antagonista 5-HT2A	3-4 semanas	Aprobado AEMPS
Venlafaxina	IR dual NA y 5-HT	3-4 semanas	Aprobado AEMPS
Otros fármacos con actividad antidepresiva			
Agomelatina	Agonista MT/Antagonista 5-HT2C	3-4 semanas	Aprobado AEMPS
Duloxetina	ISRS, agonista parcial 5-HT1A	3-4 semanas	Aprobado AEMPS
Vortioxetina	ISRS	3-4 semanas	Aprobado AEMPS
Fármacos en ensayo clínico			
Ketamina	Bloqueo canal NMDA	Horas a días	Ensayo clínico
Lanicemina	Bloqueo canal NMDA	Horas a días	Ensayo clínico
CP 101.606	MNA NMDA-NR2B	Horas a días	Ensayo clínico
GLYX-13	MNA NMDA-Glicina	Horas a días	Ensayo clínico
AV-101	Modulador NMDA-Glicina	Desconocido	Ensayo clínico
ALKS-5461	Antagonista receptor opioide k	Semanas a meses	Ensayo clínico

5-HT, serotonina; 5-HT1A y 5-HT2A, subtipos receptor 5-HT; AEMPS, Agencia Española de Medicamentos y Productos Sanitarios; DA, dopamina; H1, receptor H1 histamina; IMAO, inhibidor monoaminooxidasa; ISRS, inhibidor selectivo recaptación serotonina; ISRNA, inhibidor selectivo recaptación noradrenalina; IR, inhibidor recaptación; MNA, modulador negativo alostérico; MT, melatonina; NA, noradrenalina; RA α-2, receptor adrenérgico α-2.

2. ESTRÉS Y MODELOS ANIMALES DE DEPRESIÓN

2.1. Relación entre estrés y depresión

Como se ha comentado en apartados anteriores, el estrés es el factor de riesgo más común en el desarrollo de trastornos del comportamiento como la depresión mayor (Risch y col., 2009). En este sentido, la depresión se ha relacionado con la falta de control sobre las experiencias estresantes indeseables. De hecho, estudios experimentales han demostrado que, mientras que el estrés controlado aumenta la neurogénesis y la memoria (Parihar y col., 2009), la pérdida de control del mismo produce efectos opuestos (Kempermann y col., 2002, 2003; Kronenberg y col., 2009).

Se han llevado a cabo numerosos estudios que revelan una asociación consistente entre la exposición a eventos estresantes y la aparición de episodios de depresión mayor (Kendler y col., 1998). De hecho, se ha descrito que el 80% de los casos de depresión están precedidos por un agente estresante severo (Mazure y col., 2000).

Las primeras investigaciones acerca de la relación de la depresión con el estrés se basaban casi, exclusivamente, en el análisis de eventos episódicos. En la actualidad, el interés se centra en el estudio del estrés crónico (duración superior a 12 meses), ya que éste parece un mejor vaticinador de los síntomas depresivos que el estrés agudo (McGonagle y Kessler, 1990). Hay numerosos estudios epidemiológicos que muestran una relación entre los efectos del estrés crónico en el trabajo, la pobreza (Hu y col., 2007; Siegrist, 2008; Kub y col., 2009), las dificultades del matrimonio (Heene y col., 2007) y la ausencia de apoyo social (Paykel y col., 1996) y la depresión.

El estrés crónico tiene un "efecto a largo plazo", es decir, sus consecuencias tienen larga duración. Si bien la mayor parte de las depresiones tienen una duración de meses o pocos años, algunos pacientes sufren depresión a lo largo de toda su vida aun cuando los agentes estresantes han cesado por completo. Por ejemplo, se ha observado que las situaciones estresantes vividas durante la infancia y adolescencia pueden aumentar significativamente el riesgo de desarrollar depresión en la edad adulta (Heim y col., 2008). Esto sugiere que hay efectos del estrés que persisten en el tiempo y que pueden condicionar la probabilidad de sufrir recurrencias sucesivas.

2.2. Modelos animales de depresión basados en estrés crónico

Los modelos animales de depresión son necesarios para lograr una mejor comprensión de la neurobiología de esta enfermedad, además son indispensables para el desarrollo de nuevas dianas terapéuticas.

Un buen modelo animal de depresión humana debe cumplir, al máximo, los siguientes criterios: etiología comparable (validez constructiva), fenómenos y fisiopatología similar (validez aparente) y correspondencia de tratamiento entre la clínica y el modelo (validez predictiva).

Aunque existen varios modelos animales diferentes de depresión inducidos por estrés, a continuación se describen los dos modelos que se van a utilizar en el presente trabajo: el estrés crónico suave (CMS, de sus siglas en inglés *"Chronic Mild Stress"*) y estrés crónico social por derrota (CSDS, de sus siglas en inglés *"Chronic Social Defeat Stress"*). Ambos modelos tienen en común que inducen anhedonia, considerada como la disminución de la reactividad por estímulos placenteros y síntoma central de la depresión mayor. Sin embargo, el fenotipo neuroquímico que presentan estos modelos es muy diferente entre sí (Venzala y col., 2013). Nos asemejamos de esta manera a lo que suele ocurrir en clínica, dónde un mismo síntoma, como la anhedonia, aparece en depresiones (primarias o reactivas) desencadenadas por mecanismos etiopatogénicos distintos.

2.2.1. Modelo de estrés ambiental: estrés crónico suave (CMS)

El modelo de estrés crónico suave fue desarrollado en un intento de imitar algunos de los factores ambientales que contribuyen a la inducción de trastornos depresivos en los seres humanos. Para proporcionar una simulación realista de las situaciones estresantes de la vida diaria, Willner y col. (1987; 1992) establecieron un modelo experimental en el que los roedores eran expuestos a agentes estresantes no predecibles de intensidad suave durante seis semanas. El fundamento teórico del modelo se basa en su capacidad de producir anhedonia, que es la falta de respuesta a los estímulos placenteros o la disminución de la capacidad para experimentar placer de cualquier tipo, un síntoma principal de la depresión clínica.

El modelo de CMS se considera uno de los mejores modelos de depresión en cuanto a su validez constructiva, aparente y predictiva (Willner y Mitchell, 2002; Willner, 2005).

En cuanto a la validez constructiva, se pretendió proporcionar una simulación realista del estrés de la vida diaria (Willner y col., 1990, Willner y col., 1992) estableciendo un

modelo experimental en el que los roedores eran sometidos a distintos agentes estresantes suaves y no predecibles durante varias semanas. Así, los animales se exponen secuencialmente a una variedad de agentes estresantes suaves (por ejemplo, iluminación durante toda la noche, luz estroboscópica, ruidos intermitentes, periodos de privación de comida, inclinación de la caja, emparejamiento con un compañero desconocido…), que van cambiando cada pocas horas por un periodo de semanas o meses (Willner y col., 1992; Monleon y col., 1995; Elizalde y col., 2008).

Respecto a la validez aparente, el modelo reproduce, en gran medida la sintomatología depresiva ya que induce cambios neuroadaptativos que pueden relacionarse con los hallazgos clínicos presentes en pacientes con depresión (Sanacora y col., 2004; Lucassen y col., 2006; Gould y col., 2007; Frodl y col., 2008). Los roedores presentan conducta depresiva (D'Aquila y col., 1997), pérdida de energía (Cheeta y col., 1997), alteraciones del sueño características del trastorno depresivo (Moreau y col., 1995) y un aumento de la ansiedad (Griebel y col., 2002; Ducottet y col., 2003). El CMS también es capaz de inducir un deterioro en la memoria de reconocimiento (Orsetti y col., 2007; Elizalde y col., 2008) y en la memoria espacial (Song y col., 2006).

A nivel biológico, los animales sometidos a CMS presentan signos que indican una hiperactividad del eje HPA (Bergström y col., 2008), anormalidades del sistema inmune (Lewitus y col., 2009) y del electrocardiograma (Moreau y col., 1995). El CMS también induce una disminución de la proliferación celular y de la neurogénesis en el giro dentado del hipocampo (Mineur y col., 2007; Kong y col., 2009), una alteración en la densidad, composición y morfología de las dendritas apicales a nivel cortical (Michelsen y col., 2007) así como modificaciones en los niveles de neurotransmisores y marcadores de plasticidad neuronal, como ciertas proteínas sinápticas vesiculares (Silva y col., 2008; Bessa y col., 2009; Elizalde y col., 2010) y el factor neurotrófico BDNF (Duman y Monteggia, 2006; Li y col., 2008).

El modelo CMS es único entre los modelos de estrés ya que las diferentes alteraciones conductuales y neurobioquímicas que se generan son revertidas por el tratamiento crónico con antidepresivos aun cuando el estrés continúa presente. Por tanto, su validez predictiva es mayor que la de otros modelos de estrés, en los que a menudo el tratamiento antidepresivo es preventivo (impide la aparición de los déficits) pero no revierte los déficits una vez que han aparecido (Willner, 1991). Entre estos fármacos figuran los antidepresivos tricíclicos como la imipramina, desipramina, amitriptilina y clomipramina (Bessa y col., 2009; Stein y col., 2009), los inhibidores selectivos de la recaptación de serotonina como fluoxetina, fluvoxamina, paroxetina, citalopram y escitalopram (Grippo y

col., 2006; Elizalde y col., 2008, 2010; Jayatissa y col., 2008; Bessa y col., 2009) así como otros fármacos antidepresivos típicos y atípicos, como la agomelatina (Papp y col., 2003).

2.2.2. Modelo de estrés social: estrés crónico social por derrota (CSDS)

Teniendo en cuenta que la mayoría de los estímulos de estrés en los seres humanos, son de naturaleza social (Brown y Prudo, 1981), la investigación sobre las consecuencias del estrés social en los modelos animales es crucial. Así surgió el modelo del CSDS para poder mimetizar esos factores sociales adversos que contribuyen a la inducción de los desórdenes depresivos y trastornos de ansiedad en humanos.

La validez constructiva del modelo radica en que muchos de los estímulos estresantes que sufre el ser humano son de carácter social. Teniendo en cuenta esto, se desarrolló este modelo, que se basa en someter a los roedores a subordinación social, situación que origina una evitación social en los animales. La subordinación social se consigue sometiendo a los roedores a cortos periodos de lucha y convivencia continuada con una animal dominante (Kudriavtseva y col., 1991). El método consiste en introducir a un roedor intruso en la jaula de otro roedor, dominante, y se les deja luchar por un breve periodo de tiempo, hasta que el intruso presente una conducta de subordinación. Estas luchas se repiten durante varios días consecutivos con diferentes ratones dominantes, lo que da lugar a un estrés crónico social por derrota. Además, el roedor subordinado está sometido, durante todo el día, a señales auditivas, olfativas y visuales del roedor dominante, ya que conviven en una misma jaula, separados por una malla metálica, con lo que se consigue, además, un estrés crónico social en forma de amenaza.

En cuanto a la validez aparente de este modelo se basa en que la subordinación social a la cual se somete a los roedores induce evitación social, este comportamiento podría asemejarse al aislamiento social que sufren los humanos en enfermedades como la depresión clínica, el estrés postraumático o la fobia social (Berton y col., 2006; Tsankova y col., 2006; Wilkinson y col., 2009). Además, se ha descrito que el estrés crónico social por derrota induce otras alteraciones conductuales a corto y largo plazo, tales como desesperación conductual, anhedonia, ansiedad, disminución del acicalamiento o aumento de la vulnerabilidad para adicción a drogas de abuso (Von Frijtag y col., 2000; Krishnan y col., 2007; Covington y col., 2009).

Además de las alteraciones conductuales descritas, este modelo induce una serie de cambios a nivel neurobiológico. Se ha descrito que la evitación social inducida por el CSDS depende de un mal funcionamiento del circuito dopaminérgico mesolímbico, que está compuesto por neuronas de dopamina en el área tegmental ventral (ATV) y sus regiones de

proyección al cerebro anterior, en particular, el núcleo accumbens (NAc). Concretamente se ha observado que los ratones vulnerables al estrés presentan una hiperactividad dopaminérgica desde la ATV y un aumento en la expresión del BDNF en el núcleo accumbens. Por otro lado, se ha descrito que la conducta anhedónica inducida por el CSDS estaría relacionada con alteraciones en la corteza prefrontal (Venzala y col., 2013), y sería la causa de la conducta anhedónica (Miczek y col., 2008).

Respecto a la validez predictiva, han sido pocos los fármacos probados en este modelo. El antidepresivo tricíclico, imipramina, y el inhibidor selectivo de la recaptación de serotonina (ISRS), fluoxetina, han sido capaces de revertir la conducta antisocial de los ratones estresados socialmente (Tsankova y col., 2006; Berton y col., 2006; Wilkinson y col., 2009; Haenisch y col., 2009; Yan y col., 2009). Además, estudios previos realizados en nuestro equipo de investigación, han demostrado la eficacia de la venlafaxina y la tianeptina para revertir los síntomas depresivos como la anhedonia y la indefensión en este modelo animal (Venzala y col., 2012).

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CAPÍTULO II

CAPÍTULO 2

Planteamiento y objetivos

La depresión es una de las causas principales de discapacidad a nivel mundial. Actualmente se estima que más del 15% de la población residente en España presenta sintomatología depresiva de distinta gravedad. Teniendo en cuenta que se trata de una enfermedad crónica, estas cifras desvelan una enorme carga social en términos de sufrimiento humano para pacientes y familiares, además de un elevado coste económico para los países desarrollados.

La hipótesis de la deficiencia monoaminérgica ha constituido durante años la base de los enfoques terapéuticos para el tratamiento de la depresión. No obstante, esta hipótesis ha sido cuestionada debido a la ausencia de una eficacia inmediata de los tratamientos, al fracaso de dichos tratamientos, que suele rondar el 30-35%, así como a las numerosas recaídas que presentan los pacientes tratados. Se cree por tanto, que en la patofisiología de la enfermedad y en la acción antidepresiva se deben dar una cadena de eventos biológicos de mayor complejidad que la simple alteración de monoaminas cerebrales. Por ello, se plantean nuevas hipótesis como alteraciones en la neuroplasticidad o en los sistemas de glutamato y GABA, entre otras.

Sin embargo, en la actualidad, no hay una explicación común que aúne y reconcilie las distintas teorías. Esto pone de manifiesto la necesidad de seguir profundizando en los mecanismos moleculares implicados en el desarrollo del trastorno depresivo. En este sentido, la ausencia de claros efectos genéticos en la depresión, junto con el hecho de que numerosos factores ambientales pueden modificar de manera más o menos estable la expresión génica a través de modificaciones epigenéticas, sin necesidad de alterar la secuencia del ADN, apoyan el concepto de que los factores de riesgo biológicos de la depresión sean, en su base, epigenéticos. Además, dichas modificaciones epigenéticas podrían ser la base para explicar la enorme variabilidad interindividual hacia la adversidad o la respuesta al tratamiento. En este contexto, la depresión y el tratamiento antidepresivo se ha asociado a cambios en la plasticidad sináptica en regiones límbicas como la PFC, efecto que podría deberse a modificaciones en la acetilación de histonas. Además, la regulación epigenética del sistema glutamatérgico y el papel que éste juega en la actividad antidepresiva está siendo objeto de numerosos estudios en la actualidad.

De acuerdo con estas consideraciones, los objetivos generales de la presente Tesis Doctoral son profundizar en el estudio de una hipótesis que asocia la depresión mayor y la terapia antidepresiva a cambios epigenéticos que remodelan la cromatina y afectan a la neuroplasticidad. Se pretende con ello lograr una mejor comprensión de la neurobiología de

la depresión así como identificar nuevas dianas terapéuticas que permitan el desarrollo de nuevas moléculas con actividad antidepresiva.

Brevemente, en primer lugar, se estudiará el efecto del tratamiento antidepresivo sobre alteraciones moleculares y conductuales inducidas por un modelo animal de depresión, el estrés crónico suave (CMS), con el fin de confirmar su validez predictiva. Seguidamente, se estudiará la modificación de la expresión de las distintas enzimas histona desacetilasas (Hdacs) por el estrés crónico, utilizando el modelo de estrés crónico social por derrota (CSDS), y por el tratamiento antidepresivo, con el fin de seleccionar enzimas que regulen la expresión de marcadores de plasticidad sináptica. Por último, utilizando un inhibidor selectivo de una Hdac candidata, se estudiará el potencial valor terapéutico de la misma en el modelo del CMS.

Para la consecución de este proyecto, se plantearon los siguientes objetivos concretos:

1. Estudio de la validez predictiva del modelo del estrés crónico suave (CMS): alteraciones del comportamiento y de la expresión génica inducidas por el CMS y por el tratamiento con imipramina a largo plazo. El desarrollo de este objetivo queda reflejado en el capítulo 3.

1.1. Evaluación del efecto del CMS y del tratamiento con imipramina sobre una batería de test conductuales entre los que se incluyen la valoración de la anhedonia, del estado cognitivo y de la interacción social.

1.2. Estudio del efecto del CMS y del tratamiento con imipramina en la expresión génica en la corteza prefrontal (PFC) mediante la técnica de Microarray.

1.3. Identificación de las vías de señalización alteradas por el CMS y el tratamiento con imipramina y validación por PCR a tiempo real y Western blot.

2. Estudio de la regulación de enzimas desacetilasas (Hdacs) por el modelo del estrés crónico social por derrota (CSDS) y por el tratamiento con imipramina. El desarrollo de este objetivo queda reflejado en el capítulo 4.

2.1. Evaluación del efecto del CSDS y del tratamiento con imipramina sobre la expresión del ARNm de las diferentes Hdacs en la PFC.

2.2. Estudio del efecto del CSDS y del tratamiento con imipramina en la expresión proteica de la acetilación de la histona 3 y 4 y de los marcadores de plasticidad sináptica, CREB y proBDNF, en la PFC.

2.3. Análisis del efecto del CSDS y del tratamiento con imipramina en la expresión proteica de HDAC5 nuclear y P-HDAC5 en la PFC.

2.4. Evaluación del efecto del tratamiento antidepresivo con imipramina, fluoxetina y reboxetina sobre la expresión proteica de HDAC5 y P-HDAC5 en la PFC.

2.5. Estudio del efecto del tratamiento repetido con el inhibidor de la clase IIa de Hdacs, MC1568, sobre la expresión proteica de la acetilación de la histona 3 y 4, CREB y proBDNF, en la PFC.

2.6. Análisis del efecto del CSDS y del tratamiento con imipramina en la expresión proteica de Sirtuina 2 (SIRT2) y de uno de sus substratos citoplasmáticos, α-tubulina acetilada, en la PFC.

2.7. Evaluación del efecto del tratamiento antidepresivo con imipramina, fluoxetina y reboxetina sobre la expresión proteica de SIRT2 y α-tubulina acetilada en la PFC.

2.8. Estudio del efecto del tratamiento repetido con el inhibidor de SIRT2, 33i, sobre la expresión proteica de la acetilación de la histona 3 y 4, CREB y proBDNF, en la PFC.

2.9. Evaluación de la expresión del ARNm de las diferentes Hdacs en la PFC de pacientes deprimidos.

3. Estudio del efecto del inhibidor específico de SIRT2, 33i, sobre las alteraciones conductuales y neurobiológicas inducidas por el estrés crónico suave. El desarrollo de este objetivo queda reflejado en el capítulo 5.

3.1 Evaluación de la actividad inhibitoria del compuesto 33i sobre SIRT2 *in vivo.*

3.2 Estudio del efecto del tratamiento con 33i en la expresión proteica de diferentes marcadores de plasticidad sináptica (VGLUT1, sinaptofisina, receptor metabotrópico de glutamato, subunidades del receptor NMDA y AMPA).

3.3 Análisis del efecto del tratamiento con 33i sobre los niveles del neurotransmisor serotonina y su metabolito, el ácido hidroxiindolacético, en PFC.

3.4. Evaluación del efecto del CMS y del tratamiento con 33i sobre una batería de test conductuales entre los que se incluyen la valoración de la anhedonia, de la interacción social, la actividad motora y el estado cognitivo.

3.5. Estudio del efecto del CMS y el tratamiento con 33i sobre la expresión proteica de la subunidad del receptor de glutamato GluN2B y sobre GluA1 en la PFC.

CAPÍTULO III

CAPÍTULO 3

Chronic mild stress and imipramine treatment elicit opposite changes in behavior and in gene expression in the mouse prefrontal cortex

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ABSTRACT

Many studies suggest that the prefrontal cortex (PFC) is a target limbic region for stress response because a dysfunction here is linked to anhedonia, a decrease in reactivity to rewards, and to anxiety. It is suggested that stress-induced persistent molecular changes in this brain region could bring some light on the mechanisms perpetuating depressive episodes.

In order to address this issue, here we have studied the long-term PFC gene expression pattern and behavioral effects induced by a chronic mild stress (CMS) model and antidepressant treatment in mice.

CMS was applied to mice for six weeks and imipramine (10 mg/kg, i.p.) or saline treatment was administered for five weeks starting from the third week of CMS. Mice were sacrificed one month after CMS and following two weeks after the discontinuation of drug treatment and the PFC was dissected and prepared for gene (mRNA) and protein expression studies. Using the same experimental design, a separate group of mice was tested for anhedonia, recognition memory, social interaction and anxiety.

CMS induced a long-term altered gene expression profile in the PFC that was partially reverted by imipramine. Specifically, the circadian rhythm signaling pathway and functions such as gene expression, cell proliferation, survival and apoptosis as well as neurological and psychiatric disorders were affected. Of these, some changes of the circadian rhythm pathway (Hdac5, Per1 and Per2) were validated by RT-PCR and Western blot. Moreover, CMS induced long-lasting anhedonia that was reverted by imipramine treatment. Impaired memory, decreased social interaction and anxiety behavior were also induced by chronic stress.

We have identified in the PFC molecular targets oppositely regulated by CMS and imipramine that could be relevant for chronic depression and antidepressant action. Among these, a possible candidate for further investigation could be the circadian rhythm pathway.

Key words: chronic mild stress, imipramine, depression, microarray, circadian rhythm, anhedonia, *Hdac5*, prefrontal cortex

1. INTRODUCTION

Major depression is a chronic disabling disorder mainly characterized by anhedonia and depressed mood, and still unresolved in terms of etiology and response to treatment. Among the etiological factors, exposure to an excess of stressful life events precedes the onset of first episodes of many depressive disorders (Kendler et al., 2001; Hammen, 2005). However, the illness can be prolonged for a long time after the remission of the stressful events (Stroud et al., 2008). Similarly, acute neurochemical changes induced by antidepressants do not correspond with the requirement of two to three weeks of drug treatment for the therapeutic effects (Duman et al., 1997, 1999; Manji et al., 2001; Nestler et al., 2002). Thus, the pathophysiology and treatment of depression might induce downstream biological events of greater complexity than changes in monoamine levels. Among them, structural and ultra-structural (Drevets et al., 1997; Rajkowska et al., 1999; Bowley et al., 2002; Kang et al., 2012) as well as molecular changes affecting neuroplasticity and signal transduction pathways in the prefrontal cortex (PFC) are reported (Gilabert-Juan et al., 2012; Ota et al., 2014). Altogether, these changes may affect gene regulation through transcription factors or even chromatin modifications (Shelton et al., 2011; Li et al., 2013).

Several human *post mortem* studies have been published on mood disorders using microarrays to identify candidate genes and relevant signaling pathways (Kang et al., 2007; Chu et al., 2009; Shelton et al., 2011; Kim et al., 2012; Li et al., 2013). It has been applied to specific regions, including the PFC, hippocampus, amygdala or thalamus, to document gene expression alterations associated with depression. However, a major drawback for studies in human depression is the inaccessibility of diseased tissue. Moreover, the effects of antidepressants and the underlying diseases cannot be distinguished with certainty. Therefore, studies aiming at discovery of genes that are regulated by depression and antidepressants mainly rely on animal models where different functional states can be controlled.

The chronic mild stress (CMS) model in mice mimics in a naturalistic way the role of socio-environmental stressors in precipitating a depressive pathology and the time frame of therapeutic responses to antidepressants. Specifically, the random application of various environmental and social mild stressors for several weeks results in a syndrome that is reminiscent of symptoms of depression, including anhedonia or decreased reactivity to rewards, increased anxiety-like behavior, mild memory impairments and physiological changes (Santarelli et al., 2003; Pothion et al., 2004; Song et al., 2006; Elizalde et al., 2008, 2010a). One of the most characteristic stressors applied is the alteration of the day-

night cycle given the strong link between circadian disturbances and some characteristic symptoms of clinical depression, such as delayed sleep onset, early-morning wakening, day-time fatigue, and blunting of the normal morning peaks in subjective energy, mood, and alertness (Germain and Kupfer, 2008). Importantly, the intensity of major depressive symptoms has been correlated with the misalignment of circadian rhythms (Emens et al., 2009).

Interestingly, we have recently observed in our laboratory that CMS-induced anhedonia is long-lasting, persisting for at least one month after the termination of the stress procedure. Moreover, at this time point, CMS exposed mice that were treated with the selective serotonin reuptake inhibitor paroxetine behaved as control mice (Elizalde et al., 2008, 2010a). Here, we used the same experimental design to investigate long-term transcriptome changes in the PFC of mice exposed to CMS as well as after depressive behavior reversal by the antidepressant imipramine. Our study is focused on the PFC as a critical component of a corticolimbic circuit of mood regulation that is affected in major depression (Mayberg, 1997; Seminowicz et al., 2004). Indeed, anhedonia has been linked to abnormal function of the PFC (Keedwell et al., 2005; Tremblay et al., 2005; Germine et al., 2011). We aim to differentiate here molecular effects that might be responsible for the chronicity of depressive disorders and that could be also molecular targets for the antidepressant action. To our knowledge, no microarray study has been carried out at this time point. Indeed, microarray studies that attempted to characterize the molecular effects of CMS and antidepressant treatment in rodents (Bergstrom et al., 2007; Orsetti et al., 2008; Liu et al., 2010; Lisowski et al., 2011; Tordera et al., 2011), have been carried out shortly after the termination of the stress procedure (usually after 24 h). Moreover, up to now, perhaps due to differences in chronic stress procedures or antidepressant drugs used results have yielded to no consensus.

Here we show that CMS induces a persistent molecular pathology affecting the PFC that could have a role in the establishment of depressive disorders. Among different genes affected, the circadian rhythm pathway appears as a good candidate for further investigation in depression and antidepressant action.

2. MATERIAL AND METHODS

2.1. Animals and drug treatment

Male C57BL/6J mice (Harlan, France, 8 weeks of age) were housed in individual cages and allowed for 2 weeks to habituate before beginning experimentation. Food and water were available *ad libitum* for the duration of the experiments unless otherwise specified. Animals were maintained in a temperature (21±1°C) and humidity-controlled room (55±2%) on a 12 h light-dark cycle (lights on at 08:00 h).

Experimental procedures and animal husbandry were conducted according to the principles of laboratory animal care as detailed in the European Communities Council Directive (2003/65/EC), Spanish legislation (Real Decreto 1201/2005) and approved by the Ethical Committee of University of Navarra.

Imipramine HCl (Sigma-Aldrich, UK) was dissolved in saline (0.9%) and administered i.p. at 10 mg/kg once daily. A single daily dose was selected in order to reduce to a minimum the stress of the injection in control mice.

2.2. Experimental design

Mice were divided into control and CMS groups. CMS procedure was applied for six weeks. After three weeks, mice from control and CMS groups were subdivided into saline and imipramine groups: Saline, Imipramine, CMS-Saline and CMS-Imipramine. Mice received daily (i.p.) injections of imipramine (10 mg/kg) or saline (at 1 pm) from the third week of CMS and continued for two weeks thereafter the procedure (Fig. 1). Behavioral and neurochemical studies were carried out in separate groups of animals. The long-term effects of CMS on molecular changes were studied (n = 6 mice/group, 24 in total) one month after the CMS procedure (Fig. 1A). Mice were killed by cervical dislocation, brains were rapidly removed and the PFC (around 15 mg) was rapidly dissected in an acrylic mouse brain slicer matrix with 1.0 mm coronal slice intervals (Zivic Instruments, Pittsburgh, PA, USA). Using a mouse brain atlas (Hof et al., 2000), a 1mm slice was taken from the infralimbic section of the PFC (bregma 2.20 mm through bregma 1.20 mm) and dissected out bilaterally using a scalpel and kept at -80 °C for neurochemical studies.

In addition, using 9 mice/group (36 in total) we studied the effect of CMS on behavior. Anhedonic-like behavior was analysed by weekly monitoring of sucrose intake during the CMS procedure and in the fourth week after the termination of stress. Novel object recognition test, social interaction test and elevated plus maze test were carried out

during the three weeks after the CMS procedure (one test per week in order to reduce to a minimum any possible interference of the behavioral test on the last measure of anhedonia) (Fig. 1B).

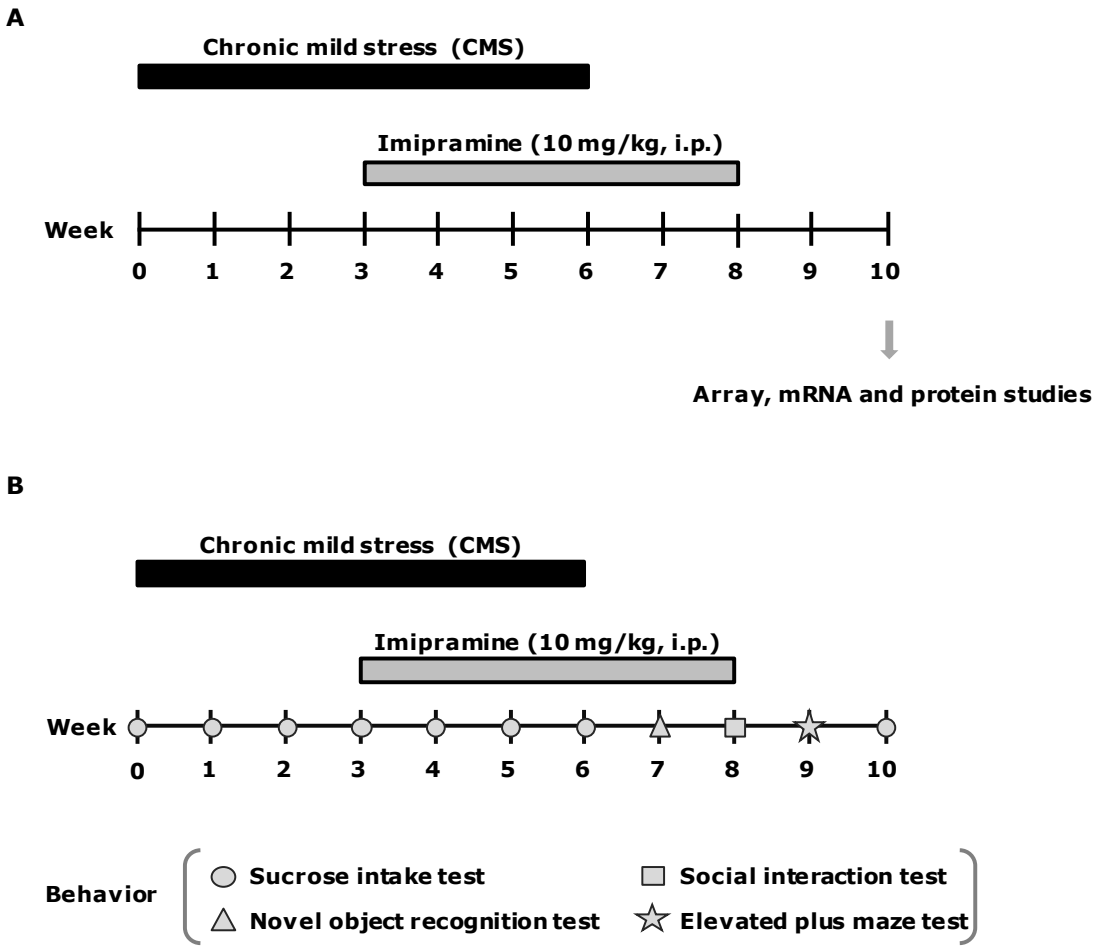

Fig. 1. Experimental design of chronic mild stress (CMS) procedure and imipramine treatment

2.3. Chronic mild stress procedure

CMS was performed according to a procedure optimized in our laboratory (Elizalde et al., 2008). The following unpredictable mild stressors (2-3 in any 24h period) were randomly applied for 6 weeks: stroboscopic illumination (8 h), intermittent bell (10 db, 1 s/10 s) or white noise (4 h), rat odor (saw dust from rat cages; 8 h), placement of novel object in the home cage (3 h), cage tilt 45° (8 h), soiled bedding (6 h), paired housing (with new partner, 2 h), overnight illumination, removal of nesting material (12 h) and confinement (1 h).

2.4. Gene expression studies

2.4.1. RNA extraction

Isolation of total RNA was carried out according to manufacturer's instructions (NucleoSpin RNA II kit, Macherey-Nagel, Germany) and previous methods (Tordera et al., 2011). Total RNA was isolated separately from each individual cortex (n=6/group). The frozen PFC samples were lysed and dounze-homogenized in the presence of a highly denaturing ß-mercaptoethanol containing buffer, which immediately inactivates RNases. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA bound to the membrane and contaminants were washed away. RNA was then eluted in 30-60 µL RNase-free water. The eluates were stored at -80°C.

2.4.2. Microarray hybridization and analysis

RNA quality control was checked with the Agilent Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA) and the RNA concentration was evaluated by using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Microarray hybridizations were outsourced to genomics and bioinformatics unit of CIMA (University of Navarra, Spain). Five animals out of six in each group (Saline, Imipramine, CMS-Saline and CMS-Imipramine group) were used for large-scale gene expression analysis. Selection of the animals was based on the RNA quality from the frontal cortex extracts. The Ambion® WT Expression Kit (Ambion Inc, Austin, TX, USA) and the Affymetrix GeneChip® WT Terminal Labeling and Hybridization Kits (Affymetrix Inc, UK) were used to synthesize biotine labeled cDNA using 300 ng of total RNA. Then, the fragmented and labeled DNA were hybridized onto GeneChip® Mouse Gene 1.0 ST Array (GeneChip®, Affymetrix Inc, UK) accordingly to manufacturer's instructions.

Samples were normalized with robust multi-array average (RMA) (Irizarry et al., 2003) and probe sets with low expression were filtered with R/Bioconductor (Gentleman et al., 2005). In the filtering process probe sets with an expression value lower than 6 in more than the 50% of the samples of all the studied conditions were discarded. The remaining 22,182 probe sets were analysed with LIMMA (Linear Models for Microarray Data) for the identification of the differentially expressed probe sets between experimental conditions (Smyth, 2004). The functional interpretation and biological knowledge extraction was performed with Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems,

www.ingenuity.com), which database includes manually curated and fully traceable data derived from literature sources.

2.4.3. Real time-PCR

RT-PCR was used to validate the differential expression of selected genes (Tordera et al., 2011). For RT-PCR experiments, 0.3 µg of total RNA was reverse transcribed to cDNA using random hexamers as primers and SuperScript reverse transcriptase III (Invitrogen, Cergy Pontoise, France). The eluates were stored at -80°C. RT-PCR was performed in an ABI PRISM 7000 HT Sequence Detection System following manufacturer's recommendations (Applied Biosystems, CA, USA). Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Primers for *Hdac5, Per1, Per2, Clock, Bhlhe40, Pdgfb* and *Pfn1* were used (Applied Byosistems, CA, USA). Each cDNA prepared was used in triplicate for the real-time PCR procedures for each gene tested, and the results were calculated as the average of triplicated results. PCR products were analysed using the SDS 2.3 and the RQ Manager 1.2 Software (Applied Biosystems, CA, USA). 18S (Hs99999901) was used as an internal control to normalize RNA amount used from different samples.

Samples were analysed by the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target genes levels with respect the internal control. Normalization was based on a single reference gene (18S). Double delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample (non treated/treated non stressed and non treated/ treated stressed mice) minus the mean ΔCT of the calibrator samples (non-treated non-stressed mice) for each target gene. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$.

2.5. Protein expression studies

2.5.1. Tissue processing

For preparation of the protein extracts previous methods were followed (Elizalde et al., 2010a). Frontal cortex from the four groups (Saline, Imipramine, CMS-Saline and CMS-Imipramine) mice were rapidly dissected and homogenized in 50 mM Tris-HCl-sucrose buffer (pH 7.4, 4 °C) and centrifuged at 900 g for 10 min. The resultant post-nuclear supernatant was centrifuged at 12,800 g for 10 min and the pellet was suspended in Tris-HCl-sucrose buffer containing 0.32 M sucrose, 1 mM phenylmethylsulfonylfluoride, 5 mg/mL aprotinine and 5 mg/mL leupeptine to a final protein concentration of 0.8-1 mg/mL and stored at -80

°C. The protein concentration was determined by the Bradford method using the Bio-Rad protein assay kit.

2.5.2. Western blotting

For Western blotting, equal amounts of protein (20 µg) per lane were denatured by boiling for 10 min in a water bath at 70°C. Subsequently, proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Hybond-ECL; Amersham Bioscience) from gel using standard technique (7.5% SDS-PAGE) (Elizalde et al., 2010b). The trans-blots were blocked for 1 h by incubation with 5% not-fat milk buffer TBS containing 0.1% Tween 20. Subsequently, membranes were incubated in a solution containing the primary antibody: rabbit polyclonal anti-phosphorylated histone deacetylase 5 (1:1,000) (Applied Biological Materials, Richmond, BC, Canada); mouse monoclonal anti-histone deacetylase 5 (1:1,000) (Sigma Aldrich, St. Louis, MO, USA) and mouse monoclonal anti-Per2 and anti-Per1 (1:1,000, Sigma Aldrich, USA) at 4°C for overnight. Membranes were then rinsed in TBS with 0.1% Tween-20 (TBS-T) and incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies (1:10,000; DAKO, Cambridgeshire, United Kingdom). Proteins were visualized using an enhanced chemiluminescence (ECL™) Western blotting detection reagent (Amersham, Buckinghamsire, England) and band intensity was estimated densitometrically on a GS-800 calibrated densitometer (Biorad One, Madrid, Spain). All protein bands were normalized for β-Actin (1:1,000) (Sigma Aldrich, St. Louis, MO, USA) levels within the same membrane.

2.6. Sucrose intake test

Anhedonic-like behavior was evaluated by monitoring of sucrose intake (Elizalde et al., 2008). Mice were first trained to drink a sucrose solution by exposing them to two standard drinking bottles, one containing 2.5% sucrose and the other tap water, for every other night during one week.

After this preliminary phase, mice were food- and water-deprived for 8 h and then exposed to the sucrose solution and water from 6:00 p.m. until 09:00 h in the morning. The intake baseline for the sucrose solution was established, which corresponded to the average of three consecutive measurements.

Then, mice were divided into two groups matched for sucrose consumption and body weight. One group of mice was exposed to the CMS for 6 weeks and the other group served as non-stressed control. Once a week, mice were given a 15-h exposure to the sucrose

solution and tap water in their home cage as described above. The position of the two bottles (right/left) was varied randomly from trial to trial. Body weight measurements were taken weekly in both stressed and no stressed groups and the relative sucrose intake and sucrose preference (sucrose intake/total intake) was calculated as absolute intake (g) per mouse body weight.

2.7. Behavioral tests

2.7.1. Novel object recognition

Visual recognition memory was assessed with this test (Elizalde et al., 2008). On day 1, mice were placed for 30 min in the empty box to habituate them to the apparatus and test room. On day 2, two identical objects (A1 and A2; two prisms 7 × 3 × 3 cm^3) were placed symmetrically 11 cm away from the wall and separated 22 cm from each other. The mouse was placed in the box at equal distance from both objects and video recorded for 5 minutes (sample phase). Then, the mouse was returned to its cage. After a delay of 1 h or 24 h the mouse was placed back in the box and exposed to the familiar object (A3) and to a novel object (B or C for 1 or 24 h retention interval respectively) a further 5 minutes (retention tests). The novel object B consisted of a ball (3.5 cm diameter) mounted on a cube (3 cm) and C was a cylindrical plastic bottle (7 × 3 cm^2). The positions of the familiar and the new object alternated between the 1 h and the 24 h retention test. Previous experiments applied in our lab have shown no preferences for any of the objects (A, B or C) used during the procedure.

Mice were video recorded and the total time spent exploring each of the two objects in the sample and retention phases were measured (Ethovision XT 10 plus multiple body point module). The exploration time was measured as the time that the nose of the mouse was in an area surrounding 2 cm the object. Percentage of exploration of the new object compared to the total exploration time in the retention test was calculated (Venzala et al., 2012).

2.7.2. Social interaction test

This test measured the approach-avoidance behavior towards an unfamiliar social target (Tsankova et al., 2006 and Venzala et al., 2012). The arena is a transparent plastic open field (45 × 45 × 45 cm^3) maintained in a very softly illuminated room (50 lx). Each experimental mouse was introduced into the open field and its trajectory was tracked for two consecutive sessions of 2.5 min. During the first session ("no target") the open field contained an empty metallic mesh cage (10.5 × 9 cm^2) located at one end of the field.

During the second session ("target") the conditions were identical except that a social target animal (a mouse from the same strain and of similar weight) had been introduced into the cage. Between the two sessions, the experimental mouse was removed from the arena, and was placed back into its home cage for approximately 1 min. The time that the experimental mouse spent in the "interaction zone" (7 cm wide corridor surrounding the little metallic cage) both in the "no target" and "target" conditions were recorded (Ethovision XT 10 plus multiple body point module) and differences ("target-no target") were calculated for each mouse (Venzala et al., 2012).

2.7.3. Elevated plus maze

This test was applied to measure unconditioned anxiety-like behavior (Lister, 1987). The elevated plus maze (EPM) consisted of two open arms (30 × 5 cm^2), two enclosed arms (30 × 5 cm^2) and a central platform (5 × 5 cm^2) elevated 38.5 cm above the ground in a softly illuminated room (50 lx). The floor of the entire EPM was covered by a gray plastic that could be easily cleansed with water and sawdust between trials. The walls of the enclosed arms were gray opaque. At the beginning of the 5-min session, each mouse was placed in the central neutral zone, facing one of the close arms. Percentage time in the open and central arms and number of head dips over the edge of open arms were calculated (Ethovision XT 10 plus multiple body point module). An arm entry was defined as a mouse having entered an arm of the maze with all four legs (Venzala et al., 2012).

2.8. Statistical analysis

For microarray studies, probes were selected as significant using p-value ($p<0.01$) and fold change cut offs ($logFC>0.19$). Significantly enriched Ingenuity functions were considered at p-value ($p<0.05$).

The different behavioral paradigms, RT-PCR and Western blotting studies were analysed using two-way ANOVA (stress, treatment) analysis followed by Tukey *post hoc*, in those cases where there was a significant interaction. When no significant interaction between two factors was found, main effects were considered.

Weight gain and sucrose intake were analysed with two-way ANOVA being time the within-subjects factor and stress the between-subjects factor. For each week, a one-way ANOVA was carried out and significant differences among groups were analysed with Tukey *post hoc* test.

3. RESULTS

3.1. Microarray studies

We considered statistical significance levels of $p<0.01$ and logarithm of fold change higher than ±0.19 when comparing patterns of gene expression on microarray between the CMS-Saline *versus* Saline treated group. In addition, in the comparison of the CMS treated with Imipramine *versus* the CMS-Saline treated group the significant level was set at $p<0.05$. Using these conditions, 759 probes were differentially expressed in the CMS-Saline *versus* Saline treated group. Subsequently, we observed that the expression of 484 of these probes was significantly changed by repeated imipramine treatment (Fig. 2).

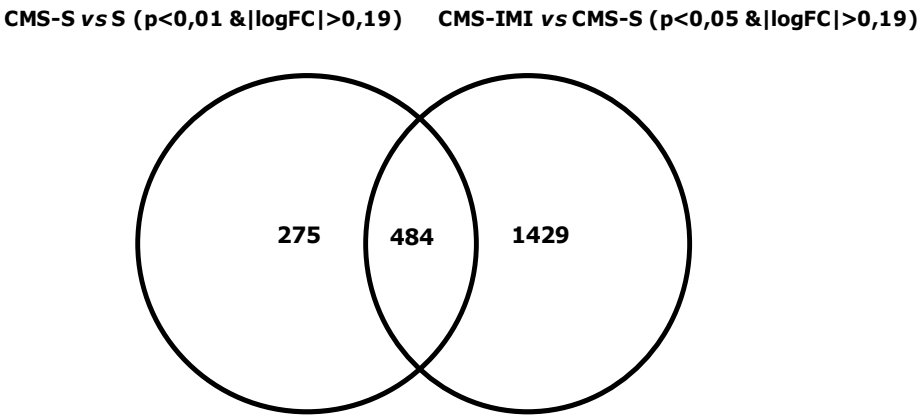

Fig. 2. Venn diagram of the differentially expressed probe sets in the comparison of CMS-Saline and CMS-Imipramine groups. Selection criteria are detailed in the figure.

A hierarchical clustering of CMS-Saline, Saline, CMS-Imipramine and Imipramine samples based on the 759 probes affected by stress revealed that CMS-Saline samples differed from Saline, CMS-Imipramine and Imipramine samples. The clusters of upregulated (206 probes) and downregulated probes (553) could also be observed (Fig. 1S). In the case of a hierarchical clustering of the same samples based only on the 484 probes affected by stress and reverted by imipramine treatment, the effect of imipramine was confirmed in the majority of the probes. All the 343 downregulated probes and 140 of 141 upregulated probes were reverted (Fig. 2S). Finally, the clustering based on the 275 probes not reverted by imipramine showed that CMS-Imipramine samples differed from Saline samples in a way that in the majority of the genes imipramine treatment only achieved a partial amelioration of the alteration in gene expression induced by CMS. Within this group, while 65 probes were

upregulated 210 were downregulated in the CMS-Saline samples compared to the Saline group (Fig. 3S).

3.1.1. Ingenuity pathway analysis (IPA)

The IPA was used to identify enriched pathways and biological functions in the list of differentially expressed genes. This analysis revealed alterations in canonical pathways such as circadian rhythm signaling and regulation of actin-based motility by Rho. In addition, some diseases or biological functions significantly over represented in the group of mice exposed to CMS were neurological and psychological disorders, behavioral alterations, gene expression, cellular growth and proliferation, cell death and survival and cell to cell signaling and interaction (summarized in Table 1). In particular, genes involved in the canonical circadian rhythm pathway downregulated by CMS and reverted by imipramine *were Bhlhe40, Hdac5, Nr1d1, Per1 and Per2* (Table 2). Genes belonging to each enriched biological function significantly affected by CMS are also represented in Table 2.

Table 1. Some of the significantly enriched pathways or biological functions affected by chronic mild stress and with a course of treatment with the antidepressant imipramine.

	Differentially expressed genes	
	CMS-S vs S	**CMS-IMI vs CMS-S**
Canonical pathways		
Circadian Rhythm Signaling	7	6
Regulation of Actin-based Motility by Rho	8	-
Diseases and Disorders		
Neurological Diseases	141	85
Psychological Disorders	82	46
Skeletal and Muscular Disorders	78	43
Behavioral Alterations	67	49
Molecular and Cellular Functions		
Gene Expression	95	17
Cellular Growth and Proliferation	93	70
Cell Death and Survival	81	49
Cell to Cell Signaling and Interaction	21	21

Data show number of genes affected in each pathway or function

Table 2. Differentially expressed genes in the prefrontal cortex affected by chronic mild stress and ameliorated by repeated imipramine treatment.

Biological function	Genes	
	Up	Down
Canonical pathways		
Circadian Rhythm	*Clock*, ***Grin3a***	***Bhlhe40***, ***Hdac5***, ***Nr1d1***, ***Per1***, ***Per2***
Gene expression		
DNA transcription	***Acvr1***, ***Ar***, ***Atad2***, ***Atrx***, ***Bclaf1***, ***Bmpr2***, ***Cask***, *Clock*, ***Hspa8***, ***Jmy***, *Pparg*, *St18*, *Tbx22*, ***Tdg***, *Wnt4*	*Abhd14b*, *Ablim1*, ***Bcl6***, ***Bhlhe40***, ***C1orf85***, *Cbfa2t3*, ***Chmp1a***, *Dlx5*, ***Dusp5***, ***Egr1***, ***Egr2***, ***Egr4***, ***Eng***, ***Etv3***, ***Etv5***, ***Etv6***, ***Fosb***, *Foxg1*, *Gmeb2*, ***Hdac5***, *Hivep3*, *Hsf1*, ***Id3***, *Irf7*, *Kctd13*, ***Kdm6b***, *Klf13*, *Mapre3*, *Mavs*, ***Med25***, ***Mef2d***, *Ncor2*, *Ndnl2*, ***Nfix***, ***Nr1d1***, ***Nr4a3***, ***Nup62***, *Olig1*, *Pcgf2*, ***Pdgfb***, ***Per1***, ***Per2***, ***Pfn1***, ***Phb2***, ***Pias4***, ***Pkig***, *Pknox1*, ***Plk3***, ***Ppp5c***, ***Ramp3***, ***Rara***, ***Rela***, ***Rnasek***, ***Sik1***, *Sirt7*, *Smad7*, ***Smarcb1***, ***Snapc2***, *Sox18*, ***Ssbp3***, *Sufu*, *Tgfb1i1*, ***Tp53***, *Trim28*, ***Zfp36l2***, ***Zmiz1***, ***Zmiz2***, ***Znf628***
Cell death and survival		
Cell viability of neurons	***Ar***, ***L1cam***	*Cc2d1a*, *Cx3cl1*, ***Efna2***, ***Egr1***, *Foxg1*, ***Hdac5***, *Herpud1*, *Hsf1*, ***Hspb1***, *Pla2g6*, ***Rara***, ***Rela***, ***Tp53***, ***Vgf***
Neuronal cell death	***Apc***, ***Ar***, ***Atrx***, *Gapdh*, ***Grik2***, ***Grin3a***, ***L1cam***, *Nova1*, *Pdk1*, ***Tcp1***	*Ache*, ***Akt1s1***, *Cc2d1a*, ***Cntfr***, ***Ctsv***, *Cx3cl1*, ***Efna2***, ***Egr1***, ***Etv6***, *Faim2*, *Foxg1*, ***Hdac5***, *Herpud1*, ***Hpca***, *Hsf1*, ***Hspb1***, ***Mef2d***, ***Mif***, ***Nr1d1***, ***Nr4a3***, ***Pigt***, *Pla2g6*, ***Ppif***, ***Rara***, ***Rela***, ***Rtn4r***, *Serpina3*, ***Sncbtp53***, *Uchl1*, ***Vegfb***, ***Vgf***
Cellular growth and proliferation	***Acvr1***, ***Acvr1c***, ***Apc***, ***Ar***, ***Atad2*** ***Bmpr2***, ***Capn6***, ***Ccdc88a***, *Chrm*, ***Csnk2a1***, ***Dcx***, ***Erbb2ip***, ***Itgb8***, ***L1cam***, ***Lrp1b***, *Mir181*, ***Mir218***, ***Mir221***, *Pparg*, *Tnfrsf8*,	*Acin1*, ***, Akt1s1***, ***, Bcl6***, ***Camk2n2***, ***Ccnd3***, *Cd151*, *Cx3cl1*, ***Dohh***, ***Dusp5***, ***Egr1***, ***Egr2***, ***Eng***, ***, Etv6***, ***Fosb***, *Foxg1*, ***Ftl***, ***Grn***, ***Hdac5***, ***Hgs***, *Hsf1*, ***Id3***, *Igfbp2*, ***Jup***, ***Mif***, *Ncor2*, *Ndnl2*, ***Oaz1***, *Pcgf2*, ***Pdgfb***, ***Pebp1***, *Pelp1*, ***Pfn1***, *Pla2g6*, ***Pmepa1***, ***Por***, ***Ppif***, ***Ppp5c***, *Ptpru*, ***Rara***, ***Rela***, *Rhoc*, ***Rnf126***, ***Rnf31***, ***Rnf5***, *Sirt7*, *Slc7a5*, *Smad7*, ***Smarcb1***, ***Sst***, ***Sstr2***, *Steap3*, *Stub1*, *Sufu*, ***Tcp1***,***Tek***, ***Tgfb1i1***, ***Tmsb4x***, ***Tp53***, *Trim28*, *Uchl1*, ***Wbp2***, ***Zmiz1***, ***Zmiz2***

Table 2 (continuation)

Cell-To-Cell Signaling and Interaction	*Apba1, Bbs10, Chrm3,* ***Grik2,*** *Il1rapl1, Lrrtm4, Mir-181, Slitrk4*	*Apba3, Bbs1,* ***Cplx1,*** *Eng, Cc2d1a, Cplx1, Eif4ebp2, Lhx6,* ***Grn,*** *Mef2d,* ***Rtn4r,*** *Slitrk3,* ***Rela,*** *Rhoc,*
Neuronal-Synaptic transmission	*Chrm3,* ***Grin3a, Grik2***	***Gpr37l1, Gpr52,*** *Htr7,* ***Ntsr2, Slc12a2***, ***Slc36a1,*** *Slc44a1, Slc7a5,* ***Sstr2***
Neurological and Psychological Disorders		
Movement disorders	*Apba1,* ***Ar,*** *Chrm3,* ***Dnajc13***, *Gapdh,* ***Grik2, Grin3a, Hspa8, Kcnc2***, ***Man1a1***, ***Pcdh7,*** *Pdk1, Pparg,* ***Slc25a22, Unc13c, Xk***	*Ache,* ***Ap1s1,*** *Atp5o,* ***Bcl6***, ***Bhlhe40***, ***Brsk2***, *Cacng2, ,* ***Cntfr***, ***Cplx1***, ***Ctsv***, *Cx3cl1, Cyc1,* ***Dusp5***, ***Eef1a2***, ***Egr1, Egr2, Egr4, Etv5, Fbl, Fosb, Foxg1***, ***Ftl,*** *,* ***Gpd1, Gstp1, Hdac5***, *Herpud1,* ***Hpca, Hspb1***, *Htr7,* ***Id3,*** *Kctd13,* ***Mef2d***, ***Mfsd2a, Ndufa8***, ***Nfix***, ***Nr1d1***, ***Ntsr2, Paqr6***, *Pdlim7,* ***Pebp1***, ***Pfn1***, *Pla2g6, Pprc1, Ppt2,* ***Rela***, *Rnf5, Rpl13, Rtn1,* ***Rtn4r***, *Serpina3,* ***Slc12a2***, ***Sncb***, ***Sst***, *Stub1,* ***Tmsb4x***, ***Tp53***, *Uchl1, Usp36,* ***Vamp2***,
Behavior	*Apba1,* ***Apc, Ar, Chd7,*** *Chrm3, Clock,* ***Csnk2a1, Grik2,*** *Jph1,* ***Kcnc2, L1cam, Neto1,*** *Pparg,* ***Slc12a2,***	*Ache,* ***Bbs1, Bscl2, Cacnb1,*** *Cacng2, Cc2d1a,* ***Ccnd3, , Chst8,*** *Cmklr1,* ***Cntfr, Cplx1, Ctsv, Cxcl14, Cyp46a1, Efna2, Egr1, Egr2, Eif4ebp2, Fezf2, Fkbp8, Fosb, Gpr37l1, Grn, Hdac5,*** *Htr7,* ***Lrrtm1, Mfsd2a, Mif,*** *Nipal3,* ***Nr4a3, Ntsr2, Pak6, Pcsk1n, Per1, Per2,*** *Pla2g6, Ptprn2,* ***Qpct, Rela, Rtn4r, Sdc3,*** *Sepn1,* ***Sst, Sstr2,*** *Stub1,* ***Tet1, Tp53, Tpgs1,*** *Uchl1,* ***Unc13c, Vgf***

In bold, genes significantly ameliorated by imipramine treatment. In grey, genes selected for RT-PCR validation

Subsequently, in order to validate by RT-PCR the microarray results, we selected just a few candidates (*Hdac5, Per1, Per2, Bhlhe40, Pdgfb and Pfn1*) downregulated by stress and reverted by antidepressant treatment to validate. These genes were representative of the circadian rhythm signaling and some of the enriched functions identified (Table 3).

Table 3. Selected differentially expressed genes mainly involved in circadian rhythm signaling and DNA transcription, significantly affected by CMS and ameliorated by imipramine treatment.

Altered genes			**CMS-S *vs* S**		**CMS-IMI *vs* CMS-S**	
RefSeq	**Symbol**	**Name**	***p***	**log FC**	***p***	**log FC**
NM_001077696	***Hdac5***	*histone deacetylase 5*	**0.00179**	**-0.21**	**0.00180**	**0.20**
NM_001159367	***Per1***	*period homolog 1*	**0.00028**	**-0.46**	**0.00370**	**0.34**
NM_011066	***Per2***	*period homolog 2*	**0.00166**	**-0.41**	**0.01064**	**0.31**
NM_011498	***Bhlhe40***	*basic helix-loop-helix family, member e40*	**0.00871**	**-0.34**	**0.02981**	**0.26**
NM_011057	***Pdgfb***	*platelet-derived growth factor beta polypeptide*	**0.00059**	**-0.34**	**0.00106**	**0.31**
NM_011072	***Pfn1***	*profilin 1*	**0.00022**	**-0.31**	**0.00018**	**0.30**

Abbreviations: S, Saline; CMS-S, Chronic Mild Stress-Saline; CMS-IMI, Chronic Mild Stress-Imipramine

3.2. RNA expression studies

RT-PCR studies confirmed that most of these genes were affected by stress and imipramine treatment. A significant interaction between stress and treatment was shown for *Per1* ($F_{1,31}$=12.263, p<0.01), *Per2* ($F_{1,31}$=15.870, p<0.01), *Bhlhe40* ($F_{1,31}$=3.270, p<0.05) and *Pdgfb* ($F_{1,31}$=2.910, p<0.05). *Post hoc* analysis confirmed that stress induced a downregulation of *Per1, Per2, Bhlhe40* and *Pdgfb* and this effect was reverted by imipramine. In addition, *Hdac5* ($F_{1,31}$=6.816, p<0.05) and *Pfn1* ($F_{1,31}$=25.269, p<0.01) expression were down regulated by stress (Fig. 3).

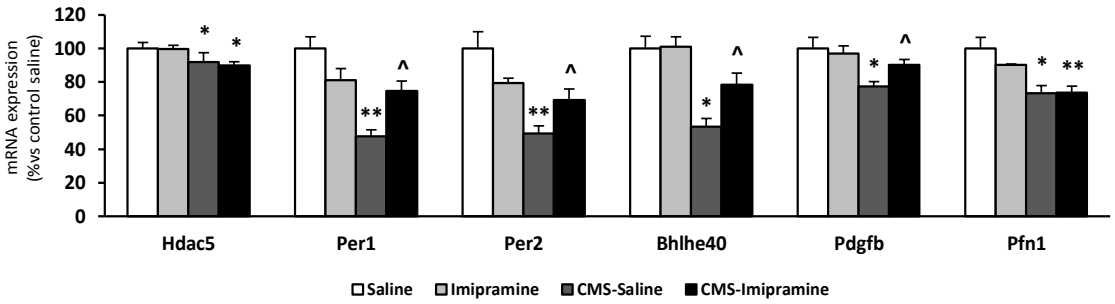

Fig. 3. Effect of CMS and imipramine treatment on the mRNA expression of *Hdac5, Per1, Per2, Bhlhe40, Pdgfb and Pfn1*. Data show mean ± SEM of % of expression *vs*. saline controls. For Per1, Per2, Bhlhe40 and Pdgf, *p<0.05 *vs*. control saline group; ^p<0.05, *vs*. CMS-Saline group (Tukey-*post hoc*). For Hdac5 and Pfn1, *p<0.05 vs. corresponding control mice (Two way ANOVA, main effects of stress).

3.3. Protein expression studies

The protein expression of histone deacetylase 5 (HDAC5) and its cytoplasmic phosphorylated at serine 498 form (P-HDAC5) were downregulated by CMS ($F_{1,31}$= 7.524, $p<0.05$ and $F_{1,31}$= 5.471, $p<0.05$, respectively) (Fig. 4A and B).

Moreover, a significant effect of stress ($F_{1,31}$= 5.251, $p<0.05$) and of imipramine treatment ($F_{1,31}$= 5.034, $p<0.05$) was observed on PER2 expression levels. Specifically, we observed a downregulation of this protein in mice exposed to CMS. Conversely, imipramine treatment upregulated PER2 (Fig. 4C). Western blot analysis of PER1 protein levels in the PFC, revealed no changes (data not shown).

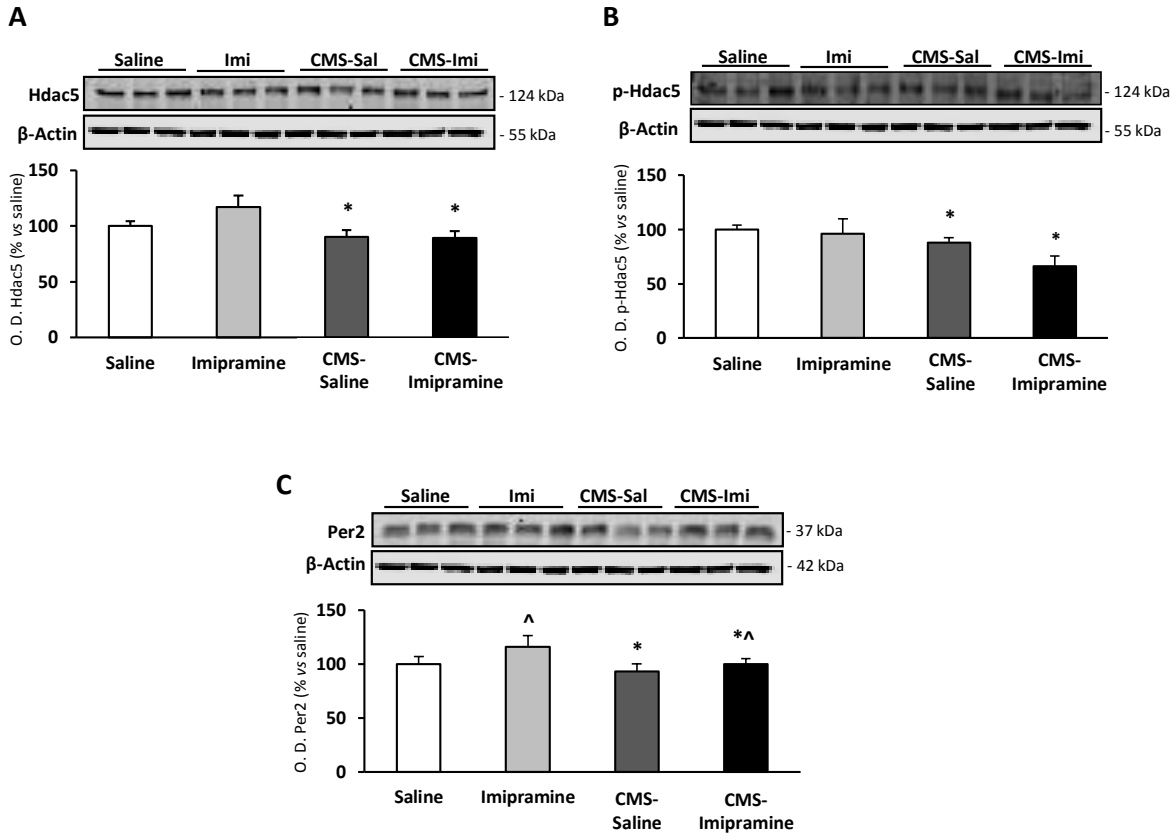

Fig. 4. Effect of CMS and imipramine treatment on expression of HDAC5 **(A)**, P-HDAC5 **(B)** and PER2 **(C).** Data show mean ± SEM of % of expression vs. saline controls. *p<0.05 *vs*. corresponding control mice; ^p<0.05 *vs*. corresponding saline groups (Two way ANOVA, main effects of stress and treatment).

3.4. Depressive-like behavior

3.4.1. Sucrose intake test

Body weight was unaffected by CMS or imipramine treatment. Before onset of CMS, sucrose and water intake were similar in all the groups. In this test, sucrose intake varied across one week time blocks and differences among the groups were shown ($F_{1,241}$ = 3.088, $p<0.05$). Subsequently, analysis of each independent week revealed significant differences on weeks 5, 6 and 10 ($F_{3, 31}$ = 3.031, $p<0.05$; $F_{3, 31}$ = 3.135, $p<0.05$; $F_{3, 31}$ = 4.099, $p<0.05$, respectively). *Post hoc* analysis revealed that stressed mice treated with saline showed decreased sucrose intake on these weeks and imipramine treatment was able to revert this effect on weeks 6 and 10 (Fig. 5A).

3.5. Recognition memory

3.5.1. Novel object recognition test

No significant differences were found in short-term recognition memory (data not shown). In the long-term memory, there was a significant interaction between stress and imipramine treatment on the exploration time of the new object ($F_{1, 31}$ = 3.778, $p<0.05$). In addition, a significant interaction between stress and imipramine treatment on the frequency of visits to the new object was also observed ($F_{1, 31}$ = 4.285, $p<0.05$). *Post hoc* analysis revealed that saline mice exposed to CMS showed a decrease in the percentage of exploration time of the new object and decreased numbers of visits compared to saline control mice ($p<0.05$) (Fig. 5B and C).

3.6. Anxiety-like behavior and social interaction

3.6.1. Social interaction test

Significant differences were observed in the time spent in the interaction zone in the presence of a mouse of the same strain. A significant effect of stress ($F_{1, 31}$ = 4.845, $p<0.05$) and treatment ($F_{1, 31}$ = 4.319, $p<0.05$) was shown. Mice exposed to CMS showed a decreased social behavior and mice treated with imipramine increased social behavior compared to corresponding controls (Fig. 5D).

3.6.2. Elevated plus maze

Similarly, latency time to first entry in the open arms was affected by stress ($F_{1,31}$ = 10.931, p<0.05) and imipramine treatment ($F_{1,31}$= 11.275, p<0.05). While imipramine decreased latency time, stress did the opposite (Fig. 5E).

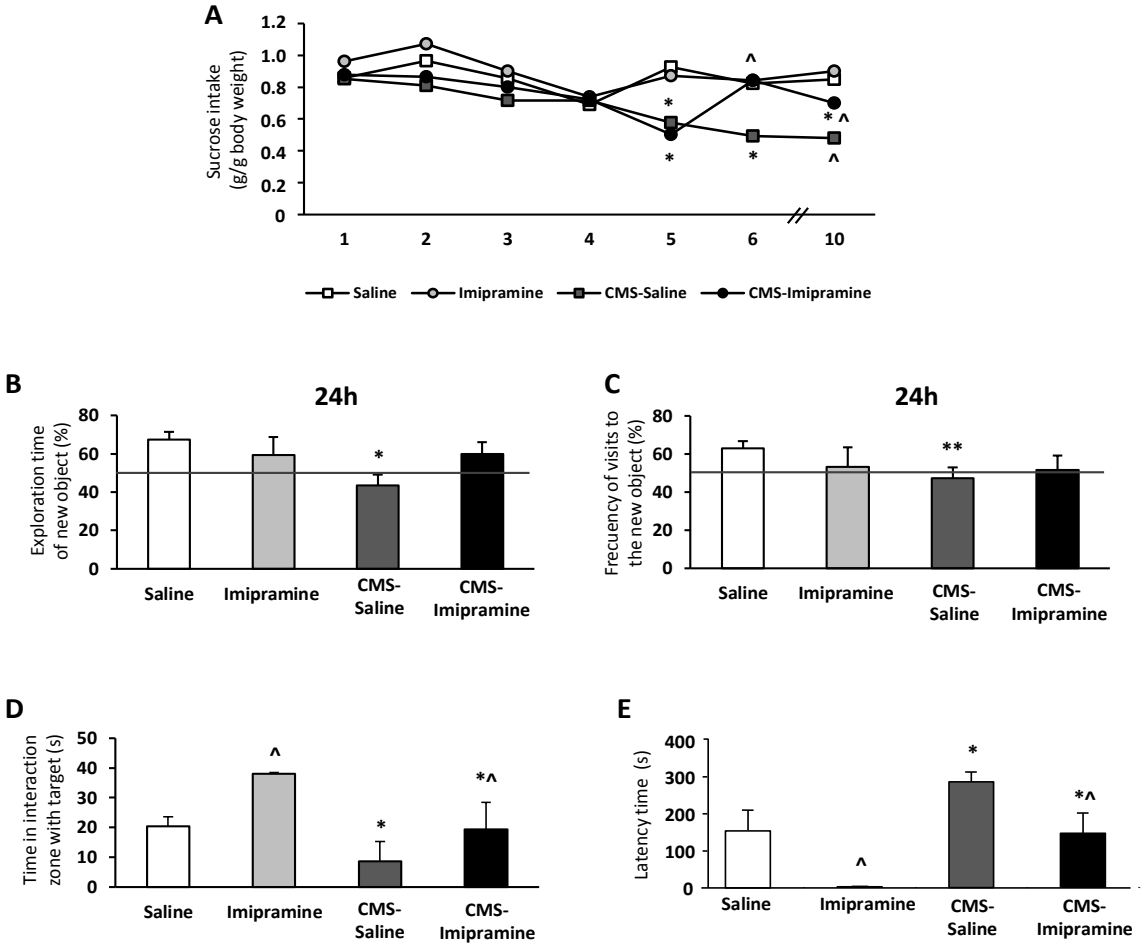

Fig. 5. Effect of CMS and imipramine treatment on depressive like behavior in the sucrose intake test **(A)**, long-term memory in the novel object recognition test **(B,C)**, social behavior in the social interaction test **(D)** and anxiety behavior in the elevated plus maze test **(E).** Data show mean ± SEM of different behavioral paradigms. In (A–C) *p<0.05 *vs*. control saline group; ^p<0.05 *vs*. CMS-Saline group (Tukey-*post hoc*). In (D, E) *p<0.05 *vs*. corresponding control mice; ^p<0.05 *vs*. corresponding saline treated groups (Two way ANOVA, main effects of stress and treatment).

4. DISCUSSION

In the present study, we show for the first time the long term altered gene expression profile induced by CMS and the effect of a course of antidepressant treatment. Changes in different biological functions were shown, including circadian rhythm signaling pathway, gene expression, cell proliferation, survival and apoptosis, synaptic neurotransmission, neurological and psychiatric disorders as well as behavior. Importantly, chronic imipramine administered for 5 weeks and after a two-week washout period reverted some of the observed gene expression changes suggesting that imipramine speeds up the recovery from the pathologically altered molecular network induced by stress. Among different genes affected, the circadian rhythm signaling pathway appeared as a candidate target for the antidepressant action.

As expected, CMS induced long-lasting anhedonic-like behavior that persisted even one month after the CMS procedure. Repeated imipramine treatment reverted these effects, in agreement with previous studies carried out in our laboratory using the antidepressant paroxetine (Elizalde et al., 2008). Moreover, along this month, other CMS induced altered behaviors were observed including impaired memory in the novel object recognition test, decreased sociability in the social interaction test and anxiety in the elevated plus maze test. Altogether, our data provide new insights on the possible molecular mechanisms that might be perpetuating episodes of depression and/or establishing subthreshold symptoms that could be increasing the vulnerability for relapse. Moreover, it brings some light on possible molecular targets for the antidepressant action.

4.1. Long-term molecular network alterations induced by CMS are reverted by imipramine

In addition to behavior, the CMS model has been extensively studied at the molecular (Airan et al., 2007; Gronli et al., 2007; Garcia-Garcia et al., 2009; Banasr et al., 2010) or cellular (Warner-Schmidt and Duman, 2006; Jayatissa et al., 2008) levels. CMS induces neuroadaptive changes that could be addressing clinical findings with depressed patients (Rajkowska, 2000; Sanacora et al., 2004; Lucassen et al., 2006; Gould et al., 2007; Frodl et al., 2008). Moreover some of these alterations persist one month after the CMS procedure (Elizalde et al., 2010a, 2010b). CMS affected the expression of transcripts involved in biological functions or pathways such as the canonical circadian rhythm pathway, gene expression, cell death and survival and neurological or psychological and behavioral disorders. For instance, Ingenuity pathway analysis (IPA) suggested that CMS promotes neuronal cell death, inhibits cell growth and proliferation and promotes altered behaviors and

neurological movement disorders (Supplemental Table 1S). Importantly, these findings could also address evidence of persistent neuroadaptive changes in the PFC in both animal models (Elizalde et al., 2010a, 2010b) and depressed patients (Drevets et al., 1997; Rajkowska et al., 1999; Bowley et al., 2002; Kang et al., 2012; Gilabert-Juan et al., 2012; Ota et al., 2014). Interestingly, some of the CMS-induced changes were reverted by imipramine suggesting the relevance of these targets for antidepressant action. On the other hand, genes not reverted by imipramine could form part of the residual neurobiological mechanisms for vulnerability to relapse. Yet, further RT-PCR and protein studies should be carried out to confirm the validity of the microarray data.

4.2. Long-term alterations in circadian rhythm induced by CMS are reverted by imipramine

Strong links exist between circadian disturbances and some of the most characteristic symptoms of clinical depression (Germain and Kupfer, 2008). The disruption of the circadian clock can manifest as changes in sleep-wake cycles (Van Cauter and Turek, 1986; Turek, 2007) as well as at the level of the molecular circadian clock (Mendlewicz, 2009). In keeping with this hypothesis, candidates of the circadian rhythm signaling pathway (*Hdac5, Per1, Per2* and *Bhlhe40*) and two other randomly selected *(Pdgfb and Pfn1*) were studied by RT-PCR. All changes induced by CMS and imipramine were confirmed with the exception of the effect of imipramine on *Hdac5* and *Pfn1*. Importantly, the regulation of Hdac5 and Per2 targets was confirmed at protein level. These results agree with previous microarray studies in the brain showing the notable reliability of arrays (Pozhitkov et al., 2010) and also underlie the importance of RT-PCR studies to confirm gene changes.

Previous studies using Per1 and Per2 knock-out mice show a key role for these proteins in the maintenance of rhythmicity and even in day-night cycle alterations (Bae et al., 2001). In line with our results, other authors have also reported that CMS reduces the function of PER2 in the suprachiasmatic nucleus and that imipramine treatment for three weeks is able to revert this effect (Jiang et al., 2011). Moreover, Spencer et al. (2013) suggests that lowered Per gene expression is associated with a heightened anxious response to stress. Importantly, clinical data show that genetic variation in *PER2* has been associated with depression vulnerability in a Swedish population-based sample (Lavebratt et al., 2010). Noteworthy, studies carried out in our laboratory (unpublished observations) have shown decreased expression of PER1 and PER2 proteins in the PFC one week after CMS indicating that this persistent change appears early after CMS. Interestingly, imipramine treatment returned PER2 decrease to control levels indicating that this target could play a key role in antidepressant action.

The HDAC5 enzyme belongs to the class IIa superfamily. The class IIa HDACs (HDAC 4, 5, 7, and 9) are unique among the Hdac family proteins in that they suffer synaptic activity-dependent nucleocytoplasmic shuttling (McKinsey et al., 2001). Under basal conditions, class IIa HDACs are unphosphorylated and located in the nucleus, where they are recruited to their target genes through interaction with transcription factors, enabling their transcriptional repressive function. However, class IIa HDACs become phosphorylated in response to specific signals, leading to disruption of the interaction with transcription factors, their export to the cytoplasmic compartment, and de-repression of their targets (Parra, 2014). CMS induced a downregulation of total and phosphorylated HDAC5 in the PFC matching with the observed *Hdac5* mRNA downregulation. In keeping with our results, Renthal et al. (2007) observed that chronic social defeat stress significantly downregulated *Hdac5* mRNA levels in the nucleus accumbens. Altogether, these data agree with studies revealing a key role for HDAC5 in regulating behavioral adaptations to chronic stress (Tsankova et al., 2006). Importantly, rhythmic changes in the acetylation status of core clock transcription factors and of histones at gene promoters play an important role in imparting circadian periodicity to physiological processes such as metabolism (Fogg et al., 2014). In this context, the contribution of several histone acetyltransferases and HDACs has been studied. HDAC4/5 inhibition would increase acetylation levels of targets of the circadian rhythm pathway that would be expected to result in a loss of rhythmicity (Fogg et al., 2014).

4.3. Long-term behavioral alterations induced by CMS are reverted by imipramine

Anhedonia, defined as a reduced ability to experience pleasure, is considered a core symptom of major depressive disorders according to the *Diagnostic and Statistical Manual of Mental Disorders*. Many studies show that CMS induces anhedonia, measured as a decrease of consumption or of preference for sucrose. In our study, CMS induced a decrease of sucrose solution intake (2.5%) per body weight, from the third week of stress up to one month after the cessation of the stress, an effect previously reported by us (Elizalde et al., 2008). Unlike the study carried out with paroxetine (Elizalde et al., 2008), the anti-anhedonic action of imipramine was manifested much earlier, from the third week of treatment (last week of CMS) and persisted even after two weeks of washout period. Thus, imipramine speeds up the recovery of the stressed-induced anhedonia, and strengthens the validity of the CMS model to study the long-term molecular effects that could be linked to this behavior and antidepressant action. Moreover, CMS mice showed along this month impaired recognition memory, decreased sociability interaction and anxiety. Importantly, this behavior was not observed in CMS mice treated with imipramine. Impaired recognition memory has been linked to abnormal function of the PFC, both in rodents (Meunier et al.,

1997; Ragozzino et al., 2002; Akirav and Maroun, 2006) and in depressed patients (Walter et al., 2007). The novel object recognition task is considered a test of episodic memory (Bertaina-Anglade et al., 2006) based on rodents' exploratory behavior and spontaneous preference for novel objects (Ennaceur and Delacour, 1988). Our results agree with clinical data showing the ability of different tricyclic antidepressant treatments (Amado-Boccara et al., 1995) to ameliorate the cognitive function in parallel to mood improvements in depressed patients. In keeping with our previous study (Venzala et al., 2012) CMS mice became less interactive toward a mouse from the same strain. Interestingly, a recent clinical study has identified in the PFC common neural substrates for anhedonia and social anxiety (Hamilton et al., 2014). Anxiety, a frequent comorbid feature of depression (Moffitt et al., 2007), was also present in the EPM in chronically stressed mice, and imipramine acted as an anxiolytic in agreement with clinical data (Nair et al., 1996). Although previous studies have shown that CMS induces anxious behavior only in the short-term using the light-dark box (Elizalde et al., 2008), here we show persistent anxiety three weeks after the stress procedure. Yet, the EPM includes, in addition to an open and illuminated area, two other anxiety-provoking environmental parameters (height and a totally open area). Thus, mild levels of anxiety might be more easily detectable in here. Many clinical evidences show that different cognitive dysfunctions, social withdrawal and anxiety are associated to anhedonia and vary as a function of the severity of the depressive disorder (Airaksinen et al., 2004; Moffitt et al., 2007; Hamilton et al., 2014). The CMS model has been extensively defined as a reliable model to test anhedonia (Willner, 2005) and the antianhedonic action of potential antidepressants. Indeed, monoaminergic antidepressants, known to increase noradrenaline and serotonin levels in the synaptic cleft, are effective to revert anhedonia (Monleon et al., 1995; Kubera et al., 2001; Harkin et al., 2002; Strekalova et al., 2006; Elizalde et al., 2008; Garcia-Garcia et al., 2009). In keeping with these studies, the underpinning chemical mechanisms for the antianhedonic action of imipramine and its counteracting effect on the altered gene expression pattern triggered by chronic stress might be somehow related. Yet, further studies should explore the link between anhedonia and other altered behaviors related to depression.

5. CONCLUSION

Persistent molecular changes observed in the PFC of a stress mouse model of long-lasting anhedonia could bring some light on the mechanisms perpetuating depressive episodes and/or establishing subthreshold symptoms that could be increasing the vulnerability for relapse. In addition, the counteracting effects of imipramine on the stress induced altered gene expression suggest molecular targets for antidepressant action. Among these, targets of the circadian rhythm pathway could be key regulators of depression and antidepressant action. Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.pbb.2015.06.001.

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Table 1S. Differentially expressed genes in the prefrontal cortex affected by chronic mild stress and ameliorated by repeated imipramine treatment contributing to the following functions.

Biological function	Genes	
	Up	Down
DNA transcription		
Decrease	***Bclaf1,Hspa8,*** *St18, Tbx22,* ***Tdg***	*Cbfa2t3, Hivep3, Hsf1, Mapre3, Mavs, Pknox1,* ***Rela, Smarcb1, Ssbp3, Zmiz2***
Activation	-	***Bhlhe40, Hdac5****,* ***Sirt7***
Decrease cell viability of neurons	-	*Cx3cl1,* ***Egr1****, Foxg1, Herpud1, Hsf1,* ***Hspb1****,* ***Rela, Vgf***
Increase neuronal cell death	***Ar,*** *Gapdh,* ***Grik2, L1cam,*** *Pdk1*	***Cntfr, Ctsv, Etv6****, Faim2, Foxg1,* ***Hdac5, Hpca,*** *Hsf1,* ***Hspb1, Mif, Nr1d1, Nr4a3, Rara, Sncb, Vegfb***
Decrease cellular growth and proliferation	***Acvr1, Apc, Csnk2, a1, Dcx, Itgb8, Lrp1b,*** *Mir181,* ***Mir218, Mir221,*** *Pparg,* ***Tek,*** *Tnfrsf8*	***Akt1s1,*** *Cd151, Cx3cl1, Foxg1, Hsf1, Igfbp2, Pcgf2, Pelp1, Pla2g6,* ***Ppp5c, Rela,*** *Rhoc,* ***Rnf5,*** *Slc7a5, Smad7,* ***Zmiz2***
Alteration movement disorders	***Grik2, Grin3a,*** *Pdk1,* ***Xk***	*Ache,* ***Brsk2****, Cacng2,* ***Cplx1****,* ***Mfsd2a, Nfix****, Pla2g6, Ppt2, Stub1, Uchl1,****Vamp2***
Alteration normal behavior	*Clock,* ***Csnk2a1***	*Cmklr1,* ***Cplx1, Ctsv, Cyp46a1, Efna2, Fosb, Hdac5,*** *Htr7,* ***Mfsd2a,Pak6, Per1, Per2, Rtn4r,*** *Sepn1,* ***Sstr2,*** *Stub1*

In bold, genes significantly ameliorated by imipramine treatment

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CAPÍTULO IV

CAPÍTULO 4

Chronic stress and antidepressant induced changes in Hdac5 and Sirt2 affect synaptic plasticity

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ABSTRACT

Changes in histone acetylation could contribute to the pathogenesis of depression and antidepressant therapy. Using the chronic social defeat stress (CSDS) model of depression and different antidepressant treatments we studied the regulation of histone deacetylases (Hdacs) and synaptic plasticity markers in the prefrontal cortex (PFC).

Mice were exposed to CSDS (10 days) followed by saline or imipramine (4 weeks). PFC Hdac's mRNA abundance was studied and compared to human's. Further, protein expression of acetylated histones (AcH3 and AcH4), neuroplasticity markers (CREB and pro-BDNF) and selected Hdacs were analysed. Moreover, other antidepressants (fluoxetine and reboxetine) and selective Hdac inhibitors were studied.

CSDS increased *Hdac5* and *Sirt2* mRNA whereas repeated imipramine did the opposite. Accordingly, stress and imipramine induced opposite changes on AcH3, AcH4 and CREB expression. At protein level, CSDS upregulated nuclear fraction of HDAC5 and repeated imipramine and reboxetine increased its phosphorylated form (P-HDAC5), mainly located in the cytoplasm. Moreover, SIRT2 was downregulated by all monoaminergic antidepressants. Further, repeated treatment with the class IIa Hdac inhibitor MC1568 and the Sirt2 inhibitor 33i for three weeks increased synaptic plasticity in the prefrontal cortex.

Our results suggest that HDAC5 and SIRT2 upregulation could constitute stable stress-induced neuronal adaptations. Noteworthy, the SIRT2 upregulation in depressed patients supports the interest of this target for therapeutic intervention. On the other hand, cytoplasmic HDAC5 export and SIRT2 downregulation induced by monoaminergic antidepressants could contribute to the well-known beneficial effects of antidepressants on brain plasticity.

Keywords: chronic social defeat stress (CSDS), histone deacetylase 5 (Hdac5), prefrontal cortex (PFC), Sirt2, imipramine, major depression.

1. INTRODUCTION

Epigenetic mechanisms regulate the gene's transcriptional potential in the absence of changes to the DNA sequence. DNA transcription can be regulated directly by methylation of DNA cytosine basis or indirectly by chemical modification of histone and non-histone proteins involved in chromatin structure (Volmar and Wahlested, 2015).

Growing evidence suggests that epigenetic mechanisms play a key role in neuronal plasticity, and, therefore, they mediate stable functional changes in the brain in response to environmental stimuli (Dulac, 2010). Specifically, it has been hypothesised that stress-mediated epigenetic changes in limbic regions, that can persist for a lifetime, could contribute to the pathogenesis of depression and other stress-related disorders such as post-traumatic stress and other anxiety disorders (Golden et al., 2013). Likewise, antidepressant therapy might be achieved in part via epigenetic mechanisms (Tsankova et al., 2006). Different epigenetic alterations have been observed in post-mortem tissue of depressed patients (Hobara et al., 2010) and of individuals who committed suicide (Keller et al., 2010). In addition, at the experimental level using animal models, stress and antidepressants induce chromatin modifications in limbic regions and induce stable changes in gene expression that affect different plasticity markers such as the brain derived neurotrophic factor (BDNF) and its upstream regulator cAMP response element binding protein (CREB) (Tsankova et al., 2006; Wilkinson et al., 2009). Moreover, epigenetic modifications could establish the basis for the interindividual variability in vulnerability to adversity and/or response to treatment (Wilkinson et al., 2009).

Among the epigenetic mechanisms, histone posttranslational modifications could contribute to the persisting abnormalities of stress-related psychopathology. Several groups have observed that, while chronic stress induce a decrease in acetylation levels of histone 3 and 4, monoaminergic antidepressants counteract this effect by decreasing the levels or the activity of some histone deacetylases enzymes (HDAC) (Tsankova et al., 2006; Covington et al., 2011; Reus et al., 2013). Interestingly several nonspecific histone deacetylase inhibitors behave as potential antidepressants in animal models (Schroeder et al., 2007; Jochems et al., 2014). Yet, little is known about the specific Hdac enzymes specially involved in mediating stress or antidepressant-induced changes in histone acetylation.

Emerging clinical interest in the mechanisms that link exposure to stressful situations to the perpetuation of depressive disorders and/or increased vulnerability to relapse (Kendler et al., 1999; Mazure et al., 2000) has stimulated the study of different animal models aiming to identify stable neuroadaptations induced by stress (Krishnan et al., 2007). The chronic

social defeat stress (CSDS) model has its theoretical rationale in the induction of "social subordination" caused by short periods of struggle with a dominant animal followed by fellowship with it in an adyacent cage. Our laboratory and others have evaluated the long term effects of CSDS in different behavioral tests (Becker et al., 2008; Venzala et al., 2012). Specifically, up to four-five weeks after the cessation of the social defeat procedure, we have observed anhedonia, a core symptom of clinical depression, i.e., loss of interest in normally rewarding stimuli, but also anxiety and social avoidance, signs also related to anxiety and posttraumatic stress disorders (Venzala et al., 2012; 2013). Interestingly, anhedonia and some of these behaviors induced by CSDS have been effectively reversed or prevented by different monoaminergic antidepressants supporting the predictive validity of this model (Venzala et al., 2012).

The behavioral deficits induced by CSDS could be related to neuroadaptive changes in areas such as the prefrontal cortex, which, is compromised in depressed patients. The prefrontal cortex is a target limbic region for stress response. Changes in this region directly affect reactivity to rewards and anxiety (Davidson, 2002). In addition, it might also promote maladaptive behavioral responses to subsequent stresses (Elizalde et al., 2010). For instance, our CSDS model induces long-lasting neurochemical alterations in this region (Venzala et al., 2013) that have been associated to anhedonia (Banasr et al., 2010). In agreement with this idea, anhedonia has been shown to correlate with a dysfunction of the prefrontal cortex in depressed patients (Keedwell et al., 2005; Germine et al., 2011).

Here we studied the regulation of the expression of the different classes of HDACs by CSDS and imipramine treatment. Functional implications of HDAC regulation by stress, antidepressant treatments and specific HDAC inhibitors were explored. Comparatively, mRNA expression of Hdac superfamily was studied in human post-mortem prefrontal cortex of depressed patients.

2. EXPERIMENTAL PROCEDURES

2.1. Human samples

Human mRNA samples were obtained from the Stanley Foundation. Total mRNA from prefrontal cortex of thirty patients (nine controls, eleven depressed patients and ten depressed with psychosis patients) was studied. Those samples whose mRNA was degraded was excluded from the study.

All individuals were of white race. The averages of the ages were 45.3 ± 4.3, 44.5 ± 2.5 and 40.1 ± 3.3 for controls, depressed and depressed with psychosis respectively. The duration of the illness showed an average of 12.5 ± 2.6 and of 11.8 ± 1.5 years for both depressed groups (Supplementary table S1). The cause of death was cardiovascular disease for controls and suicide for both depressed groups. The group of depressed patients with psychosis was being treated with different antipsychotics. The intervals from estimated time of death to refrigeration of body and tissue extraction (post-mortem interval, PMI) were 7.7± 1.1 and 27.7 ± 1.9 hours respectively. The possible influence of the PMI on the analysis, as well as the use of antipsychotics in some patients was studied as possible confounding variables. Data analysis was performed based on the diagnosis of the disease and on the use of antipsychotics.

2.2. Animals and drug treatment

Male C57BL/6 mice (Harlan, France, 8-10 weeks of age) were housed in individual cages and allowed for one week to habituate before beginning experimentation. Food and water were available ad libitum for the duration of the experiments unless otherwise specified. Animals were maintained in a temperature (21±1°C) and humidity-controlled room (55±2%) on a 12 h light-dark cycle (lights on at 08:00 h).

Male CD1 retired breeders (Charles River, UK, older than 5 months of age) were used as residents for the CSDS procedure.

Experimental procedures and animal husbandry were conducted according to the principles of laboratory animal care as detailed in the European Communities Council Directive (2003/65/EC), Spanish legislation (Real Decreto 1201/2005) and approved by the Ethical Committee of University of Navarra.

Imipramine HCl (Sigma-Aldrich, USA) was dissolved in saline (0.9%) and administered i.p. at 10 mg/kg once daily. Fluoxetine HCl (Interchim, Montluçon, France) and

reboxetine HCl (kindly donated by Servier SL, Paris, France) were dissolved in saline (0.9 %) and administered i.p. at 15 and 10 mg/kg respectively once daily. At these doses, imipramine has shown to effectively counteract the effects of social defeat stress in mice (Berton et al., 2006) and both fluoxetine and reboxetine show antidepressant-like action in the forced swimming test (Dhir and Kulkami, 2007; Rogóz, 2009).

The compound 33i, 2-{3-(3-fluorophenethyloxy)phenylamino}benzamide, (kindly donated by Dr Suzuki from Kyoto Prefectural University of Medicine, Japan) and the compound MC1568, 3-[5-(3-(3-fluorophenyl)-3-oxopropen-1-yl)-1-methyl-1H-pyrrol-2-yl]-N-hydroxy-2-propenamide, (kindly donated by Dr Mai, Sapienza University of Rome, Italy) were prepared in suspension using 0.5% tween 80 in water, and administered i.p. at 1 mg/kg and 10 mg/kg once daily. The compound MC1568 is a class II-selective histone deacetylase inhibitor (Mai et al., 2005), able to inhibit HDAC4-6 at 5 μM without changes in HDAC1-3 activities. The compound 33i is a selective and specific class III (SIRT2) Hdac inhibitor with IC50 of 570 nM towards SIRT2 and no affinity for SIRT1 or SIRT3 (Suzuki et al., 2012). The dose was chosen based on a preliminary study carried out in our laboratory that showed an increase in acetylation levels of histone 4 (AcH4) in the hippocampus of the same mouse strain after the treatment (data not shown).

2.3. Experimental design

Three independent experiments were carried out with mice (C57BL/6) (n=8-9 mice/group).

First experiment: Before starting the stress model, mice were assigned to four different groups (saline, imipramine, CSDS-saline, CSDS-imipramine). Mice were exposed to the chronic social defeat stress (CSDS) procedure for 10 days. During this time, CSDS mice were housed individually in cages with two compartments separated by a metallic mesh, with a dominant mouse (CD1) in the second compartment. On the other hand, control mice were singly housed in similar cages with a mouse of the same strain in the other half of the cage. Twenty-four hours after the last session, both control and defeated mice were housed individually for the following four weeks and mice received daily (i.p.) injections of imipramine (10 mg/kg) or saline (at 1 p.m.) (Figure 1). Mice were killed by cervical dislocation 21-24 h after the last drug injection.

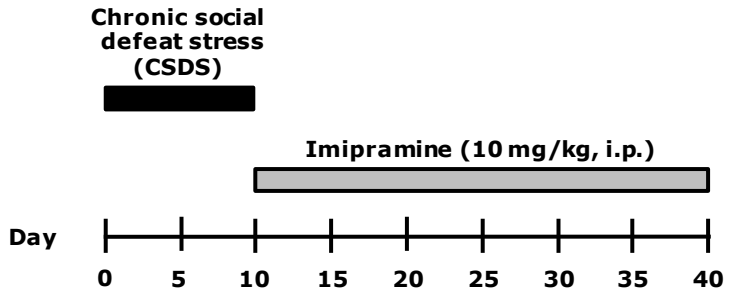

Fig. 1. Experimental design of chronic social defeat stress CSDS procedure and imipramine treatment

Second experiment: The second experiment comprised four groups of mice (n=8/group) treated once daily (at 10 a.m.) with saline (i.p.), imipramine (10 mg/kg), fluoxetine (15 mg/kg, i.p.) or reboxetine (10 mg/kg, i.p.) for three weeks. Mice were killed by cervical dislocation 2 h after the last drug injection.

Third experiment: In the third experiment, mice were treated once daily (at 10 a.m.) with either saline (i.p.), MC1568 (10 mg/kg, i.p.) or 33i (1 mg/kg, i.p.) for three weeks. Mice were killed by cervical dislocation 2 h after the last drug injection.

For the dissection, an acrylic mouse brain slicer matrix was used with 1.0 mm coronal slice intervals (Zivic Instruments, Pittsburgh, PA, USA). Using a mouse brain atlas (Hof et al., 2000), 1 mm slice containing the infralimbic section of the PFC (bregma 2.20 mm through bregma 1.20 mm) was taken and dissected out bilaterally using a scalpel and kept at -80 ºC for neurochemical studies.

2.4. Chronic social defeat stress procedure

CSDS procedure was carried out using a similar method described by Tsankova et al. (2006). Briefly, mice were submitted to social defeat stress for ten consecutive days. Every day, each mouse was introduced into the home cage of an unfamiliar resident. Resident mice were CD1 retired breeders selected for their attack latencies reliably shorter than 30s upon three consecutive screening tests. Once the experimental mouse had been physically defeated by three attacks, both animals (defeated and aggressor) were maintained in sensory contact for 24h using a metallic mesh dividing the resident home cage in two halves. During the ten days, control mice were individually housed in equivalent cages but with members of the same strain in the opposite half. On day 11, all mice were individually housed and treatments were initiated.

2.5. RT-PCR studies

2.5.1. Extraction of RNA and RT-PCR

Total RNA was isolated separately from each individual frozen prefrontal cortex sample. Isolation of total RNA was carried out according to manufacturer's instructions (NucleoSpin RNA II kit, Macherey-Nagel, Germany). Reverse transcription was performed using random hexamers as primers and Superscript reverse transcriptase III (Invitrogen, Cergy Pontoise, France). The eluates were stored at -20 °C.

In order to study the expression of selected genes in the prefrontal cortex of mice and patients TaqMan Low Density Arrays (TLDA) microfluidic card technology from Applied Biosystems (Foster City, CA, USA) was used. As previously described by Tordera et al., 2011), for each tissue sample, 500 ng of reverse-transcribed RNA were diluted to 50 µL with sterile water, combined with an equal volume of TaqMan Universal PCR Master Mix (2x; Applied Biosystems, Foster City, CA, USA), mixed by inversion, and spun briefly in an Eppendorf® 5415C microcentrifuge (Brinkmann Instruments, Westbury, NY, USA). After TLDAs were brought to room temperature, 100 µL master mix were loaded into each port connected to reaction wells. TLDAs were placed in Sorvall®/Heraeus® Custom Buckets (Applied Biosystems, Foster City, CA, USA) and centrifuged in a Sorvall Legend™ centrifuge (Kendro Scientific, Asheville, NC, USA) for 1 min at 331 x g followed closely by a second 1-min centrifugation at 331 x g. Cards with excess sample in the fill reservoir were spun for an additional 1 min. Immediately following centrifugation, the cards were sealed with a TaqMan LDA Stylus Staker (Applied Biosystems, Foster City, CA, USA), and the loading ports excised. Quantitative RT-PCR amplifications were run on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a TaqMan LDA cycling block and an automation accessory upgrade. Thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each test sample was processed in duplicate on individual TLDA cards, thus allowing eight samples to be processed on each card.

2.5.2. Analysis of quantitative RT-PCR data

RT-PCR TaqMan instrumentation monitors gene-specific products with fluorescent dye chemistry. A cycle threshold (CT) for each reaction is the number of cycles at which the reaction crosses a selected threshold. The threshold is defined as a straight line drawn above noise/baseline and positioned within the linear region of the semi-log amplification plot. The fewer cycles required to reach threshold fluorescence intensity, the lower the CT value and

the greater the initial amount of input target. Results for each target on TLDAs were quantified concurrently using the same baseline and threshold for a target gene in order to limit interplate errors in the analysis.

Samples were analysed by a double delta CT (ΔΔCT) method (Livak and Schmittgen, 2001). Delta CT (ΔCT) values represent normalized target genes levels with respect the internal control. Normalization was based on a single reference housekeeping gene (18s). Among other housekeeping genes, 18s has shown to be very little affected by acute and/or chronic stress in the central nervous system (Derks et al. 2008). Delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample (imipramine, CSDS-saline and CSDS imipramine, in CSDS; depressed and depressed with psychosis, in patients) minus the mean ΔCT of the calibrator samples (control saline in CSDS; control in patients) for each target gene. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$.

2.6. Western blotting studies

2.6.1. Preparation of the protein extracts

Prefrontal cortex was homogenized in a cold lysis buffer with protease inhibitors (0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 200 mM NaF, 2 mM Na4P2O7, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 1 mM Na3VO4, 1 mM benzamidine, 10 mg/mL leupeptin, 400 U/mL aprotinin) as described elsewhere Cuadrado-Tejedor et al., (2010). The homogenate was centrifuged at 14,000 x g at 4 ºC for 20 min and the supernatant aliquoted and stored at -80 ºC. Total protein concentrations were determined using the BioRad Bradford protein assay (BioRad Laboratories).

For determination of nuclear HDAC5 abundance, nuclear fractions were prepared as previously described by Barros-Minones et al. (2013). Briefly, tissue were gently homogenized by 20 strokes in a glass-Teflon Potter homogenizer on ice in 200 µL of Buffer A (10 mM Tris, pH 7.4, 320 mM sucrose, 1 mM EDTA and 1 mM DTT) containing phosphatase inhibitors (Phosphatase Inhibitor Cocktail I, Sigma-Aldrich, Madrid, Spain) and protease inhibitors (Protease Inhibitor Cocktail Set I, Animal-Free Aprotinin, Calbiochem, Darmstadt, Germany). Homogenates were then centrifuged at 1500g for 5 min at 4°C. Pellets were resuspended in 80µL of Buffer B (150mM NaCl, 10 mM Tris, pH 8.5, 1.5 mM MgCl , 0.5% Nonidet, 1 mM DTT, containing phosphatase and protease inhibitors) and were centrifuged twice at 1500g for 5 min at 4°C to obtain the nuclear fraction. Lamin A/C (2032 Cell Signaling Technology, Beverly, MA) was used as a nuclear marker. The absence of α-tubulin detected by Western blot indicated successful nuclear fractionation.

2.6.2. Western blot

Equal amounts of protein (20 µg per lane) were separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel (8%) under reducing conditions and transferred onto a nitrocellulose membrane (Hybond- ECL; Amersham Bioscience). The trans-blots were blocked for 1 h with 10% not-fat milk in buffer TBS containing 0.1% Tween 20 and then probed overnight at 4 ºC with one of the following primary antibodies: rabbit polyclonal anti-phosphorylated histone deacetylase 5 (1:1,000, Applied Biological Materials, Richmond, BC, Canada); mouse monoclonal anti-histone deacetylase 5 (1:1,000), rabbit monoclonal anti-sirtuin 2 (1:1,000) and mouse polyclonal anti-acetylated α-tubulin (1:10,000) (Sigma-Aldrich, St. Louis, MO, USA). rabbit polyclonal anti-acetylated histone 3 (1:1,000) and histone 4 (1:2,000) and rabbit polyclonal anti-brain derived neurotrophic factor, pro (1:1,000) (Merck Millipore, Billerica, MA, USA), rabbit monoclonal anti-CREB (1:1,000) (Cell Signaling Technology, Danvers, MA, USA) and rabbit polyclonal anti-β-actin (1:10,000, Sigma- Aldrich, St. Louis, MO, USA). The specificity of epigenetic antibodies was tested previously (Beirowski et al., 2011; Sando et al., 2012). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies (1:10,000; DAKO, Cambridgeshire, United Kingdom) were used. Proteins were visualized using an enhanced chemiluminescence (ECL™) Western blotting detection reagent (Amersham, Buckinghamsire, England) and band intensity was estimated densitometrically on a GS-800 calibrated densitometer (Biorad One, Madrid, Spain). β-actin was used as internal control.

Results were calculated as the percentage of optical density values of the control saline-treated mice.

2.7. Statistical analysis

In the first experiment, both RT-PCR and western-blot studies were analysed using two-way ANOVA (stress, treatment) analysis followed by Tukey *post hoc*. When no significant interaction between two factors was found, main effects were considered.

The effect of different antidepressants in protein expression studies (experiment 2) was analysed by one-way ANOVA followed by Dunnett *post hoc*.

The effect of MC1568 and 33i in protein expression studies compared to saline treated group was analysed by Student *t* test.

The mRNA abundance in post-mortem prefrontal cortex from human samples (controls, depressed patients and depressed with psychosis patients) was analysed using

one-way ANOVA followed by Dunnett test. The possible influence of the post-mortem interval (hours) and the use of antipsychotics (months of treatment) in some patients in Hdac´s mRNA abundance was studied using these variables as confounding variables in an univariate analysis.

Group differences were considered statistically significant at $p<0.05$. Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 15.0, SPSS Chicago, IL, USA).

3. RESULTS

3.1. Effect of CSDS and imipramine on Hdac's mRNA superfamily

The mRNA expression of the Hdac's superfamily was studied on prefrontal cortex of control mice and mice sacrificed one month after CSDS exposure, one day after the last drug administration. Both control and CSDS mice received a chronic treatment of either saline or imipramine (10 mg/kg, i.p.) for four weeks. We observed a significant effect of stress in the expression of the histone deacetylases of class I, *Hdac8* ($F_{1,31}$=8.23, p<0.01) (Figure 2A), class II, *Hdac5* ($F_{1,31}$=24.36, p<0.01) and *Hdac7* ($F_{1,31}$=5.98, p<0.05) (Figure 2B) and class III, *Sirt2* ($F_{1,31}$=6.71, p<0.05), *Sirt3* ($F_{1,31}$=5.30, p<0.05) and *Sirt6* ($F_{1,31}$=8.19, p<0.01) (Figure 2C). Specifically, we observed that CSDS induced a significant upregulation of the mRNA encoding these enzymes when compared to their corresponding non stressed groups.

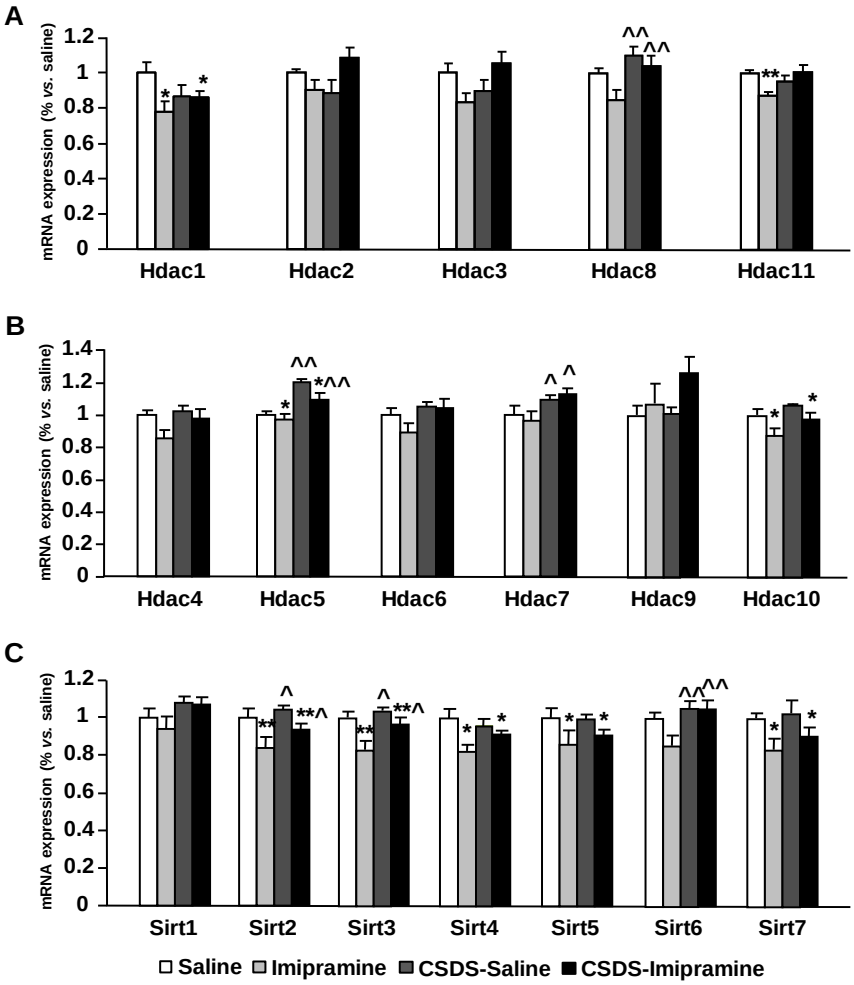

Fig. 2. Effect of chronic social defeat stress (CSDS) and imipramine treatment (10 mg/kg, i.p., 4 weeks) on mRNA abundance of histone deacetylase enzymes (Hdacs) of **(A)** class I and IV, **(B)** class II and **(C)** class III in the prefrontal cortex. Data show average±SEM of relative abundance of mRNA expression compared to control saline group values. ^^p<0.01, ^p<0.05 main effect of stress; **p<0.01, *p<0.05, main effect of treatment (Two-way ANOVA).

In addition, a significant effect of imipramine treatment was shown on the mRNA abundance of the histone deacetylases of class I and IV, *Hdac1* ($F_{1,31}$=4.99, p<0.05) and *Hdac11* ($F_{1,31}$=9.91, p<0.01), respectively (Figure 2A), class II, *Hdac5* ($F_{1,31}$=5.77, p<0.05) and *Hdac10* ($F_{1,31}$=7.04, p<0.05) (Figure 2B) and class III, *Sirt2* ($F_{1,31}$=10.54), *Sirt3* ($F_{1,31}$=11.04, p<0.01), *Sirt4* ($F_{1,31}$=8.08, p<0.05), *Sirt5* ($F_{1,31}$=4.35, p<0.05) and *Sirt7* ($F_{1,31}$=8.15, p<0.05) (Figure 2C). In all these enzymes the mRNA levels appeared to be downregulated by repeated imipramine treatment compared to their corresponding saline treated groups.

3.2. Acetylation levels of histone 3 and 4 by CSDS and imipramine

In order to investigate the functional implications of the modifications induced by CSDS and imipramine treatment observed above, the acetylated levels of histones 3 (AcH3) and histone 4 (AcH4) were measured by Western blot. We observed an effect of stress in the abundance of acetylation levels of both histone 3 ($F_{1,31}$=4.48, p<0.05) and histone 4 ($F_{1,31}$=5.6, p<0.05). In addition, a significant effect of imipramine treatment in both AcH3 ($F_{1,31}$=21.8, p<0.01) and AcH4 ($F_{1,31}$=22.7, p<0.01) levels was observed. While, mice exposed to CSDS showed a decrease in cortical AcH3 and AcH4 when compared to their corresponding non stressed mice, repeated imipramine induced a marked upregulation in the acetylated levels of these histones (Figures 3A and 3B).

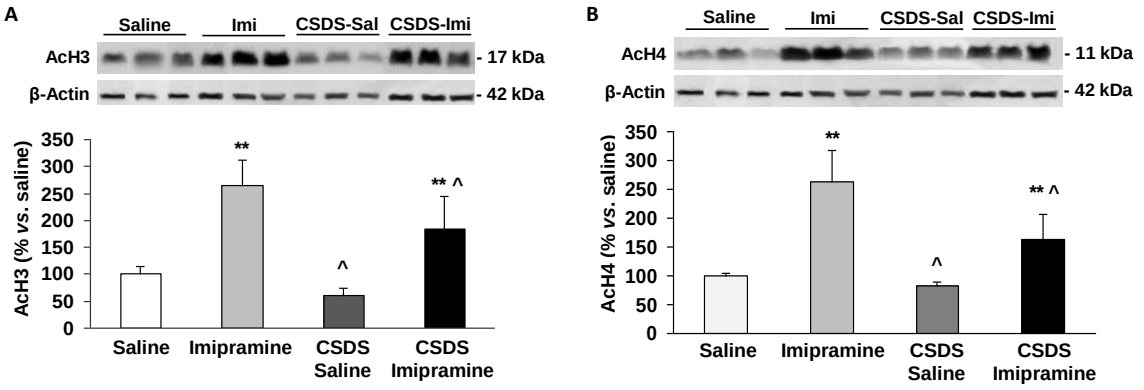

Figure 3. Quantitative measurements and representative blots showing the expression of the acetylation of histone 3 (AcH3) **(A)** and histone 4 (AcH4) **(B)** following CSDS and imipramine treatment. β-actin was used as equal loading control. Data show average ± SEM of relative abundance of protein expression compared to control saline group values. ^p<0.05, main effect of stress; **p<0.01, main effect of treatment. (Two-way ANOVA).

3.3. Regulation of CREB and pro-BDNF by CSDS and imipramine

Subsequently, it was checked whether changes in histone acetylation correlate with changes in the expression of two synaptic plasticity markers, CREB and pro-BDNF. Western blot analysis of CREB protein levels in the prefrontal cortex, revealed an effect of stress ($F_{1,31}$=5.77, p<0.05) and imipramine treatment ($F_{1,31}$=26.82, p<0.01) (Figure 4A). Similarly, an effect of imipramine treatment was observed in pro-BDNF protein expression levels ($F_{1,31}$=15.70, p<0.01) (Figure 4B). While CSDS decreased CREB levels, imipramine treatment induced an upregulation of both CREB and pro-BDNF protein levels.

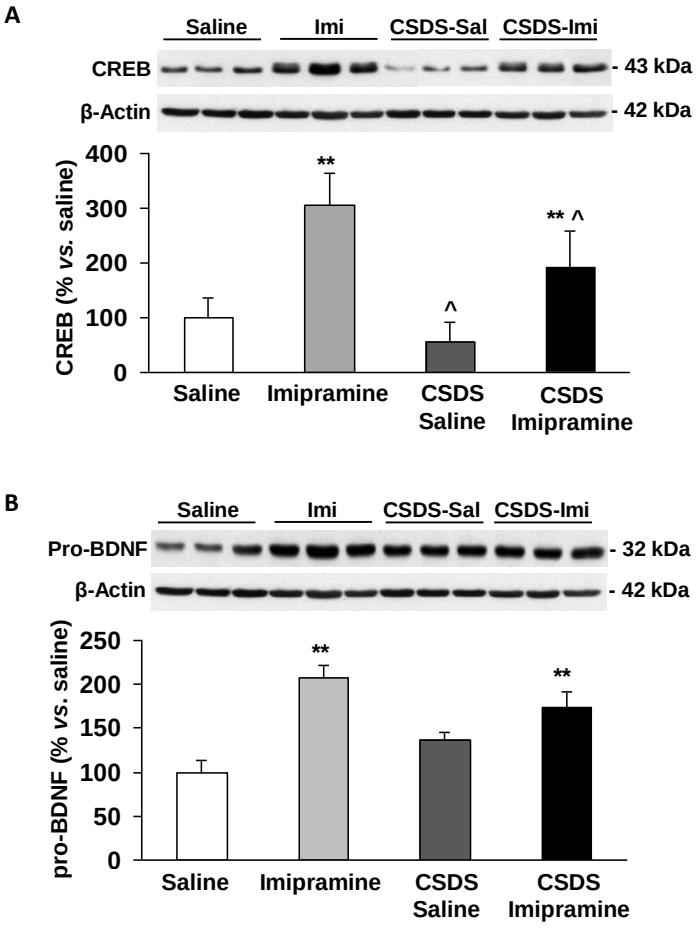

Figure 4. Quantitative measurements and representative blots showing the expression of CREB **(A)** and pro-BDNF **(B)** after CSDS and imipramine treatment. β-actin was used as equal loading control. Data show average ± SEM of relative abundance of protein expression compared to control saline group values. ^p<0.05, main effect of stress; **p<0.01 main effect of treatment. (Two-way ANOVA).

3.4. Effect of stress and antidepressants on HDAC5 expression

Given the observed opposite regulation of *Hdac5* mRNA by CSDS and imipramine treatment we further studied this regulation at protein level. No significant changes were observed in the expression levels of HDAC5 in stressed (CSDS) or imipramine treated mice (data not shown). However, when measured the abundance of this protein in nuclear protein extracts, a significant effect of stress ($F_{1,31}$=5.76, p<0.05) was shown. Specifically, CSDS upregulated nuclear HDAC5 content in the prefrontal cortex of mice. This increase was more evident in mice exposed to CSDS and treated with saline (Figure 5A) than in those treated with imipramine. In addition, Western analysis of the phosphorylated form of this protein (P-HDAC5), showed a significant effect of imipramine treatment ($F_{1,31}$=9.3, p<0.01). Imipramine treatment induced a marked upregulation of P-HDAC5 compared to their corresponding saline treated groups (Figure 5B).

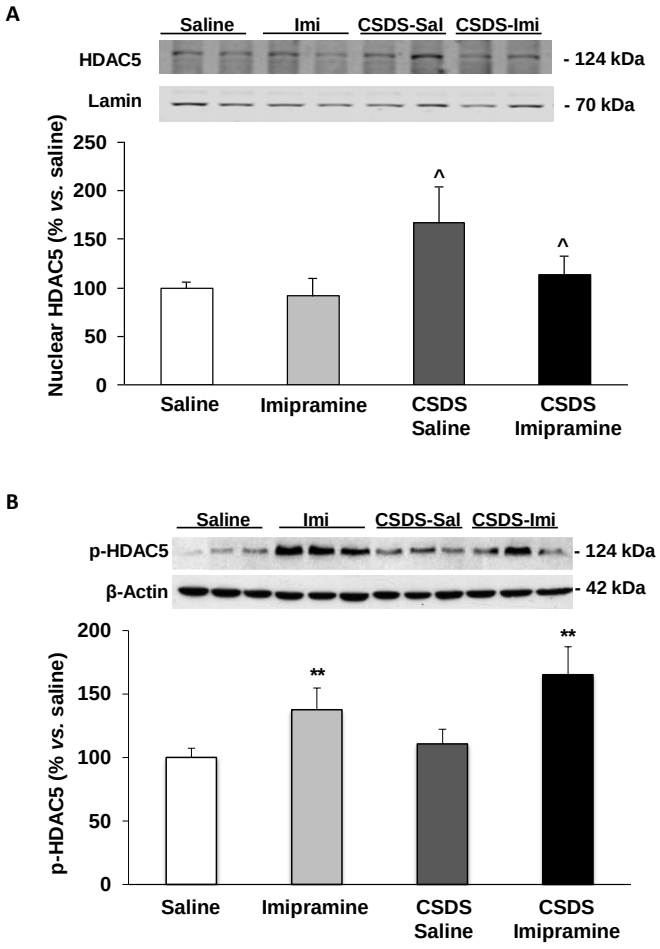

Figure 5. Quantitative measurement and representative blots showing the expression of **(A)** nuclear HDAC5 and **(B)** P-HDAC5 after CSDS and imipramine treatment. Lamin and β-actin were used as equal loading controls respectively. Data show average ± SEM of relative abundance of protein expression compared to control saline group values. ^p<0.05 main effect of stress; **p<0.01 main effect of treatment (Two-way ANOVA).

In addition, we studied the effect of the selective serotonin reuptake inhibitor fluoxetine (15 mg/kg, i.p.), noradrenaline reuptake inhibitor reboxetine (10 mg/kg, i.p.) and the tryciclic imipramine (10 mg/kg, i.p.), once daily for 21 days, on the PFC expression of HDAC5 and its phosphorylated form (P-HDAC5) (Figure 6). While no effect of drug treatment was observed in HDAC5 expression (Figure 6A), a significant increase ($p<0.05$) in the abundance of P-HDAC5 in mice treated with imipramine and reboxetine was observed ($F_{3,28}=3.48$, $p<0.05$) (Figure 6B).

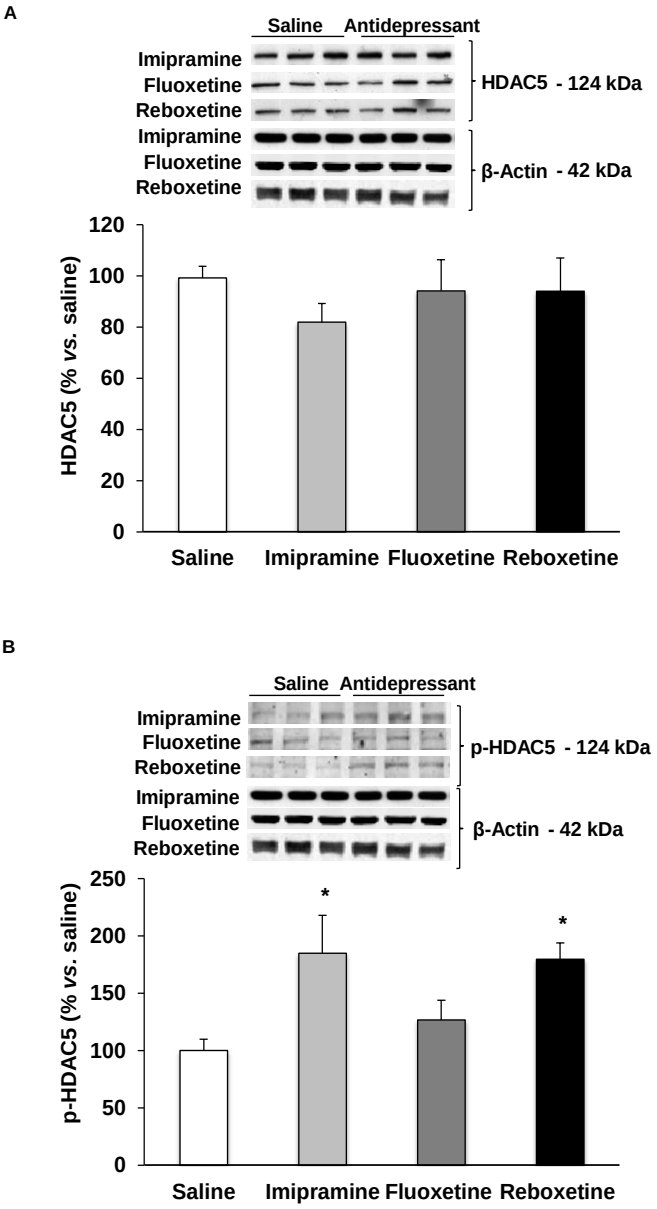

Figure 6. Quantitative measurement and representative blots showing the effect of repeated imipramine, fluoxetine and reboxetine on **(A)** HDAC5 and **(B)** P-HDAC5 expression in the prefrontal cortex. Data show average ± SEM of relative abundance of protein expression compared to saline group values. *p<0.05 *vs* saline treated group (One-way ANOVA followed by Dunnett test).

3.5. Effect of the class II HDAC inhibitor (MC1568) on synaptic plasticity

Subsequently, we aimed to study the possible functional implication of Hdac5 inhibition. Since no selective compound towards this enzyme exist yet, the specific class II HDAC inhibitor MC1568 (Mai et al., 2005) was selected. The effect of MC1568 (10mg/kg, i.p. once daily for 21 days) in AcH3 and AcH4 as well as the expression of CREB and pro-BDNF in the prefrontal cortex of mice was studied (Figure 7). It can be observed that, this compound induced a significant upregulation ($p<0.05$) of AcH3, AcH4 and pro-BDNF in the prefrontal cortex of mice. Moreover, a tendency to increase CREB was observed (Student *t* test).

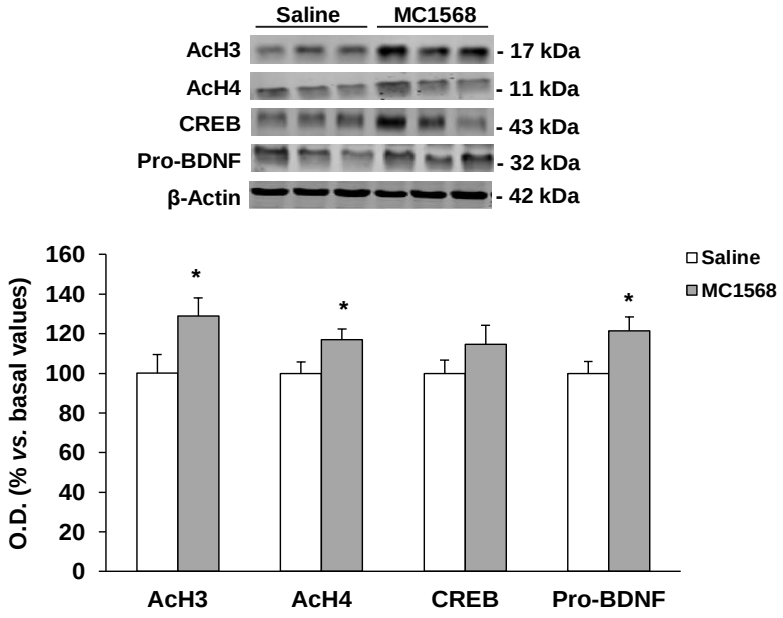

Figure 7. Quantitative measurement and representative blots showing the effect of repeated treatment with MC1568 on AcH3, AcH4, CREB and pro-BDNF expression in the prefrontal cortex. Data show average ± SEM of relative abundance of protein expression compared to saline group values. *p<0.05 *vs* saline treated group (Student *t* test).

3.6. Effect of stress and antidepressants on SIRT2 expression

The opposite regulation of *Sirt2* mRNA by CSDS and imipramine treatment was further studied at protein level. A significant effect of stress ($F_{1,31}=4.51$, $p<0.05$) and of imipramine treatment ($F_{1,31}=13.9$ $p<0.01$) was observed on SIRT2 expression levels. Specifically, we observed an upregulation of this sirtuin in mice exposed to CSDS. Conversely, imipramine treatment downregulated SIRT2 (Figure 8A).

The acetylation levels of α-tubulin, a substrate of SIRT2, revealed an interaction between stress and treatment ($F_{1,31}$=6.95, p<0.05) (Figure 8B). Repeated imipramine treatment upregulated (p<0.01) the expression of acetylated α-tubulin in non-stressed mice.

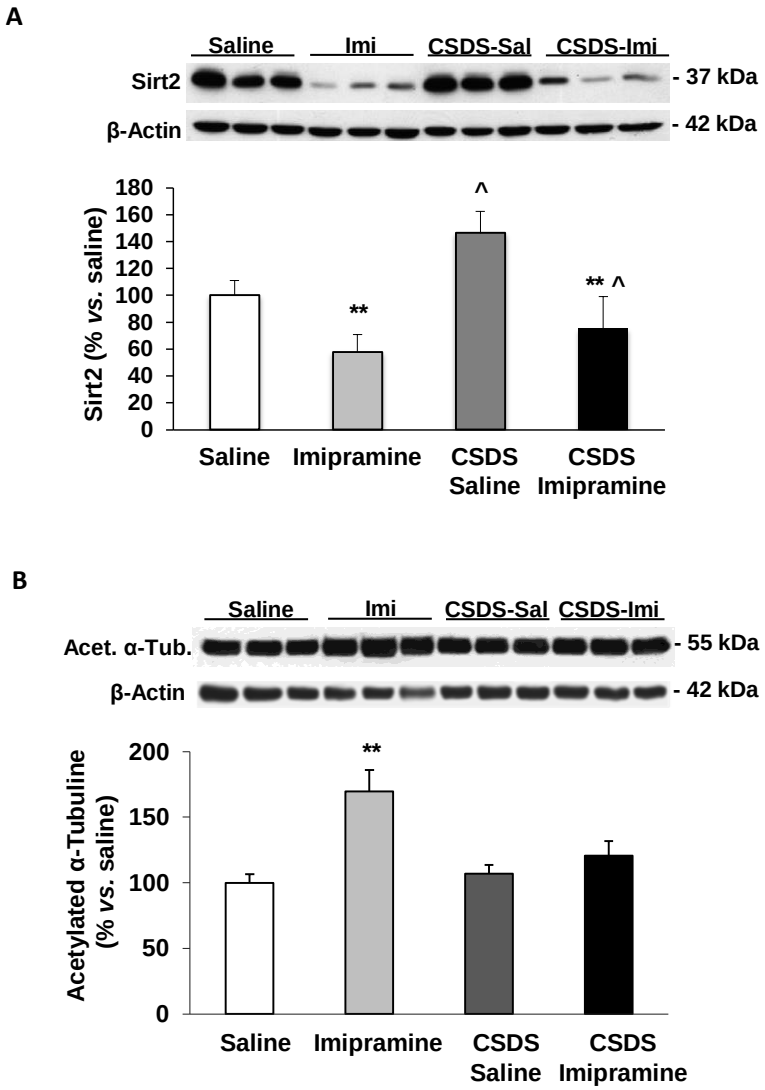

Figura 8. Quantitative measurement and representative blots showing the effect of chronic social defeat stress (CSDS) and imipramine treatment on **(A)** sirtuin 2 (SIRT2) and **(B)** acetylated α-tubulin protein expression levels in prefrontal cortex. Data show average ± SEM of relative abundance of protein expression compared to control saline group values. In A, ^p<0.05 main effect of stress; **p<0.01 main effect of treatment (Two-way ANOVA). In B, **p<0.01 *vs* saline control (Two-way ANOVA followed by Tukey-*post hoc* test).

In addition, the effect of the three antidepressant treatments (imipramine, fluoxetine and reboxetine) in the abundance of SIRT2 and acetylated α-tubulin in the prefrontal cortex of mice was studied. One-way ANOVA revealed a significant effect of antidepressant treatment on SIRT2 ($F_{3,28}$=5.6, p<0.01) and acetylated α-tubulin ($F_{3,28}$= 5.7, p<0.01) expression levels. Specifically, the repeated administration of the three antidepressants induced a significant downregulation of SIRT2 expression (p<0.05) compared to saline treated mice (Figure 9A). Acetylated α-tubulin expression was upregulated (p<0.01) by repeated reboxetine. Although no statistically significant, a tendency to increase acetylated α-tubulin was observed in both imipramine and fluoxetine treated groups (Figure 9B).

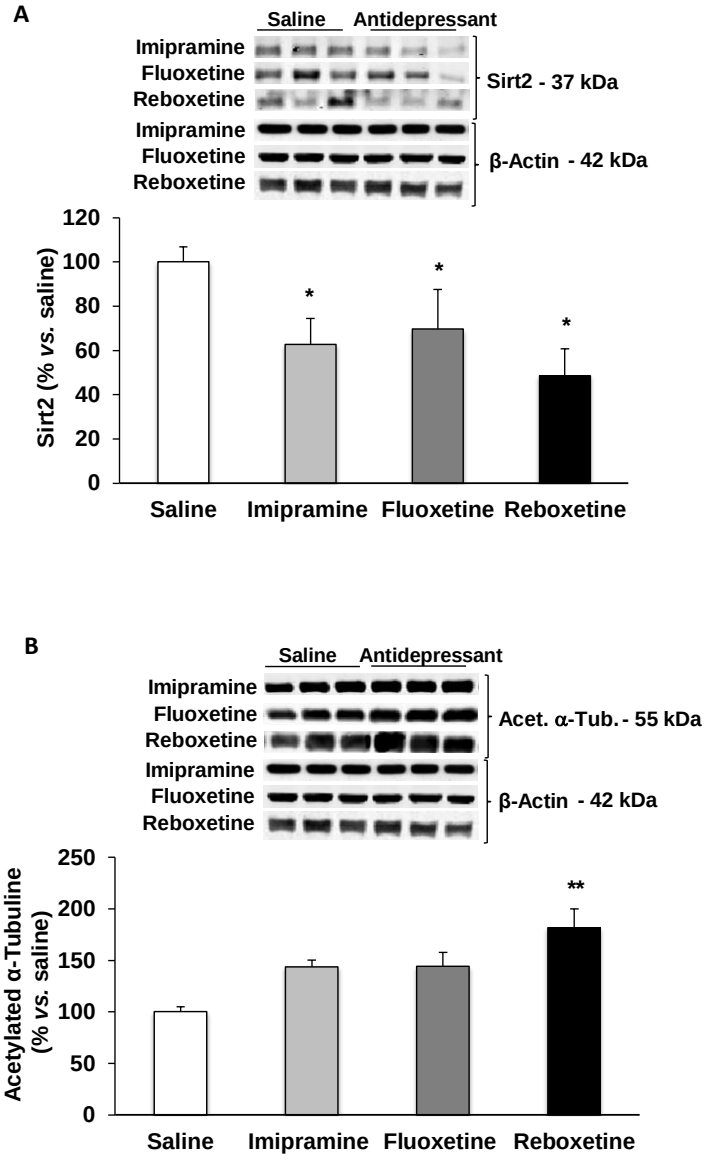

Figure 9. Quantitative measurement and representative blots showing the effect of repeated imipramine, fluoxetine and reboxetine on **(A)** SIRT2 and **(B)** acetylated α-tubulin expression in the prefrontal cortex. Data show average ± SEM of relative abundance of protein expression compared to saline group values. β-actin was used as equal loading control **p<0.01, *p<0.05 *vs* saline treated group. (One-way ANOVA followed by Dunnett test).

3.7. Effect of SIRT2 inhibition on synaptic plasticity

The possible functional implication of SIRT2 inhibition was studied here. The effect of the Sirt2 inhibitor 33i (Suzuki et al., 2012) (1 mg/kg, i.p. once daily for 21 days) on the expression levels of acetylated histones 3 (H3) and 4 (H4) as well as the expression of CREB and pro-BDNF in the prefrontal cortex of mice was studied. We observed that 33i induced a significant upregulation ($p<0.05$) of AcH4, CREB and pro-BDNF expression (Student *t* test) (Figure 10).

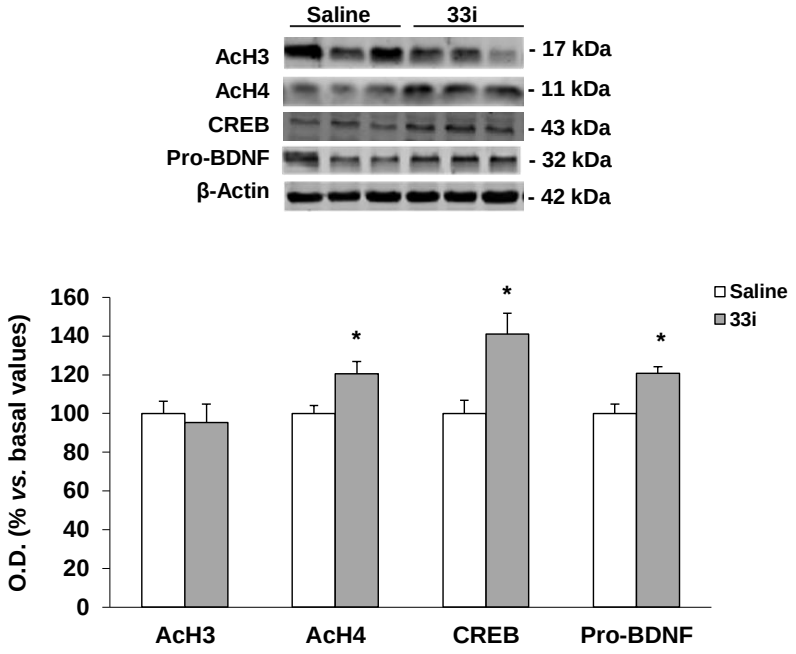

Figure 10. Quantitative measurement and representative blots showing the effect of repeated treatment with 33i on AcH3, AcH4, CREB and pro-BDNF expression in the prefrontal cortex. Data show average ± SEM of relative abundance of protein expression compared to saline group values. *$p<0.05$ *vs* saline treated group. (Student *t* test).

3.8. Expression of Hdac's mRNA superfamily on depressed patients

RT-PCR studies on prefrontal cortex of depressed patients (*post mortem* tissue) with or without psychosis showed significant changes among the groups for the expression of *HDAC11* ($F_{2,27}= 5.57$, $p<0.01$) and for *SIRT2* ($F_{2,27}= 3.56$, $p<0.05$). Posterior analysis revealed an upregulation ($p<0.05$) of the mRNA abundance for these enzymes in the group of depressed patients (Figure 11). However, the group of depressed patients with psychosis showed similar values of expression to the control group. We next studied whether the use of antipsychotics in most of the depressed patients with psychosis could be a confounding variable affecting the mRNA expression of Hdac's. Univariate analysis using months of treatment with antypsychotics as a confounding variable showed that expression of *HDAC11*

($F_{2,29}$=5.51, p<0.05) and SIRT2 ($F_{2,29}$=3.73, p<0.05) was affected by the use of antypsychotics. On the other hand, the mRNA expression of the rest of Hdacs studied was not affected by this factor.

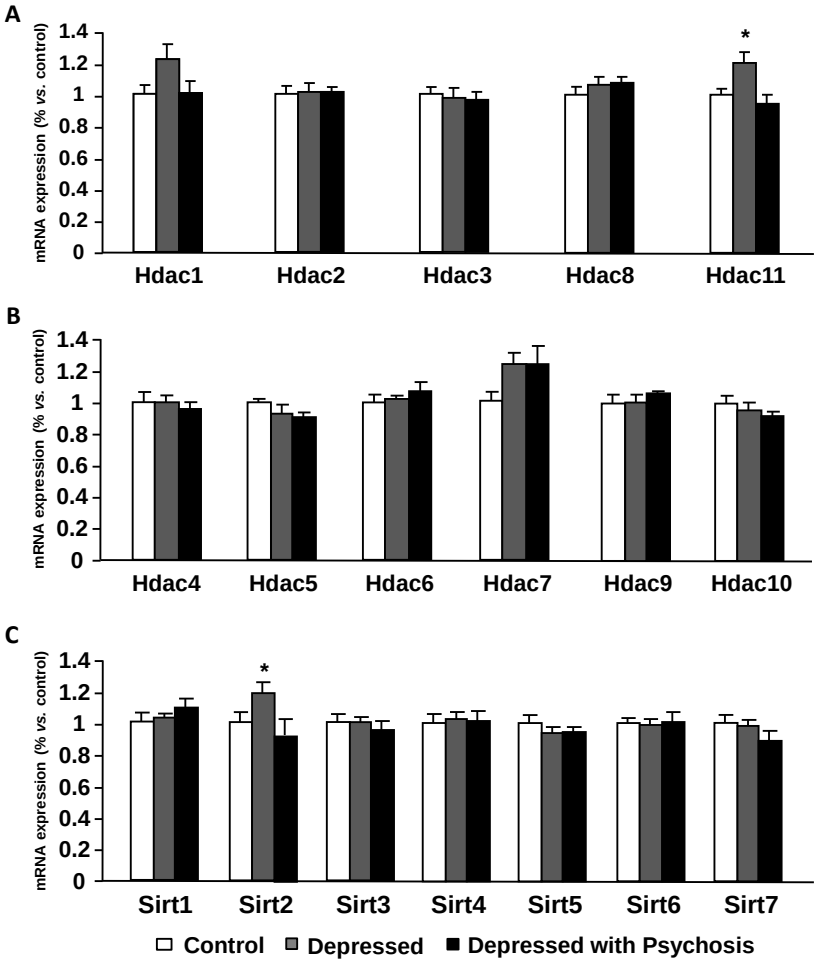

Figure 11. Abundance of mRNA of the histone deacetylase enzymes of **(A)** class I and IV, **(B)** class II and **(C)** class III in the prefrontal cortex of depressed patients. Data show average ± SEM of relative abundance of mRNA expression compared to control values. *p<0.05 *vs* control group. (One-way ANOVA followed by Dunnett *post hoc* test). Data analysis was performed based on the diagnosis of the disease.

The possible impact of *post-mortem* interval (PMI) on mRNA expression was also studied. Only in the case of *HDAC11* expression, a significant effect was found ($F_{2,29}$=3.77, p<0.05). Correlation studies showed that the higher PMI the lower expression of this enzyme. No effect was observed in the rest of enzymes.

Moreover, depressed patients using antipsychotics (depressed or depressed with psychosis) did not show a different regulation in Hdac's expression compared to patients free of antipsychotic treatment.

4. DISCUSSION

These results show that the chronic social defeat stress (CSDS) model and antidepressant treatment induced epigenetic changes in histone remodeling affecting the expression of genes involved in neuronal plasticity. Specifically, we identified two enzymes, HDAC5 (class II) and SIRT2 (class III) that were regulated by stress and antidepressant drugs in the prefrontal cortex of mice. It is suggested that the observed HDAC5 and SIRT2 upregulation could contribute to stable stress-induced neuronal adaptations. Noteworthy, the SIRT2 upregulation in depressed patients supports the interest of this target for therapeutic intervention. In addition, repeated reboxetine and imipramine increased the phosphorylated form of HDAC5 (P-HDAC5), indicating that noradrenaline mediates cytoplasmic export of this enzyme. Moreover, SIRT2 was downregulated by all monoaminergic antidepressants. It is suggested that these effects could contribute to the well-known beneficial effects of antidepressants on brain plasticity. Finally, correlating with what it happens with antidepressant treatment, repeated treatment with specific Hdac inhibitors for three weeks increased synaptic plasticity in the prefrontal cortex.

4.1. CSDS and imipramine treatment regulates Hdac's superfamily

In general, CSDS induced an increase in the mRNA expression of different classes of Hdac enzymes including *Hdac8* (Class I), *Hdac5* and *Hdac7* (Class II) and *Sirt2*, *Sirt3* and *Sirt6* (Class III). On the other hand, repeated imipramine treatment reduced mRNA expression of *Hdac1* (Class I), *Hdac5* and *Hdac10* (Class II) and *Sirt2, Sirt3*, *Sirt4, Sirt5* and *Sirt7* (Class III). Although transcriptional regulation is very complex, broadly it could be said that an increase in Hdacs would lead to a decrease in the levels of histone acetylation and increased DNA compaction, leading to a silencing of gene expression. Similarly, decreased Hdac protein expression would stimulate gene transcription (Gallinari et al., 2007). In agreement with this, CSDS induced a decrease in acetylation levels of histones 3 and 4 (AcH3 and AcH4), as well as the synaptic plasticity marker c-AMP response element binding protein (CREB). Conversely, imipramine treatment enhanced significantly AcH3, AcH4, CREB and the immature form of the brain derived neurotrophic factor (pro-BDNF). Interestingly, recent studies have also reported a downregulation of different HDACs following antidepressant treatment and increased histone acetylation (Tsankova et al., 2006).

Yet, it should be acknowledged that further studies should be carried out to establish a link between the epigenetic effects observed here and the behavioral adaptations induced by stress and the antidepressant action. Moreover, another possible limitation is the fact that control mice used in the CSDS model were housed individually for six weeks and received

daily i.p. injections constituting *per se* a model of social isolation or increased vulnerability to stress (Venzala et al. 2012). Thus, results obtained from this model, should be interpreted with caution.

Among the enzymes of the Hdac superfamily, we selected HDAC5 and SIRT2, whose mRNA expression was oppositely regulated by chronic stress and imipramine treatment.

4.2. Functional implication of HDAC5 regulation

The HDAC5 enzyme belongs to the class IIa superfamily. The class IIa Hdacs (HDAC 4, 5, 7, and 9) are unique among the Hdac family proteins because they suffer synaptic activity-dependent nucleo-cytoplasmic shuttling (McKinsey et al., 2001). The amino-terminal domain is subject to reversible phosphorylation, which controls their nucleo-cytoplasmic distribution. Unphosphorylated class IIa HDACs remains in the nucleus, bound to chromatin, and repress transcription. Upon phosphorylation, they shuttle out of the nucleus, allowing the expression of their target genes (Parra and Verdin, 2010).

Further studies should explore whether long-term anhedonia and other depression-related behaviors induced by CSDS (Venzala et al., 2012; 2013) could be associated to upregulation of nuclear Hdac5 in the prefrontal cortex. In agreement with this, nuclear HDAC5 in the nucleus accumbens inhibits cocaine experience-dependent reward sensitivity (Renthal et al., 2007), suggesting that it plays an active role to repress gene expression involved in reactivity to rewards. Moreover, a relevant study has demonstrated that HDAC5 over-expression blocks the behavioral effects of antidepressants in the CSDS model (Tsankova et al., 2006). Interestingly, an upregulation of cortical *HDAC5* mRNA has been observed in subjects with major depressive disorder and bipolar disorder, compared to controls and patients in remission, supporting a clinical relevance for this change (Hobara et al., 2010). Yet, our human studies did not reveal significant changes in the mRNA expression of this enzyme.

On the other hand, imipramine treatment increased the content of the phosphorylated form of HDAC5 (P-HDAC5), suggesting that this drug treatment induces the nuclear export of HDAC5 to the cytoplasm, in where it cannot exert its deacetylase function. However, no changes in nuclear HDAC5 were observed following imipramine treatment. Yet, it should be acknowledged that our HDAC5 antibody recognizes both the phosphorylated and unphosphorylated form of this enzyme and therefore even in the nuclear fraction some of the HDAC5 detected could be in its phosphorylated form ready to be transferred to the cytoplasm. Thus, nuclear export of HDAC5 could be a mechanism, by which, repeated

imipramine treatment stimulates the expression of specific genes involved in synaptic plasticity, such as pro-BDNF and its upstream regulator CREB.

The nucleocytoplasmic shuttling of HDAC5 in neurons is regulated by neuronal activity. Different kinases have been reported for their ability to increase HDAC5 phosphorylation at two conserved sites, S259 and S498, and to promote cytoplasmic localization of this enzyme (Vega et al., 2004). The tricyclic antidepressant imipramine is a serotonin and noradrenaline reuptake inhibitor that elevates levels of noradrenaline and serotonin in the synaptic cleft. Interestingly, a role for noradrenaline receptors in nucleocytoplasmic shuttling of HDAC5 has been described. Specifically, while activation of β adrenoceptors induces nuclear accumulation of HDAC5 (Sucharov et al., 2011), phosphorylation and translocation to the cytoplasm is induced by activation of α1 adrenoceptors (Sucharov et al., 2011) most likely via calcium influx and subsequent activation of kinase activity (Meucci et al., 1995). In keeping with this, repeated treatment with imipramine could induce cytoplasmic P-HDAC5 accumulation by downregulating postsynaptic β2 adrenoceptors (Sethy et al., 1988) and/or increasing α1 dependent noradrenergic transmission (Nomura et al., 1987). The idea that this effect mediated by imipramine is noradrenaline mediated is further supported by the fact that while repeated treatment with the noradrenaline reuptake inhibitor reboxetine upregulated P-HDAC5 expression, no changes were observed with the selective serotonine reuptake inhibitor fluoxetine. On the other hand, matching also with our results, the antidepressant action of imipramine in the CSDS model has been associated with an increase in histone acetylation at the promoter of BDNF, which, is specifically caused by a decrease in the hippocampal *Hdac5* mRNA enzyme (Tsankova et al., 2006).

Subsequently, we aimed to check whether a maintained inhibition of HDAC5 over a period of three weeks, upregulates synaptic plasticity markers. Given the lack of specific compounds towards this enzyme, we selected the class IIa Hdac inhibitor MC1568 to study the effect of repeated treatment with this compound in synaptic plasticity markers. Mice were treated for 21 days matching with our study carried out with antidepressant treatment. As expected, MC1568 was able to increase histone acetylation as well as pro-BDNF, suggesting that inhibition of the enzymatic activity of this subfamily during this period is able to modulate neuroplasticity. Moreover, this further strengths the idea that the effect noradrenergic antidepressants on synaptic plasticity could be mediated in part by a decreased Hdac5 function (Tsankova et al., 2006). Yet, in order to establish a causal link between class IIa Hdac inhibition and antidepressant action, further behavioral studies should be carried out to demonstrate the antidepressant action of this compound.

4.3. Functional implication of SIRT2 regulation

Sirtuin genes have roused a growing interest in neurodegenerative disorders because of their involvement in metabolic homeostasis and in brain aging. Among all sirtuins, SIRT2 expression is found strongest in the brain (Pandithage et al., 2008), although, compared to SIRT1, its function is less well understood. SIRT2 resides predominantly in the cytoplasm and it can also interact with nuclear proteins (North et al., 2003). In the cytoplasm, SIRT2 is involved in cytoskeleton organization by targeting the cytoskeletal protein α-tubulin (North et al., 2003). Specifically, NAD^+-mediated deacetylation activity of SIRT2 influences axonal degeneration (Suzuki and Koike, 2007) and microtubule acetylation (Southwood et al., 2007), thus influencing neuronal function. In neurons, SIRT2 is rather uniformly expressed in all neurites and their growth cones (Pandithage et al., 2008) and overexpression promotes neurodegeneration. Particularly, a polymorphism of SIRT2 gene has been associated with depression in Alzheimer's disease patients (Porcelli et al., 2013). On the other hand, inhibition of SIRT2 exerts neuroprotective effects in diverse models of neurodegenerative disease, among them, Parkinson's (Outeiro et al., 2007) and Huntington's disease (HD) (Taylor et al., 2011).

In our studies we observed an increase of mRNA and protein expression of *Sirt2* in the PFC of mice exposed to CSDS. However, no changes in the acetylation levels of α-tubulin in CSDS mice could be observed. Perhaps, this could be in part to the high variability found within the CSDS groups (both saline and imipramine treated) in the Western studies as well as to the fact that the function of this enzyme could be masked by other acetylating or deacetylating enzymes using α-tubulin as a substrate (Friedmann et al., 2012). Accordingly, a previous study shows that a robust increase in SIRT2 is associated with only a modest reduction in overall levels of acetylated α-tubulin (Maxwell et al., 2011). On the other hand, the observed imipramine-induced increase in acetylation levels of the cytoskeletal protein α-tubulin in control mice suggests that SIRT2 downregulation was functional. Furthermore, the downregulation of SIRT2 could be linked to the well-known neuroprotective action of imipramine, via stabilization of the microtubule cytoskeleton (Creppe et al., 2009). Moreover, both the selective noradrenaline reuptake inhibitor reboxetine and the selective serotonin reuptake inhibitor fluoxetine downregulated the expression of SIRT2, suggesting that this could be a shared mechanism for monoaminergic antidepressants in general. Further, specific inhibition of SIRT2 by repeated administration of the 33i compound for three weeks, following the same experimental design than for antidepressant drugs, induced an upregulation of AcH4, CREB and pro-BDNF showing that maintained inhibition of SIRT2 modulates synaptic plasticity. Importantly, preliminary studies carried out in our laboratory

have shown that repeated treatment with the 33i compound shows an antianhedonic action in another stress model of anhedonia (unpublished observation).

Altogether, these results match with the observed changes in histone acetylation, CREB and pro-BDNF following imipramine treatment and suggest that inhibition of SIRT2 could be another mechanism, by which this antidepressant stimulates the expression of specific genes involved in synaptic plasticity. Noteworthy, the *SIRT2* upregulation of mRNA expression found in the PFC of depressed patients supports the interest of this target for therapeutic intervention. However, no changes were found in the psychotic group. In this sense, it could be suggested that the antipsychotic treatment received by these patients could be reverting the increase in *SIRT2* observed in the depressed group. Indeed the use of antipsychotics appears as a confounding variable on the expression of SIRT2. It is important to note that although recent studies suggest the involvement of Hdac dysfunction in the pathophysiology of mood disorders, few studies investigates in human post-mortem brain tissue the expression of the different Hdacs (Covington et al., 2009).

In summary, our results suggest a role for HDAC5 and SIRT2 in neuronal adaptations induced by chronic stress and antidepressant treatment and highlight the therapeutic potential of these targets in the treatment of depression. Yet, much work is needed in order to identify the genes involved in the "depressive" or the antidepressant-like effects linked to HDAC5 or SIRT2 dependent histone acetylation in limbic brain regions.

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CAPÍTULO V

CAPÍTULO 5

SIRT2 inhibition modulates glutamate and serotonin system in the prefrontal cortex and induces antidepressant-like action

Manuscript submitted

ABSTRACT

Growing evidence suggests that changes in histone acetylation in specific sites of the chromatin modulate neuronal plasticity and contribute to antidepressant-like action. Sirtuin 2 (SIRT2) is a class III NAD^+-dependent histone deacetylase involved in transcriptional repression of genes that regulate synaptic plasticity. Importantly a key role for the glutamate system in the prefrontal cortex (PFC) synaptic plasticity changes induced by antidepressants has been suggested. Here, we addressed the question whether SIRT2 could be a new pharmacological target for the treatment of major depression. The compound 2-{3-(3-fluorophenethyloxy)phenylamino}benzamide (33i), a selective SIRT2 inhibitor in vitro, was studied in mice (C57Bl6). Firstly, the effect of a subchronic (10 days) treatment of 33i (5-15 mg/kg ip) on the PFC expression of pharmacodynamics markers for SIRT2 inhibitory activity and of some synaptic plasticity markers linked to glutamate transmission (VGLUT1, synaptophysin, mGluR4, GluA1, GluN2B, GluN2A) as well as on serotonin levels were studied. Further, neurochemical and behavioural effects of chronic (5 weeks) 33i (15 mg/kg) on the CMS model of depression were analysed. Subchronic 33i showed SIRT2 inhibitory activity in vivo and increased both GluN2A and GluN2B NMDA receptor subunits as well as serotonin levels in the PFC. In addition, chronic 33i treatment (15 mg/kg ip) reverted anhedonia and social avoidance induced by CMS. Moreover, 33i upregulated postsynaptic GluN2B and the phosphorylated form of GluA1 (p-GluA1) in both control and CMS mice suggesting that SIRT2 inhibition enhance synaptic strength. On the other hand, chronic stress induced a significant decrease of GluN2B in the cytosolic fraction. Taken together, these results suggest that Sirt2 inhibition induce antidepressant-like action and this effect could be mediated by modulation of glutamate and serotonin system in the PFC. Moreover, it highlights the therapeutic potential of SIRT2 as a new pharmacological target for the treatment of major depression and suggests the importance of further investigate the role of SIRT2 inhibitors as new antidepressant agents.

1. INTRODUCTION

Growing evidence suggests that changes in histone acetylation play a key role in neuronal plasticity and mediate stable functional changes in the brain in response to environmental stimuli (Dulac, 2010). Acetylation and deacetylation are important posttranslational regulators of chromatin compaction and gene expression. Acetylation, mediated by histone acetyltransferases (Hats), lowers the affinity between histones and DNA allowing chromatin to adopt a more relaxed structure and favouring transcription. Deacetylation, mediated by histone deacetylases (Hdacs), is generally associated with transcriptional repression. Among the 18 class of Hdacs described the class III, called sirtuins, is a NAD^+-dependent protein deacetylase group of enzymes harnessing ability to adapt to various cellular situations (Watroba and Szukiewicz, 2016).

Among sirtuins, human Sirtuin 2 (SIRT2) can act both in the nucleus and in the cytoplasm. In the nucleus, SIRT2 plays a role in transcriptional repression of genes encoding for DNA binding proteins as well as transcription factors that participate in synaptic plasticity, cell proliferation, differentiation and cell survival (Eskandarian et al., 2013). In the cytoplasm, it deacetylates proteins of the cytoskeleton such as α-tubulin, being involved in microtubule stabilization (Michan and Sinclair, 2007). Further, changes in Sirt2 have been linked to neurodegenerative disease (Theendakara et al., 2013) and depressive disorders (Porcelli et al., 2013; Erburu et al., 2015). Interestingly, selective SIRT2 inhibitors are effective at reducing tau phosphorylation (Green et al., 2008), α-synuclein toxicity (Outeiro et al., 2007) and brain cholesterol levels (Taylor et al., 2011) in cell cultures as well as at increasing brain derived neurotrophic factor (BDNF) in the mouse brain (Erburu et al., 2015). Thus, selective SIRT2 inhibitors show neuroprotective actions and appear as of great interest to study its therapeutic potential in neuropsychiatric and neurological diseases.

A previous study carried out in our laboratory (Erburu et al., 2015) shows that chronic stress and antidepressant treatment regulate histone acetylation in the PFC. Specifically, chronic stress upregulated SIRT2, which, could contribute to stable stress-induced neuronal adaptations. Conversely, SIRT2 was downregulated by monoaminergic antidepressants (fluoxetine, reboxetine and imipramine) suggesting that these effects could contribute to the well-known beneficial effects of antidepressants on brain plasticity. Interestingly, major depression and antidepressant action has been linked to changes in synaptic plasticity in limbic areas such as the PFC, in which histone acetylation could play an important role (Covington et al., 2015). Importantly, a key role for the glutamate system in PFC synaptic plasticity changes induced by antidepressants has been suggested (Li et al.,

2010; Koike et al., 2011; Wolak et al., 2013). In this context it becomes interesting to study the epigenetic regulation of synaptic plasticity markers linked to glutamate system and how it could be involved in the antidepressant action. Importantly, activation of glutamate receptors in the PFC has been linked to antidepressant-like effects (Li et al., 2010; Koike et al., 2011). In addition, SSRIs, that elevate 5-HT transmission in the forebrain, enhance glutamate transmission and synaptic strength in the PFC (Koike et al., 2011; Wolak et al., 2013).

Anhedonia has been shown to correlate with a dysfunction of the PFC in depressed patients (Germine et al., 2011; Keedwell et al., 2005) and in animal models (Davidson, 2002; Banasr et al., 2010). In agreement with this, we have shown that our chronic mild stress (CMS) model induces long-lasting anhedonia and neurochemical alterations in this brain region that can be partially reverted by antidepressants (Elizalde et al., 2008; 2010; Erburu et al., 2015).

Here, we aim to answer to the question whether SIRT2 could be a new pharmacological target for the treatment of depression. Firstly, we will study if the compound 33i, a selective SIRT2 inhibitor in vitro (Suzuki et al., 2012), shows evidences of SIRT2 inhibitory activity in vivo in the mouse PFC and if so, how SIRT2 inhibition regulates the expression of several synaptic plasticity markers linked to glutamate neurotransmission as well as serotonin levels. Subsequently, we will investigate neurochemical and behavioural effects of 33i on a validated mouse model of depression, the chronic mild stress (CMS) (Willner, 2005; Elizalde et al., 2008; 2010).

2. MATERIAL AND METHODS

2.1. Animals and drug treatment

Male C57BL/6J mice (Harlan, France, 8 weeks of age) were housed (4 mice per cage) and allowed for 2 weeks to habituate before beginning experimentation. Food and water were available ad libitum for the duration of the experiments unless otherwise specified. Animals were maintained in a temperature (21±1°C) and humidity-controlled room (55±2%) on a 12 h light-dark cycle (lights on at 08:00 h).

Experimental procedures and animal husbandry were conducted according to the principles of laboratory animal care as detailed in the European Communities Council Directive (2013/53/EC) and approved by the Ethical Committee of University of Navarra.

The compound 33i, 2-{3-(3-fluorophenethyloxy)phenylamino}benzamide, (kindly donated by Dr. Suzuki from Kyoto Prefectural University of Medicine, Japan) was prepared in suspension using 18% tween 80 and 5% DMSO in saline and administered i.p. at 5 or 15 mg/kg once daily. A single daily dose was selected in order to reduce to a minimum the stress of the injection in control mice.

2.2. Experimental design

Two different experiments were carried out. In the first one, mice (n=8/group) received daily (i.p.) injections of different doses of 33i (5 or 15 mg/kg) or saline during 10 days (at 1 pm). Mice were killed by cervical dislocation, brains were rapidly removed and the prefrontal cortex (PFC) (around 15 mg) was rapidly dissected in an acrylic mouse brain slicer matrix with 1.0 mm coronal slice intervals (Zivic Instruments, Pittsburgh, PA, USA). Using a mouse brain atlas (Hof et al., 2000), a 1mm slice was taken from the infralimbic section of the PFC (bregma 2.20 mm through bregma 1.20 mm) and dissected out bilaterally using a scalpel and kept at -80 °C for neurochemical studies.

In the second experiment, mice were divided into control and CMS groups. While control mice were kept in standard conditions, CMS mice were placed in individual cages and sent to a separate room fully equipped for the administration of different stressors. CMS procedure was applied for six weeks. After three weeks, mice from control and CMS groups were subdivided into saline and 33i groups: Saline, 33i, CMS-Saline and CMS-33i (n=15 animals/group). Mice received daily (i.p.) injections of 33i (15 mg/kg) or saline (at 1 pm) from the third week of CMS and continued for two weeks thereafter the procedure (Figure 1).

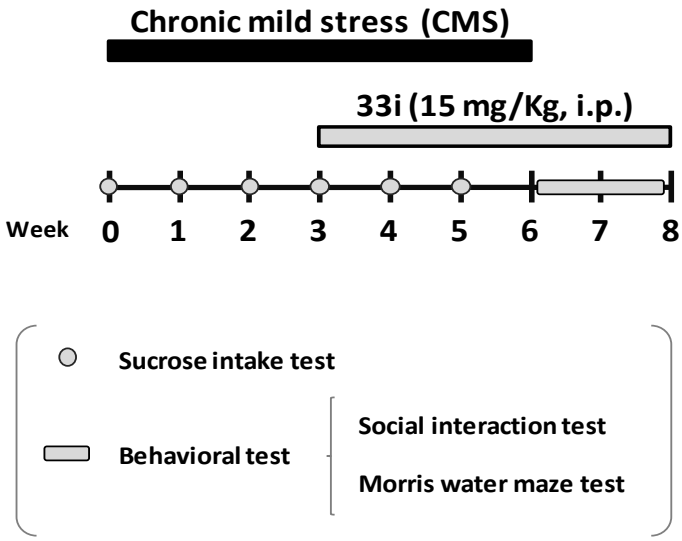

Fig. 1 Experimental design of chronic mild stress (CMS) procedure, 33i treatment and behavioral tests

Anhedonic-like behavior was analyzed by weekly monitoring of sucrose intake. After the termination of the CMS procedure, behavioral tests were applied. Test were performed in the following order: social interaction test (day 1) and Morris water maze test (day 3-10).

Mice were killed by cervical dislocation, brains were rapidly removed and the PFC (around 15 mg) was rapidly dissected as described above and kept at -80°C for neurochemical studies.

2.3. Chronic mild stress procedure

CMS was performed according to a procedure optimized in our laboratory (Elizalde et al., 2008). Mice were placed in individual cages and unpredictable mild stressors (2-3 in any 24 h period) were randomly applied for 6 weeks: stroboscopic illumination (8 h), intermittent bell (10 db, 1 s/10 s) or white noise (4 h), rat odor (saw dust from rat cages; 8 h), placement of novel object in the home cage (3 h), cage tilt 45° (8 h), soiled bedding (6 h), paired housing (with new partner, 2 h), overnight illumination, removal of nesting material (12 h) and confinement (1 h).

2.4. Sucrose intake test

Anhedonic-like behavior was evaluated by monitoring of sucrose intake (Elizalde et al., 2008). Mice were first trained to drink a sucrose solution by exposing them to two standard drinking bottles, one containing 2.5% sucrose and the other tap water, for every other night during one week.

After this preliminary phase, mice were food deprived and then exposed to the sucrose solution and water from 6:00 p.m. until 09:00 h in the morning. In order to be able to measure sucrose intake for each individual mouse, on the day of the test, a plastic mesh dividing the cage in four identical spaces and allowing visual and olfactory contact was adapted in the cage. Each space was occupied by one of the four mice. The intake baseline for the sucrose solution was established, which corresponded to the average of three consecutive measurements of sucrose intake carried out every 4-5 days.

Then, mice were divided into two groups matched for sucrose consumption and body weight. Subsequently, one group of mice was exposed to the CMS for 6 weeks and the other group served as non-stressed control. Once a week, mice were given a 15-h exposure to the sucrose solution and tap water in their home cage as described above. The position of the two bottles (right/left) was varied randomly from trial to trial. Body weight measurements were taken weekly in both stressed and no stressed groups and the relative sucrose intake and sucrose preference (sucrose intake/total intake) was calculated as absolute intake (g) per mouse body weight.

2.5. Behavioral tests

2.5.1. Social interaction test

This test measures the approach-avoidance behavior towards an unfamiliar social target (Tsankova et al., 2006 and Venzala et al., 2012). The arena is a transparent plastic open field (45 × 45 × 45 cm^3) maintained in a very softly illuminated room (50 lx). Each experimental mouse was introduced into the open field and its trajectory was tracked for two consecutive sessions of 2.5 min. During the first session ("no target") the open field contained an empty metallic mesh cage (10.5 × 9 cm^2) located at one end of the field. During the second session ("target"), the conditions were identical except that a social target animal (a mouse from the same strain and of similar weight) had been introduced into the cage. Between the two sessions, the experimental mouse was removed from the arena, and was placed back into its home cage for approximately 1 min. The time that the experimental mouse spent in the "interaction zone" (7 cm wide corridor surrounding the little metallic

cage) both in the "no target" and "target" conditions were recorded (Ethovision XT 11 plus multiple body point module, Noldus Information Technology, Wageningen, The Netherlands) and differences ("target-no target") were calculated for each mouse (Venzala et al., 2012).

2.5.2. Morris water maze test

The Morris water maze test was used to test spatial memory and to evaluate the working and reference memory functions in response to treatment, as previously described (Orejana et al., 2012).

The water maze was a circular pool (diameter of 145 cm) filled with water (21-22 °C) and virtually divided into four equal quadrants identified as northeast, northwest, southeast and southwest. Mice underwent visible-platform training for 8 trials in one day in which a platform was located in the southwest quadrant raised above the water. Mice that were unable to reach the platform, exhibiting abnormal swimming patterns or persistent floating were excluded from data analyses.

Hidden-platform training was conducted with the platform placed in the northeast quadrant 1cm below the water surface over 6 consecutive days (4 trials/day). Several large visual cues were placed in the room to guide the mice to the hidden platform. Each trial was finished when the mouse reached the platform (escape latency) or after 60 s, whichever came first. Mice failing to reach the platform were guided onto it. After each trial mice remained on the platform for 15 s.

To test memory retention, two probe trials were performed at the beginning of the day 4 and the last day of the test (day 7). In the probe trials the platform was removed from the pool and mice were allowed to swim for 60s. The percent of time spent in the target quadrant was recorded. All trials were monitored by a video camera set above the center of the pool and connected to a video tracking system (Ethovision XT 10 plus multiple body point module).

2.6. SIRT2 activity assay

SIRT2 activity was measured in PFC homogenates pre-incubated with different concentrations of 33i (0.1 μM, 1 μM, 10 μM and 100 μM) using a deacetylase fluorometric assay kit (SIRT2 Activity Assay kit, Abcam 156066) following the manufacturer's instructions. Fluorescence intensity was read for 60 min at 1 min intervals with excitation at 480 nm and emission at 520 nm using POLARstar Galaxy Multi-functional Microplate Readers (MTX Lab Systems, Virginia, USA).

2.7. Gene expression studies

2.7.1. RNA extraction

Isolation of total RNA was carried out according to the manufacturer's instructions (NucleoSpin RNA II kit, Macherey-Nagel, Germany). Total RNA was isolated separately from each individual cortex (n=8/group). The frozen PFC samples were lysed and dounze-homogenized in the presence of a highly denaturing β-mercaptoethanol containing buffer, which immediately inactivates RNAses. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA bound to the membrane and contaminants were washed away. RNA was then eluted in 30–60 µL RNase-free water. The eluates were stored at -80°C.

2.7.2. Real time-PCR

For RT-PCR experiments, 0.3 µg of total RNA was reverse transcribed to cDNA using random hexamers as primers and SuperScript reverse transcriptase III (Invitrogen, Cergy Pontoise, France). The eluates were stored at -80°C. RT-PCR was performed in an ABI PRISM 7000 HT Sequence Detection System following the manufacturer's recommendations (Applied Biosystems, CA, USA). Thermal cycling conditions were 2 min at 50 °C and 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Primers for Abca1, Glun2b and GluA1 were used (Applied Biosystems, CA, USA). Each cDNA prepared was used in triplicate for the real-time PCR procedures for each gene tested, and the results were calculated as the average of triplicated results. PCR products were analysed using the SDS 2.3 and the RQ Manager 1.2 Software (Applied Biosystems, CA, USA). 18S (Hs99999901) was used as an internal control to normalize the amount of RNA used from different samples.

Samples were analysed by the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target gene levels with respect to the internal control. Normalization was based on a single reference gene (18S). Double delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample (non-treated/treated non-stressed and non-treated/treated stressed mice) minus the mean ΔCT of the calibrator samples (non-treated non-stressed mice) for each target gene. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$.

2.8. Western blotting studies

2.8.1. Preparation of the protein extracts

Prefrontal cortex of mice were rapidly dissected and homogenized in a cold lysis buffer with protease inhibitors (0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 200 mM NaF, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 1 mM Na_3VO_4, 1 mM benzamidine, 10 mg/mL leupeptin, 400 U/mL aprotinin). The homogenate was centrifuged at 14,000 x g at 4 ºC for 20 min and the supernatant aliquoted and stored at -80 ºC. To obtain the postsynaptic membrane-enriched protein fraction, a previously described method (Gardoni et al., 2006), with only few modifications, was used. Briefly, prefrontal cortex were homogenized in ice-cold buffer (pH 7.4) containing 0.32 M sucrose, 1 mM Hepes, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 1 mM EDTA and 0.1 phenylmethanesulfonylfluoride (PMSF) in the presence of protease and phosphatase inhibitors. The samples were centrifuged at 1000 g for 10 min. The resulting supernatant was spun at 13,000 g for 15 min at 4°C to obtain a crude membrane fraction as a pellet which was resuspended in 1 mM Hepes supplemented with protease and phosphatase inhibitors. The samples were centrifuged at 100,000 g for 1 h at 4°C. The pellet was resuspended in 75 mM KCl, 1% Triton X-100 and protease and phosphatase inhibitors and then stored at -80°C. Total protein concentrations were determined using the BioRad Bradford protein assay (BioRad Laboratories).

2.8.2. Western blotting

Protein extracts from one hemisphere of the prefrontal cortex each individual were loaded in each lane of the blot. Equal amounts of protein (20 µg per lane) were separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (8%) under reducing conditions and transferred onto a nitrocellulose membrane (Hybond- ECL; Amersham Bioscience). The trans-blots were blocked for 1 h with 10% not-fat milk in buffer TBS containing 0.1% Tween 20 and then probed overnight at 4 ºC with one of the following primary antibodies: rabbit polyclonal anti-SGK1 (1:1,000) (Abcam, Cambridge, UK); rabbit polyclonal anti-acetylated histone 3 (AcH3) (1:2,000) and histone 4 (AcH4) (1:2,000), rabbit polyclonal anti-glutamate receptor 1 (AMPA) (GluA1) (1:1,000), rabbit monoclonal anti-phospho glutamate receptor 1 (AMPA) (p-GluA1) (1:1,000), rabbit polyclonal anti-glutamate receptor 4 (mGlut4) (1:1,000), rabbit polyclonal anti-NMDA receptor subunit 2A (GluN2A) (1:1,000) and 2B (GluN2B) (1:1,000), mouse monoclonal anti-synaptophysin (1:10,000) and mouse monoclonal anti-vesicular glutamate transporter 1 (VGLUT1) (1:1,000) (Merck Millipore, Billerica, MA, USA) and mouse polyclonal anti-acetylated α-tubuline (1:10,000, Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies conjugated to IRDye 800CW or

IRDye 680CW (LI-COR Biosciences, Lincoln, NE, USA) were diluted to 1/15,000 in TBS with 5% BSA. Bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β-actin (1:10,000, Sigma-Aldrich, St. Louis, MO, USA) was used as internal control. Results were calculated as the percentage of optical density values of the control vehicle mice.

2.9. Determination of serotonin (5-HT) and hydroxyindolacetic (5-HIAA) acid brain levels

Concentrations of 5-HT and 5-HIAA in different brain regions of the mice were determined by high performance liquid chromatography with electrochemical detection as previously described (Puerta et al., 2009). Briefly, samples were injected using an automatic sample injector (Waters 717 plus; Waters, Barcelona, Spain) onto a Spherisorb ODS-2 reverse phase C18 column (5 lm, 150· 4.6 mm; Teknokroma, San Cugat del Valles, Spain) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to a Ag/AgCl reference electrode. The mobile phase consisted of 0.1 M citric acid; 0.1 M Na_2HPO_4; 0.74 mmol/L octanesulphonic acid; 1 mmol/L EDTA and 16% methanol (pH 3.4), pumped at a flow rate of 1 mL/min.

2.10. Statistical analysis

In the first experiment, Western blotting and RT-PCR studies, were analysed using one-way ANOVA, followed by Tukey *post hoc* test. Student *t*-test was used in HPLC studies. In the second experiment, behavioral test and Western blotting studies, were analysed using two-way ANOVA (stress, treatment) analysis followed by Tukey *post hoc*.

Weight gain and sucrose intake were analysed with two-way ANOVA repeated measures being time the within-subjects factor and stress and treatment the between-subjects factors. For each week, a two-way ANOVA was carried out and significant differences among groups were analysed with Tukey *post hoc* test.

When no significant interaction between two factors was found, main effects were considered.

3. RESULTS

3.1. SIRT2 inhibitory activity of 33i

Firstly, we tested whether 33i would inhibit Sirt2 activity in vitro in PFC homogenates. As it can be seen in Figure 2A, 33i markedly reduced Sirt2 activity at 10 and 100 μM.

RT-PCR studies showed that Abca1 expression was affected by 33i treatment ($F_{2,21}$=5.815, p<0.01). At both 5 and 15 mg/kg, 33i increased significantly the expression of this gene (p<0.01) compared to control vehicle mice (Figure 2B).

The mRNA expression of the cholesterol transporter ATP-binding cassette transporter Abca1 and acetylation levels of histone 3 (AcH3) and histone 4 (AcH4) measured by Western blot were studied as pharmacodynamic markers of SIRT2 inhibitory activity in the PFC.

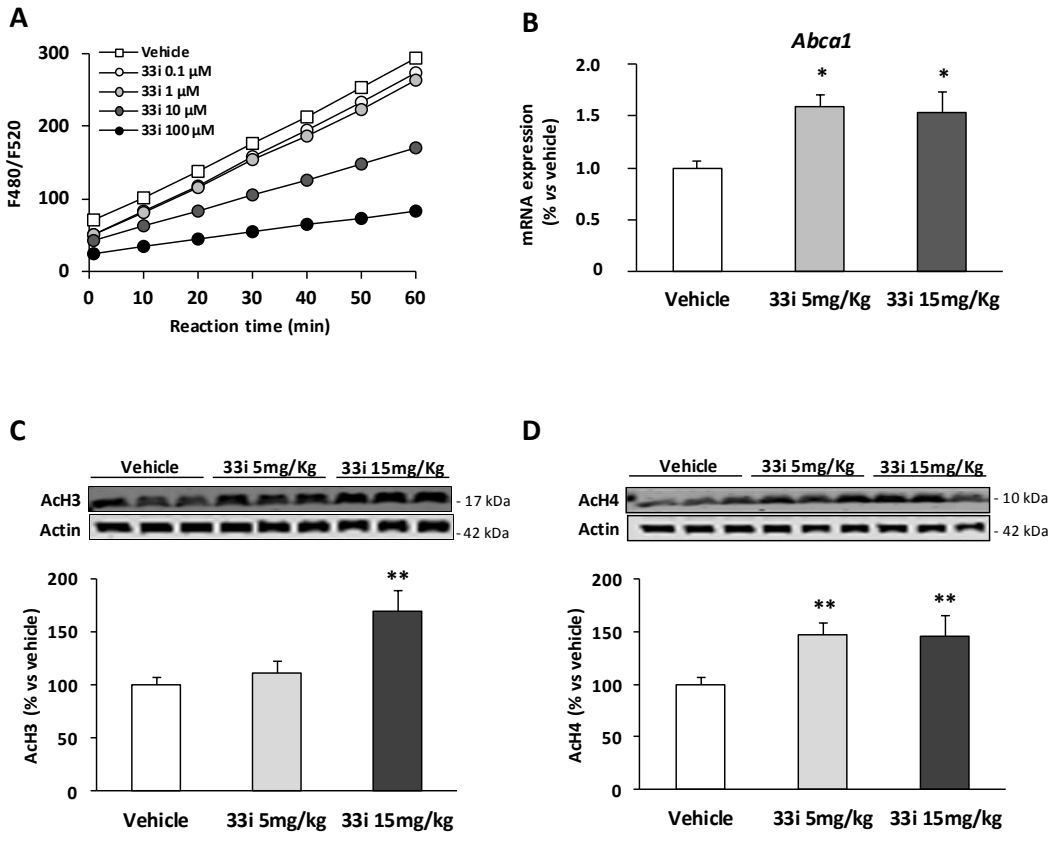

Figure 2. Inhibitory activity of the compound 33i on SIRT2 protein. Effect of 33i (0.1-100 μM) on SIRT2 activity in prefrontal cortex brain homogenates **(A)**. Effect of repeated treatment with 33i (5-15 mg/kg, i.p.) over 10 days on *Abca1* mRNA expression **(B)** and on protein expression levels of acetylated histone 3 (AcH3) **(C)** and acetylated histone 4 (AcH4) **(D)** in the prefrontal cortex. Data show the mean ± SEM of relative abundance of mRNA or protein expression compared to vehicle group values (n=8 animals/group). **p<0.01; *p<0.05 *vs* vehicle group (One-way ANOVA).

In addition, both AcH3 and AcH4 expression levels were affected by the 33i treatment ($F_{2,21}$=7.644 and 8.258, p<0.01 for AcH3 and AcH4 respectively). While AcH3 was significantly increased by 33i at 15 mg/kg (Figure 2C), AcH4 was significantly increased by both 5 and 15 mg/kg of 33i (Figure 2D).

3.2. Effect of subchronic 33i on synaptic plasticity markers

No changes were observed in the PFC expression levels of the synaptic vesicle proteins VGLUT1 and synaptophysin as well as of the presynaptic metabotropic glutamate receptor 4 (mGluR4) (Figure 3A-C).

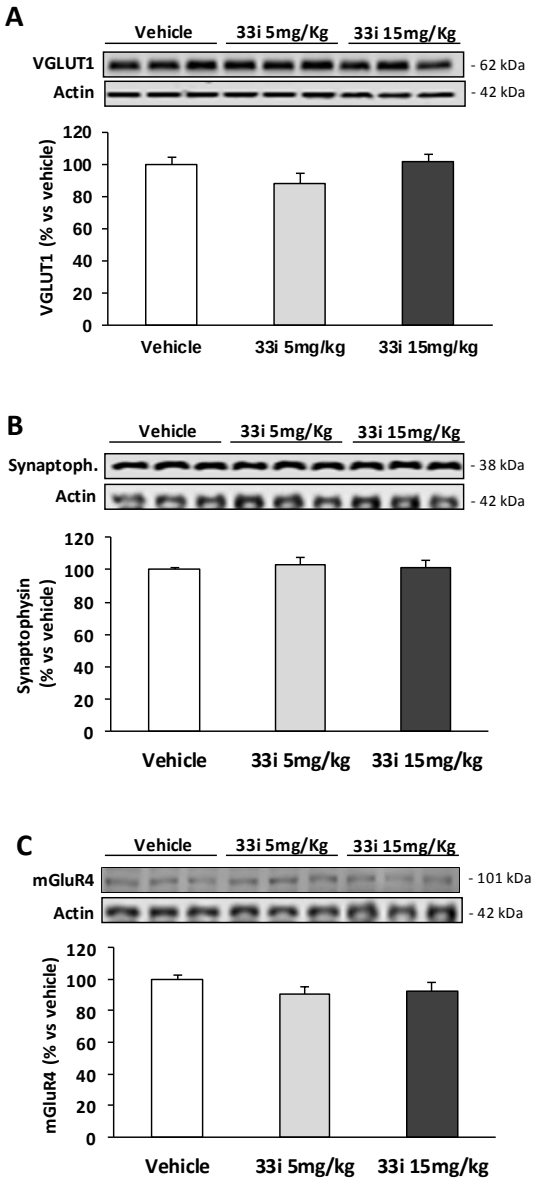

Figure 3. Effect of Sirt2 inhibition on synaptic proteins expression. Effect of SIRT2 inhibition on vesicular glutamate transporter 1 (VGLUT1) **(A)**, synaptophysin **(B)** and metabotropic glutamate receptor 4 (mGluR4) **(C)** protein expression levels in prefrontal cortex. Data show the mean ± SEM of relative abundance of protein expression compared to vehicle group values (n=6-8 mice/group).*p<0.05 *vs* vehicle group (One-way ANOVA).

On the other hand, the expression of the NMDA receptor subunits, GluN2A and GluN2B, was affected by 33i treatment ($F_{2,21}$=5.046, p<0.05 and $F_{2,21}$=3.736, p<0.05, respectively). At 15 mg/kg 33i increased GluN2A protein expression in the mouse PFC compared to vehicle treated mice (p<0.05) (Fig. 4A). As shown in figure 4B, GluN2B expression was significantly increased by both 5 mg/kg and 15 mg/kg of 33i (p<0.05). Moreover, a significant increase in GluN2A/GluN2B ratio was found at 15 mg/kg. Yet, AMPA receptor subunit GluA1 expression was unaltered (Figure 4D).

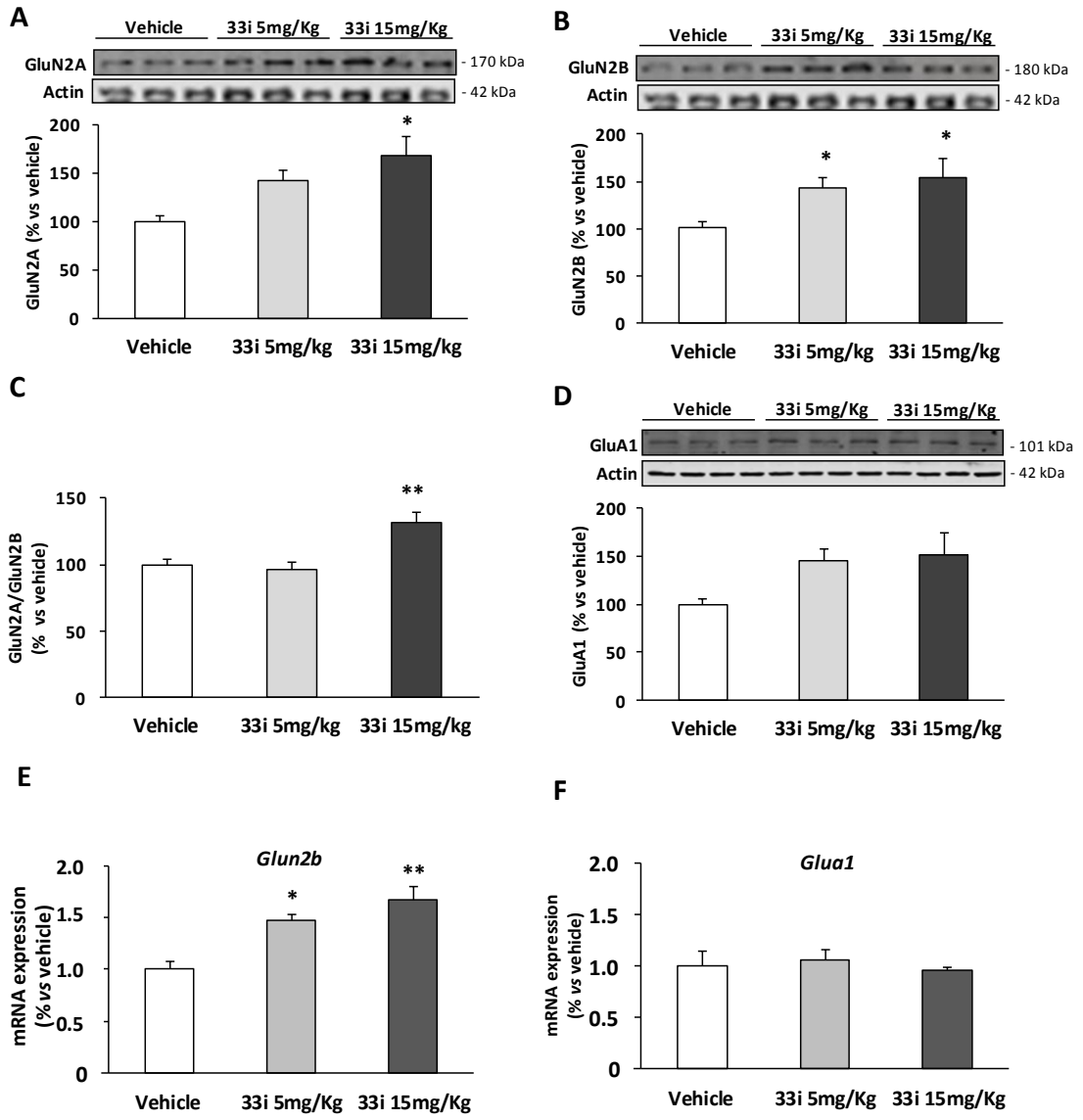

Figure 4. Sirt2 inhibition upregulates GluN2B expression. Effect of SIRT2 inhibition on GluN2A **(A)** and GluN2B (**B)** expression, on GluN2A/GluN2B ratio (**C**), AMPA receptor subunit GluA1 **(D)** expression levels and on mRNA abundance of *Glun2b* **(E)** and *Glua1* **(F)** receptor subunits in the prefrontal cortex. Data show the mean ± SEM of relative abundance of protein expression compared to vehicle group values (n=6-8 mice/group).**p<0.01, *p<0.05 vs vehicle group (One-way ANOVA).

Real-time PCR studies also showed that *Glun2b* expression was affected by 33i treatment ($F_{2,18}$=9.088, p<0.01). At both 5 (p<0.05) and 15 mg/kg (p<0.01) 33i increased *Glun2b* mRNA abundance (Fig. 4E). However, *Glua1* mRNA expression was not changed (Figure 4F).

3.3. Effect of subchronic 33i on 5-HT and 5-HIAA brain levels

Ten days of 33i treatment (15 mg/kg) induced a significant increase (p<0.05) in PFC 5-HT levels (Figure 5A), while no changes in 5HIAA levels were observed (data not shown). In addition, at this dose, the serotonin turnover or ratio 5-HIAA/5-HT in the PFC and brain stem containing the raphe nuclei were significantly (p<0.05) decreased (Figure 5B). However, at 5 mg/kg no changes in 5-HT levels were observed in any brain area.

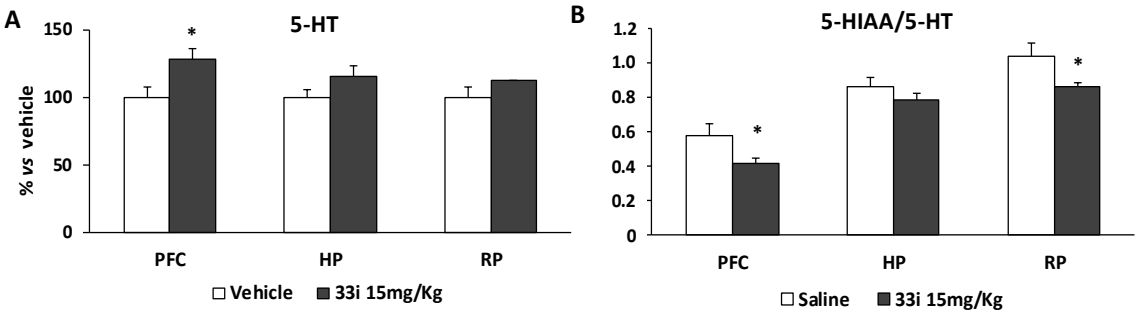

Figure 5. Sirt2 inhibition increases serotonin levels in prefrontal cortex. Effect of 33i treatment on serotonin (5-HT) levels **(A)** and 5-Hydroxyindoleacetic acid/serotonin ratio **(B)** in the prefrontal cortex (PFC), hippocampus (HP) and raphe nucleus (RP). Data show the mean ± SEM of relative abundance of mRNA expression compared to vehicle group values (n= 6-8). *p<0.05 vs vehicle group (Student *t* test).

3.4. Effect of chronic 33i on behavioral alterations induced by CMS

3.4.1. Sucrose intake test

Mice body weight was not affected neither by CMS nor 33i treatment (data not shown). Before onset of CMS, sucrose and water intake was similar in all groups. Sucrose intake was significantly affected by time, stress and treatment ($F_{1,420}$=5,1; p<0.05). Two-way ANOVA for each week showed a significant effect of stress on sucrose intake for week 3 ($F_{1,56}$=6.775; p<0.05). Moreover, there was a significant interaction between stress and treatment in week 6 ($F_{1,56=}$5.701; p<0.05). *Post hoc* analysis revealed that vehicle mice exposed to CMS showed decreased sucrose intake and this effect was reverted by 33i (15 mg/kg) treatment (Figure 6A).

3.4.2. Social interaction test

In this test, a significant interaction between stress and 33i treatment on the time spent in the interaction zone was observed ($F_{1,56=}$5.660; p<0.05). *Post hoc* analysis revealed that control vehicle mice exposed to CMS showed a decreased social behavior towards an unfamiliar mouse of the same strain compared to control vehicle mice (p<0.05). Moreover, 33i (15 mg/kg) treatment full reverted this effect (p<0.05) (Figure 6B).

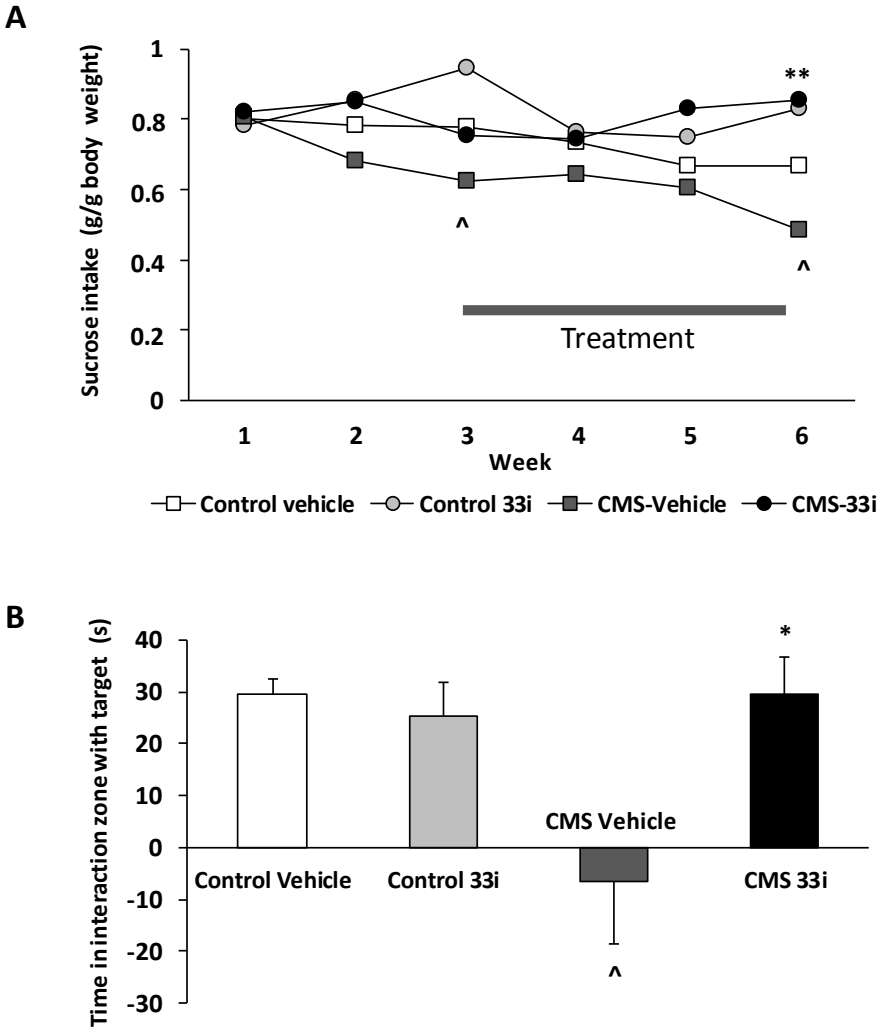

Figure 6. Effect of CMS and 33i (15 mg/Kg) treatment on anhedonia in the sucrose intake test **(A)** and social behavior in the social interaction test **(B)**. Data show the mean ± SEM of different behavioral paradigms (n=12-15 mice per group). ^p<0.05 vs control vehicle group; **p<0.01, *p<0.05 vs CMS vehicle group (Two-way ANOVA followed by Tukey test).

3.4.3. Morris Water Maze

During the hidden platform trial (acquisition trial), there were no differences in the escape latencies among the different groups (Figure 7A).

The probe trial took place at the beginning of the 4th and 7th days of the MWM test. In this trial, the platform was removed from the pool where mice swam during 1 minute to evaluate memory retention. As shown in Supplementary Figure 7B, no significant differences were found among all four groups.

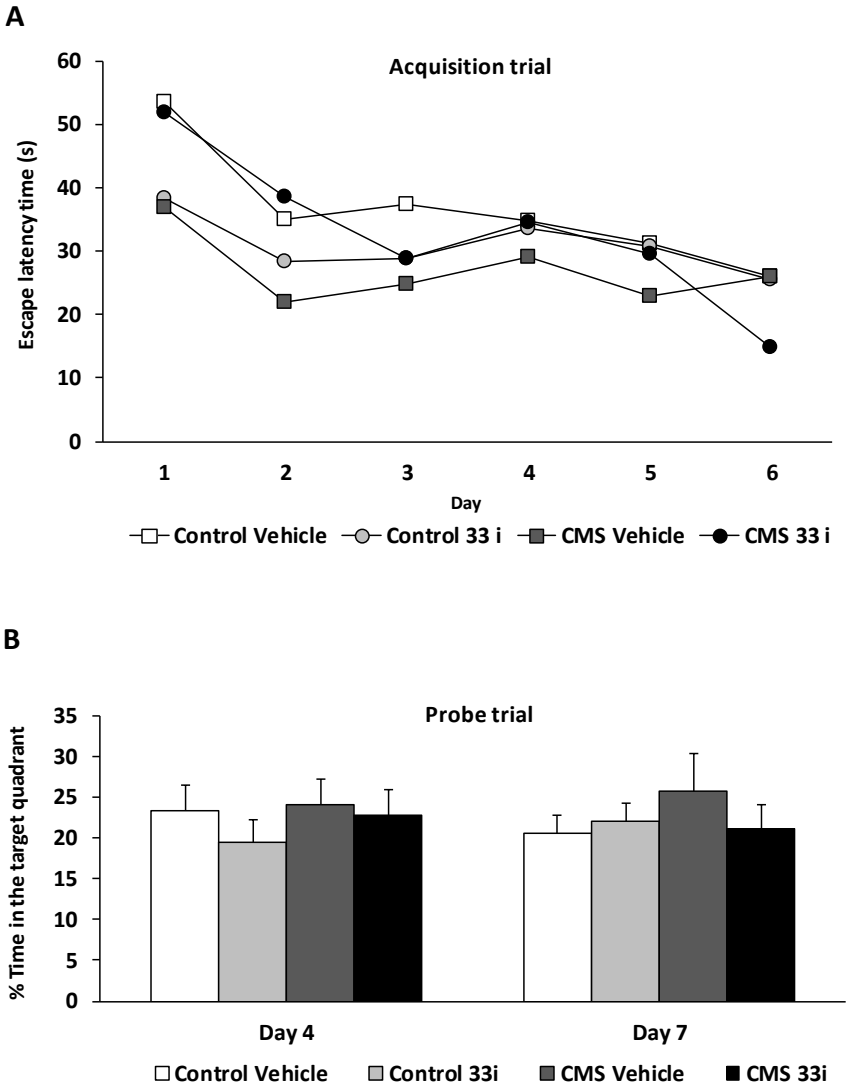

Figure 7. Effect of CMS and 33i (15 mg/Kg) treatment on the performance in the Morris water maze test. (A) Escape latency in the hidden platform phase. **(B)** In the probe trial data are presented as percentage of time spent in the target quadrant. Results are shown as mean ± SEM (n= 12-15). No differences were found among all four groups.

3.5. Effect of chronic 33i and CMS on the glutamate receptor subunits GluN2B and GluA1

While GluN2A levels were affected by stress ($F_{1, 25}$= 5,736, p<0.05), GluN2B levels were affected by stress ($F_{1,25=}$10.797, p<0.01) and a by the 33i treatment ($F_{1,25}$=5.971, p<0.05). Specifically, CMS induced an increase of GluN2A and GluN2B subunits and 33i treatment induced an increase of GluN2B (Figure 8).

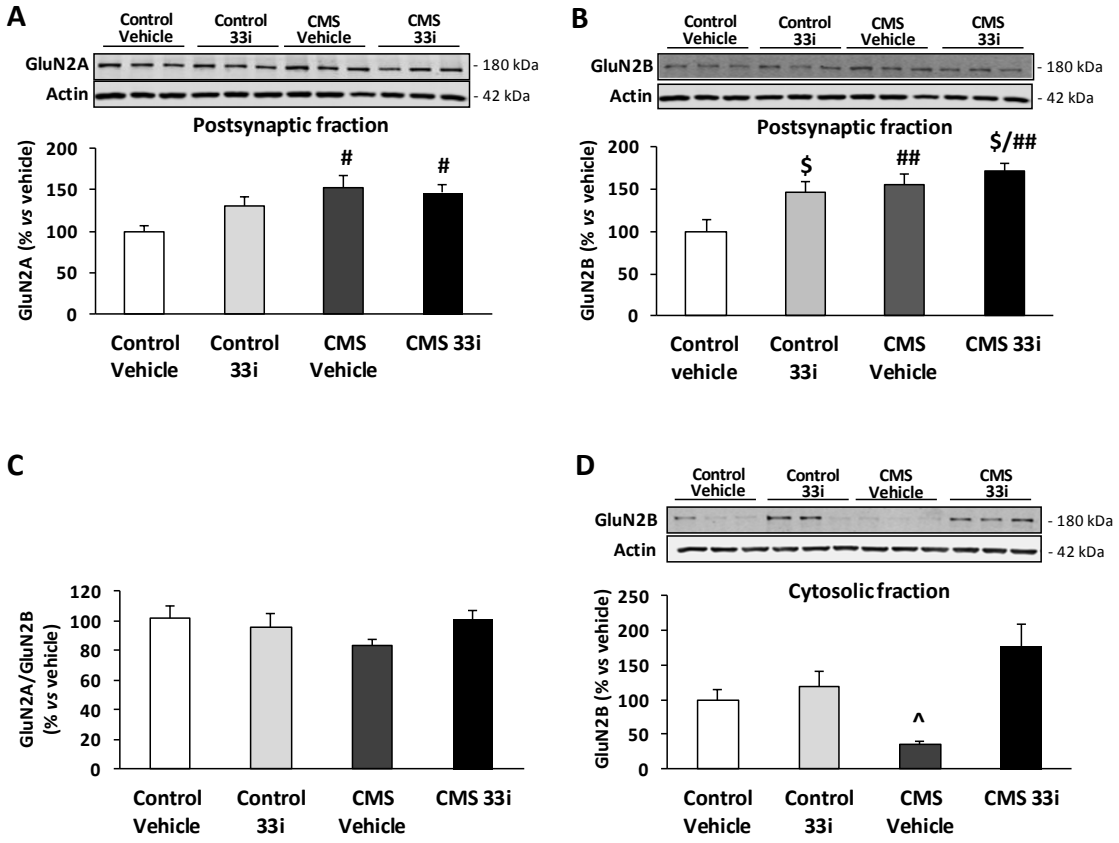

Figure 8. Effect of CMS and 33i (15 mg/Kg) treatment on postsynaptic fraction of NMDA receptor GluN2A **(A)**, GluN2B **(B)**, GluN2A/GluN2B ratio **(C)** and cytosolic fraction of GluN2B **(D)** protein expression levels in prefrontal cortex. Data show the mean ± SEM of relative abundance of protein expression compared to control saline group values (n= 8-10 mice/group). ^p<0.05 vs control vehicle group; *p<0.05 *vs* CMS vehicle group; ## p<0.01 main effect of stress; $ p< 0.05 main effect of treatment (Two-way ANOVA followed by Tukey test).

The study of the cytosolic fraction of GluN2B protein levels showed a significant interaction between stress and treatment ($F_{1,25}$=6.197; p<0.05). *Post hoc* analysis revealed that CMS mice showed a decrease in GluN2B expression compared to controls (p<0.05) and this effect was reverted by 33i treatment (p<0.05) (Figure 8B).

Postsynaptic GluA1 protein levels were unchanged in the PFC of mice exposed to CMS and 33i treatment (Figure 9A). Nevertheless, the analysis of the phosphorylated form of GluA1, P-GluA1, showed a significant main effect of treatment ($F_{1,25}$=7.097, p<0.05). The compound 33i at 15 mg/kg increased P-GluA1 expression compared to vehicle treated mice (Figure 9B). Moreover, the analysis of the ratio P-GluA1/GluA1 showed a significant effect of treatment ($F_{1,25}$=5.119, p<0.05). Again, 33i treatment increased the ratio P-GluA1/GluA1 compared to corresponding vehicle treated mice (Figure 9C). Finally, cytosolic GluA1 protein levels remained unaltered (Figure 9D).

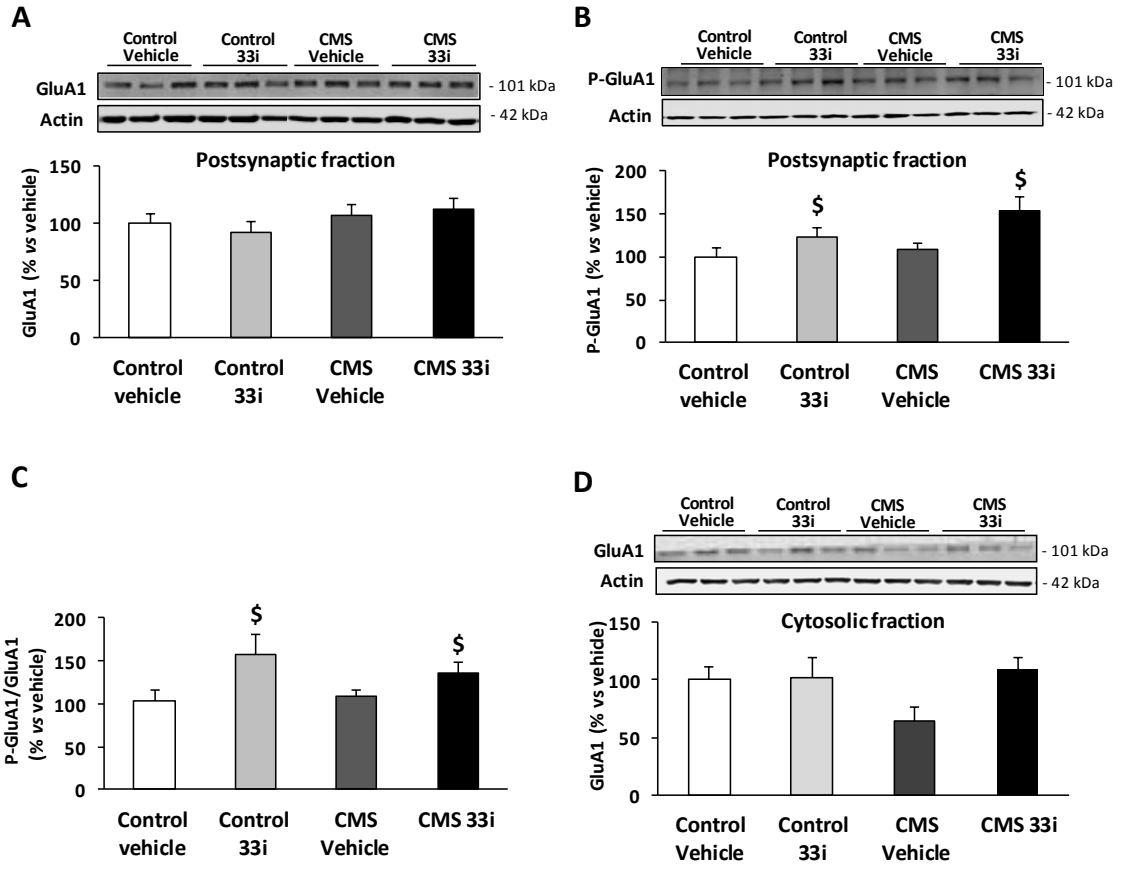

Figure 9. Effect of CMS and 33i (15 mg/Kg) treatment on postsynaptic fraction of GluA1 **(A)**, P-GluA1 **(B)** and P-GluA1/GluA1 ratio **(C)** and cytosolic fraction of GluA1 **(D)** protein expression levels in prefrontal cortex. Data show the mean ± SEM of relative abundance of protein expression compared to control saline group values (n= 8-10 mice/group). $ p<0.05, main effect of treatment (Two-way ANOVA followed by Tukey test).

On the other hand, cytosolic and postsynaptic SGK1 protein levels remained unaltered (Fig. 9).

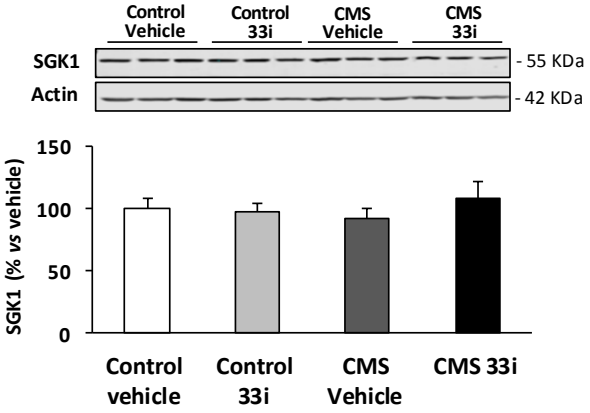

Figure 10. Effect of CMS and 33i (15 mg/Kg) treatment on SGK1 protein expression levels in prefrontal cortex.

4. DISCUSSION

This study suggests that the class III histone deacetylase SIRT2 could be a new pharmacological target for the treatment of major depression. Using the compound 33i, reported as a selective SIRT2 inhibitor *in vitro* (Suzuki et al., 2012) we show that SIRT2 inhibition modulates glutamate and serotonin system in the mouse prefrontal cortex (PFC) and induces an antidepressant-like action.

4.1. Sirt2 inhibitory activity of 33i in vivo

The compound 33i is a 3'-phenethyloxy-2-anilinobenzamide analogue representing a new class of SIRT2-selective inhibitors (Suzuki et al., 2012). Enzyme assays using human recombinant SIRT1 and SIRT2 have revealed that 33i is a potent and selective SIRT2 inhibitor, showing more than 35-fold greater SIRT2-selectivity compared to AGK2 (Suzuki et al., 2012), a previously reported SIRT2-selective inhibitor (Outeiro et al., 2007). In our study, we confirmed that 33i is also able to inhibit SIRT2 deacetylase activity in mouse PFC homogenates.

We next studied the effect of a subchronic administration of 33i (5-15 mg/kg) over 10 days on different pharmacodynamic markers that could give us evidence of 33i successfully reaching the brain and exerting an inhibitory SIRT2 deacetylase function. The first evidence was the observation of increased histone 3 (AcH3) and histone 4 (AcH4) acetylation levels, which are natural substrates of all the histone deacetylases (Hdacs). *In vitro* enzymatic assays have previously shown the ability of SIRT2 to deacetylate both histone 3 (H3) and 4 (H4). Of these, a strong preference for histone H4 specifically on lysine 16 (H4K16Ac) has been reported followed by H3 on lysine 9 (H3K9Ac) (Vaquero et al, 2006). Our results suggest that the reported *in vitro* preference of SIRT2 for H4 is also maintained *in vivo*, in the PFC, since AcH4 levels were upregulated by subchronic 33i at 5 and 15 mg/kg, while, only the highest dose increased significantly AcH3. However, recent studies suggest that this enzyme resides predominantly in the cytoplasm and that only specific stimulus such as cellular mitosis (Vaquero et al., 2006) or bacterial infection (Eskandarian et al., 2013) shuttle SIRT2 to the nucleus where it acts as an Hdac. However, as previously reported (North and Verdin., 2007) our studies reveal a constant role for SIRT2 in the nucleus.

In the cytoplasm, both the histone deacetylases SIRT2 and the NAD^+-independent class II HDAC6 play an important role in regulating microtubule dynamics through deacetylation of α-tubulin on lysine 40 (Noth et al., 2003; Hubbert et al., 2002). Cell cultures studies have previously reported hyperacetylation of α-tubulin using selective SIRT2

inhibitors such as AGK2 (Mangas-Sanjuan et al., 2014), 33i (Suzuki et al., 2012) or SirReal2 (Rumpf et al., 2015). Here, subchronic 33i treatment failed to increase PFC α-tubulin acetylation levels. Acetylation of α-tubulinK40 is one of the most important posttranslational modification of microtubules and is tightly controlled by several N-acetyltransferases (Wloga and Gaertig, 2010). Thus, N-acetyltransferases could be counteracting the inhibitory action of 33i on SIRT2 (Rumpf et al, 2015). In keeping with this, reduction or loss of SIRT2 has also failed to increase acetylated α-tubulin in the mouse brain (Bobrowska et al., 2012).

In addition, selective SIRT2 inhibitors upregulate Abca1 (Taylor et al., 2011: Spire-Jones et al., 2012), a gene encoding for the brain cholesterol transporter and a major regulator of cholesterol homeostasis (Zhang et al., 2005; Luthi-Carter et al., 2010). In agreement with this, we observed that mRNA abundance of *Abca1* was upregulated by subchronic 33i. Taken together, increased histone acetylation and *Abca1* mRNA abundance suggest that subchronic 33i exerts a SIRT2 inhibitory function in the mouse PFC.

4.2. Subchronic SIRT2 inhibition targets NMDA receptors and increase serotonin

SIRT2 is a histone deacetylase playing a role in transcriptional repression of genes encoding for DNA binding proteins as well as transcription factors that participate in synaptic plasticity, cell proliferation, differentiation and cell survival (Eskandarian et al., 2013). We studied here how gene expression changes induced by SIRT2 inhibition could modulate glutamate and serotonin system in the PFC. Subchronic treatment with 33i increased the expression of GluN2A and GluN2B NMDA receptor subunits. Moreover, it increased GluN2A/GluN2B ratio suggesting that SIRT2 inhibition upregulates NMDA receptors and favors glutamate synaptic transmission (Paoletti et al., 2013). Moreover, mRNA abundance of GluN2B gene was also upregulated suggesting that SIRT2 inhibition stimulates the synthesis of this subunit.

In addition, we observed that subchronic 33i treatment increased serotonin tissue levels in the PFC. Noteworthy, a reciprocal interaction between glutamate and serotonin occurs in the PFC, a brain area playing a primary role in affective disorders as well as in working memory and attention. In this region, NMDA receptors have been shown to stimulate serotonin release from fibers originating in the raphe nuclei (Fink et al., 1996). Similarly, serotonin in the PFC enhances glutamate transmission acting through postsynaptic 5-HT2A receptor activation (Aghajanian and Marek, 1999). In keeping with this, furture studies should explore whether GluN2B increase induced by SIRT2 inhibition could lead to an increase in NMDA dependent glutamate transmission in the PFC and subsequently to an increase in 5-HT release.

4.3. Chronic SIRT2 inhibition shows antidepressant-like activity

The therapeutic value of SIRT2 inhibition was studied in the chronic mild stress (CMS), a validated model of depression (Willner, 2005; Elizalde et al., 2008; 2010). We firstly analyzed anhedonia, defined as a reduced ability to experience pleasure and considered a core symptom of major depressive disorders according to the diagnostic and statistical manual of mental disorders (DSMMD-V). Interestingly, anhedonic behavior induced by CMS was fully reverted by 33i after three weeks of treatment.

Social withdrawal is a psychiatric phenomenon common to depression (Friedlander et al., 2004; Berton et al., 2006). Using the social interaction test, we found a significant decrease in the time spent in the interaction zone in CMS mice, a clear symptom of decreased sociability or social anxiety. This result agrees with a previous study in our lab and shows that CMS mice become aversive toward social stimuli (Venzala et al., 2012). Repeated treatment with 33i for more than three weeks fully reverted the decreased social interaction induced by CMS. Taken together these results suggest that selective inhibition of SIRT2 in the PFC induces an antidepressant-like action in this depression mouse model.

NMDA receptor-dependent synaptic plasticity is a strong candidate to mediate learning and memory processes (Bannerman et al., 2012). Given the previous effect of 33i on NMDA receptor subunits we explored the effect of possible memory enhancing action of SIRT2 inhibition on spatial memory using the Morris water maze (MWM). However, our data showed that neither CMS nor 33i treatment had an effect on spatial memory retention in the MWM test.

4.4. Chronic SIRT2 inhibition targets NMDA and AMPA receptors

While chronic SIRT2 inhibition increased GluN2B protein expression in the postsynaptic fraction, no changes in the cytosolic fraction were observed. These results suggest that SIRT2 inhibition promotes the synthesis of this subunit as well as its localization to the postsynaptic membrane. Regarding the AMPA receptor, although GluA1 subunit was not changed either in the postsynaptic or in the cytosolic fraction, we could see an increase of the phosphorylated form of this protein (P-GluA1) at Ser831 in the postsynaptic fraction, both in control and CMS mice treated with 33i. Thus, SIRT2 inhibition might stimulate the activation of AMPA receptors in the membrane.

NMDA receptors are molecular mediators of plasticity and among the different subunits GluN2B is particularly important to plasticity, because it can make a synapse bidirectional malleable (Shipton and Paulsen., 2014). Current evidences suggest that the

presence of GluN2B subunit-containing NMDARs at the postsynaptic density might be necessary for the synaptic plasticity strengthening. Specifically, GluN2B interact with calcium/calmodulin-dependent protein kinase II (CaMKII) and mediate AMPA receptors insertion through phosphorylation of Ser831 on GluA1 subunit (Shipton and Paulsen., 2014). Thus, SIRT2 inhibition could increase synaptic strength through GluN2B mediated increase in AMPA glutamate transmission. On the other hand, future studies should explore the effect of chronic stress on synaptic strength and the role of NMDA receptors.

Activation of glutamate receptors in the PFC has been linked to antidepressant like effects (Li et al., 2010; Koike et al., 2011). Antidepressant-like action exhibited by the SIRT2 inhibitor 33i could be associated to activation of AMPA receptors in the PFC. Importantly, a growing number of studies are now reporting that a NMDA receptor targeting drugs induce a rapid antidepressant response in patients and that this is due to a transient activation of AMPA receptors (Koike et al., 2011). In line with this, recent studies have shown that SSRIs and other antidepressants enhance glutamate transmission and synaptic strength in the PFC (Koike et al., 2011; Wolak et al., 2013).

On the other hand, the CMS induced increase in both GluN2A and GluN2B subunit expression in the postsynaptic fraction together with the significant decrease in the GluN2B cytosolic fraction suggests that chronic stress promotes the export of NMDA subunits from the cytosol to the cell membrane. Yet, in our study, the expression of the SGK1 enzyme, reported to be involved in stress induced NMDA receptor trafficking (Yuen et al., 2011), was not affected by CMS. Further studies should investigate how chronic stress might affect to NMDA expression.

Taken together, these results suggest that SIRT2 inhibition in the PFC modulates glutamate and serotonin system and induces antidepressant-like action. It is highlighted the therapeutic potential of SIRT2 as a new pharmacological target for the treatment of major depression and the need to further investigate the role of SIRT2 inhibitors as antidepressant agents.

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CAPÍTULO VI

CAPÍTULO 6

Discusión general

La depresión es un desorden mental caracterizado por humor depresivo, pérdida de interés por estímulos placenteros o anhedonia. Frecuentemente se encuentra asociado a otros síntomas psicológicos o somáticos tales como trastornos del sueño, baja autoestima, sentimientos de culpa e inclusive, en los casos más graves, tendencias suicidas (Wong y Licinio, 2001).

Es el trastorno afectivo más común, lo que supone una importante carga socioeconómica, sobre todo para países desarrollados. Debido a ello, se ha puesto un gran esfuerzo desde hace varias décadas en investigar, tanto a nivel clínico como experimental, los factores biológicos que contribuyen a su desarrollo. Sin embargo, a día de hoy, los estudios no han encontrado una causa que explique su etiopatología. Una hipótesis reciente sugiere que los factores de riesgo biológicos de la depresión podrían ser, en su base, epigenéticos (Oh y col., 2008). La ausencia de claros efectos genéticos en la depresión, junto con el hecho de que numerosos factores ambientales pueden modificar de manera más o menos estable la expresión génica a través de modificaciones epigenéticas, sin necesidad de alterar la secuencia del ADN, apoyan esta idea. Además, dichas modificaciones epigenéticas podrían ser la base para la enorme variabilidad interindividual hacia la adversidad o la respuesta al tratamiento (Wilkinson y col., 2009).

Siguiendo esta hipótesis reciente, este trabajo ha pretendido lograr una mejor comprensión de la neurobiología de la depresión así como identificar nuevas dianas farmacológicas implicadas en la remodelación de la cromatina, que permitan el desarrollo de nuevas moléculas con actividad antidepresiva.

Entre los diferentes agentes causantes de la depresión, numerosos estudios revelan una asociación consistente entre la exposición a eventos estresantes y la aparición de episodios de depresión mayor (Kendler y col., 1998; Mazure y col., 2000; Pittenger y Duman, 2008). Por ello, a nivel experimental, se han desarrollado modelos de estrés crónico en ratón, con la intención de mimetizar situaciones de la vida cotidiana en humanos. Algunos se basan en el estrés ambiental, como el estrés crónico suave (CMS, de sus siglas en inglés "*Chronic Mild Stress*"), mientras que otros, como el estrés crónico social por derrota (CSDS, de sus siglas en inglés *"Chronic Social Defeat Stress"*) se basan más en factores sociales. En el presente trabajo se han utilizado ambos modelos.

1. El tratamiento antidepresivo con imipramina revierte alteraciones conductuales y moleculares inducidas por el modelo CMS

El objetivo general del primer estudio era identificar los cambios moleculares que podrían causar episodios de depresión y/o aumentar la vulnerabilidad para la recaída así como confirmar la validez predictiva del modelo de CMS para estudiar nuevos compuestos con actividad antidepresiva. Para ello se analizaron cambios en la expresión génica producidas por el CMS a largo plazo y por el tratamiento con el antidepresivo imipramina. Igualmente, se estudió el efecto de este antidepresivo sobre el fenotipo depresivo inducido por el CMS.

1.1. Acción antidepresiva de la imipramina en el modelo de CMS a largo plazo

La anhedonia, definida como disminución de la capacidad para experimentar placer, es considerada un síntoma clave de los trastornos depresivos mayores. El modelo CMS ha sido ampliamente definido como un modelo robusto para estudiar la anhedonia (Willner, 2005) y la acción antianhedónica de posibles antidepresivos (Monleón y col., 1995; Kubera y col., 2001; Harkin y col., 2002; Strekalova y col., 2006; Elizalde y col., 2008; 2010; Garcia-Garcia y col., 2009). Como era de esperar, en el presente estudio, el CMS indujo un comportamiento anhedónico, medido como una disminución del consumo o de preferencia para la sacarosa, que persistió incluso un mes después de finalizar el procedimiento. El tratamiento crónico con imipramina fue capaz de revertir estos efectos, resultado que concuerda con estudios previos realizados en nuestro laboratorio utilizando el antidepresivo paroxetina (Elizalde y col., 2008). Sin embargo, a diferencia del estudio realizado con paroxetina (Elizalde y col., 2008), la acción anti-anhedónica de la imipramina se manifestó mucho antes, desde la tercera semana de tratamiento (última semana de CMS) y persistió incluso dos semanas después del período de lavado. Por tanto, la imipramina acelera la recuperación de la anhedonia inducida por estrés y fortalece la validez del modelo CMS para estudiar los efectos moleculares a largo plazo que podrían estar vinculados al comportamiento y a la acción antidepresiva.

Numerosas evidencias clínicas han demostrado que las diferentes disfunciones cognitivas, la evitación social y la ansiedad están asociadas a anhedonia y varían en función de la severidad del trastorno depresivo (Airaksinen y col., 2004; Moffitt y col., 2007; Hamilton y col., 2014). En línea con estos estudios, se observó que el CMS produjo alteraciones en el comportamiento, incluyendo deterioro de la memoria en el test de reconocimiento de nuevo objeto, disminución de la sociabilidad en el test de interacción social y ansiedad en el test del laberinto elevado (EPM, de sus siglas en inglés *"Elevated Plus*

Maze"). Este comportamiento, sin embargo, no se observó en los ratones sometidos al CMS tratados con imipramina. Nuestros resultados concuerdan con datos clínicos que muestran la capacidad que tienen diferentes antidepresivos tricíclicos para mejorar la función cognitiva a la vez que mejoran el humor de los pacientes deprimidos (Amado-Boccara y col., 1995), y confirman los efectos ansiolíticos de la imipramina observados en otros estudios (Nair y col., 1996).

1.2. Alteraciones moleculares a largo plazo inducidas por el CMS: Efecto de la imipramina.

El modelo CMS ha sido ampliamente estudiado a nivel molecular (Airan y col., 2007; Gronli y col., 2007; Banasr y col., 2010; Garcia-Garcia y col., 2009) y celular (Warner-Schmidt y Duman, 2006; Jayatissa y col., 2008). Además, estudios previos han demostrado que algunas de estas alteraciones persisten incluso un mes después del procedimiento (Elizalde y col., 2010a, 2010b). En el presente estudió se demuestra que el CMS afecta a largo plazo a la expresión de genes implicados en diferentes procesos como la vía canónica del ritmo circadiano, la supervivencia y muerte celular así como en trastornos neurológicos, psicológicos y conductuales. En concreto, el análisis *Ingenuity pathway analysis* (IPA) sugirió que el CMS estimula la muerte celular neuronal e inhibe la proliferación y el crecimiento celular. Resulta interesante que algunos de los cambios inducidos por el CMS fueron revertidos por la administración crónica durante cinco semanas con imipramina y después de un periodo de lavado de dos semanas. De entre los diferentes genes afectados por el CMS y revertidos por la imipramina, candidatos de la vía de la señalización del ritmo circadiano, entre los que se encuentra Hdac5 y Per2, fueron estudiados por RT-PCR y Western blot.

En su conjunto, nuestros resultados sugieren que la imipramina acelera la recuperación de algunas funciones celulares patológicamente alteradas por el estrés y subraya la relevancia de estas dianas para la acción antidepresiva. Por otro lado, los genes no revertidos por la imipramina podrían formar parte de los mecanismos neurobiológicos residuales de vulnerabilidad a la recaída. Así pues, el efecto restaurador de la imipramina, sobre las alteraciones moleculares y el fenotipo depresivo del CMS, confirma la validez predictiva del modelo del CMS para estudiar nuevos compuestos con potencial acción antidepresiva.

2. El CSDS y el tratamiento con imipramina regulan la superfamilia de Hdacs

Varios estudios sugieren que cambios en la acetilación de histonas pueden contribuir a la patogénesis de la depresión y a la terapia antidepresiva (Tsankova y col., 2006). El segundo objetivo de este trabajo fue analizar el efecto del estrés crónico, con el modelo del

CSDS, y del tratamiento antidepresivo sobre la expresión de distintas histonas desacetilasas (Hdacs). En general, el CSDS indujo un aumento en la expresión del ARNm de diferentes Hdacs, incluyendo H*dac8* (clase I), *Hdac5* y *Hdac7* (clase II) y *Sirt2*, *Sirt3* y *Sirt6* (clase III). Por otro lado, el tratamiento repetido con imipramina redujo la expresión del ARNm de *Hdac1* (clase I), *Hdac5* y *Hdac10* (clase II) *y Sirt2, Sirt3, Sirt4, Sirt5* y *Sirt7* (clase III). Aunque la regulación transcripcional es muy compleja, se podría decir que un aumento en la expresión de Hdacs daría lugar a una disminución en los niveles de acetilación de histonas y, por tanto, a una mayor compactación del ADN, produciendo un silenciamiento de la expresión génica. Del mismo modo, una disminución en la expresión de la proteína de Hdac estimularía la transcripción genética (Gallinari y col., 2007).

En consonancia con esto, el CSDS provocó una disminución en los niveles de acetilación tanto de la histona 3 como de la 4 (AcH3 y AcH4), así como del marcador de plasticidad sináptica CREB. Por el contrario, el tratamiento con imipramina aumentó significativamente la expresión de AcH3, AcH4, CREB y pro-BDNF. Estos resultados confirman estudios previos que también han mostrado una regulación a la baja de diferentes Hdacs después del tratamiento antidepresivo y consecuentemente, un aumento en la acetilación de histonas (Tsankova y col., 2006).

De entre todas las enzimas de la superfamilia de Hdacs, seleccionamos Hdac5 y Sirt2, ya que su expresión de ARNm resultó regulada opuestamente por el estrés crónico y por el tratamiento con imipramina. Concretamente, el CSDS aumentó la expresión del ARNm de *Hdac5* y *Sirt2* mientras que el tratamiento con imipramina lo revirtió.

2.1. Implicación funcional de la regulación de HDAC5

HDAC5 es una histona desacetilasa que pertenece a la clase IIa. Esta clase, a la que también pertenecen HDAC 4, 7 y 9, es única entre las proteínas de la familia de Hdacs ya que sufre transporte desde el núcleo al citoplasma, dependiente de la actividad sináptica (McKinsey y col., 2001). Esto es posible debido a que el dominio amino terminal de la enzima está sujeto a una fosforilación reversible que controla su distribución núcleo-citoplasmática. La fracción no fosforilada permanece en el núcleo, unida a la cromatina, reprimiendo la transcripción mientras que la fracción fosforilada, sale fuera del núcleo, permitiendo la expresión de sus genes diana (Parra y Verdin, 2010).

Estudios previos han demostrado que el HDAC5 nuclear en el núcleo accumbens inhibe la sensibilidad a la recompensa de la cocaína (Renthal y col., 2007), lo cual sugiere que desempeña un papel importante reprimiendo la expresión de genes involucrados en la

reactividad a la recompensa. Por otra parte, un estudio relevante ha demostrado que la sobre-expresión de HDAC5 bloquea los efectos conductuales de los antidepresivos en el modelo CSDS (Tsankova y col., 2006). En línea con estos estudios, se ha observado un aumento de ARNm de *HDAC5* en la corteza cerebral de sujetos con trastorno depresivo mayor y trastorno bipolar, en comparación con controles y pacientes en remisión, apoyando la relevancia clínica de este cambio (Hobara y col., 2010).

Por otro lado, el tratamiento con imipramina aumentó el contenido de la forma fosforilada de HDAC5 (P-HDAC5), lo que sugiere que este tratamiento favorece el transporte de HDAC5 desde el núcleo al citoplasma, donde no puede ejercer su función desacetilasa. Así, la salida de HDAC5 desde el núcleo podría ser el mecanismo por el cual el tratamiento con imipramina estimula la expresión de genes específicos involucrados en la plasticidad sináptica, como pro-BDNF y su regulador CREB.

El antidepresivo tricíclico, imipramina, es un inhibidor de recaptación de serotonina y noradrenalina y, curiosamente, se ha descrito un papel de los receptores de noradrenalina en el transporte nucleocitoplasmático de HDAC5. En concreto, mientras que la activación de los β-adrenoceptores induce la acumulación nuclear de HDAC5 (Sucharov y col., 2011), la fosforilación y translocación al citoplasma son inducidas por la activación de los receptores adrenérgicos α (Sucharov y col., 2011), probablemente como consecuencia de una afluencia del calcio y la consiguiente activación de la actividad quinasa (Meucci y col., 1995). Nuestros resultados posteriores confirmaron la implicación de la noradrenalina, ya que, mientras que el tratamiento repetido con el inhibidor de la recaptación de noradrenalina, reboxetina, aumentó la expresión de P-HDAC5, no se observaron cambios con el inhibidor selectivo de la recaptación de serotonina, fluoxetina. Por otra parte, de acuerdo con nuestros resultados, la acción antidepresiva de la imipramina en el modelo de CSDS se ha asociado con un aumento en la acetilación de histonas en el promotor de BDNF, que es, específicamente, causado por una disminución del ARNm de *Hdac5* en el hipocampo (Tsankova y col., 2006).

Posteriormente, se quiso comprobar si una inhibición mantenida de HDAC5, durante un período de tres semanas, aumentaba los marcadores de plasticidad sináptica. Seleccionamos el compuesto MC1568, un inhibidor de la clase IIa de Hdacs, para estudiar su efecto en los marcadores de plasticidad sináptica. Los ratones fueron tratados durante 21 días, imitando nuestro estudio con tratamiento antidepresivo. Como era de esperar, MC1568 fue capaz de aumentar la acetilación de las histonas, así como pro-BDNF, lo cual sugiere que la inhibición de la actividad enzimática de esta subfamilia es capaz de modular la plasticidad neuronal. Por otra parte, esto refuerza la idea de que el efecto de los antidepresivos

noradrenérgicos sobre la plasticidad sináptica podría estar mediado, en parte, por una disminución de la función HDAC5 (Tsankova y col., 2006).

2.2. Implicación funcional de la regulación de SIRT2

Debido a su participación en la homeostasis metabólica y en el envejecimiento del cerebro, las sirtuinas han despertado un creciente interés en las enfermedades neurodegenerativas. Entre todas las sirtuinas, SIRT2 es una de las que más se expresa a nivel cerebral (Michishita y col., 2005). SIRT2 reside en el núcleo realizando su función de histona desacetilasa pero también de manera importante en el citoplasma donde participa en la organización del citoesqueleto teniendo como diana a α-tubulina (North y col., 2003). Particularmente, se ha relacionado un polimorfismo del gen de *SIRT2* con la depresión en pacientes de la enfermedad de Alzheimer (Porcelli y col., 2013). Por otra parte, la inhibición de SIRT2 ejerce efectos neuroprotectores en diversos modelos de enfermedades neurodegenerativas, entre ellas la enfermedad de Parkinson (Outeiro y col., 2007) y de Huntington (Taylor y col., 2011).

En nuestros estudios se observó un aumento de expresión de ARNm y proteína de Sirt2 en la corteza prefrontal (PFC) de ratones expuestos a CSDS. Sin embargo, no se pudo observar ningún cambio en los niveles de acetilación de α-tubulina en ratones CSDS. Tal vez, esto podría deberse, en parte, al hecho de que la función de esta enzima podría estar enmascarada por otras enzimas acetil-transferasas que también utilizan α-tubulina como sustrato (Friedmann y col., 2012; Maxwell y col., 2011). Por otro lado, el tratamiento con imipramina disminuyó la expresión de SIRT2 tanto en ratones control como en los sometidos a CSDS, efecto que podría estar ligado a la conocida acción neuroprotectora de la imipramina, a través de la estabilización de los microtúbulos del citoesqueleto (Creppe y col., 2009).

Respecto al mecanismo de acción implicado en este efecto de la imipramina, tanto el inhibidor selectivo de la recaptación de noradrenalina, reboxetina, como el inhibidor selectivo de la recaptación de serotonina, fluoxetina disminuyeron la expresión de SIRT2, sugiriendo que esto podría ser un mecanismo compartido de los antidepresivos monoaminérgicos en general. Además, la inhibición especifica de SIRT2 por la administración repetida de 33i durante tres semanas, siguiendo el mismo diseño experimental que con fármacos antidepresivos, produjo un aumento en la expresión de AcH4, CREB y pro-BDNF, sugiriendo que la inhibición de SIRT2 modula la plasticidad sináptica.

En conjunto, estos resultados coinciden con los cambios observados en la acetilación de histonas, CREB y pro-BDNF tras el tratamiento con imipramina, y sugieren que la inhibición de SIRT2 podría ser otro mecanismo por el cual este antidepresivo estimula la expresión de genes involucrados en la plasticidad sináptica. En línea con esta hipótesis, nuestros resultados mostraron un aumento en la expresión del ARNm de *SIRT2* en la PFC de pacientes deprimidos confirmando el potencial interés de esta enzima para la intervención terapéutica. Sin embargo, no se encontraron cambios en el grupo de pacientes con psicosis. En este sentido, podría sugerirse que el tratamiento antipsicótico recibido por estos pacientes podría revertir el incremento de *SIRT2* observado en el grupo deprimido. De hecho, el uso de antipsicóticos aparece como una variable de confusión en la expresión de *SIRT2*.

En resumen, nuestros resultados sugieren un papel de Hdac5 y Sirt2 en las adaptaciones neuronales inducidas por estrés crónico y el tratamiento antidepresivo y destacan el potencial terapéutico de estas dianas en el tratamiento de la depresión.

3. Implicación de SIRT2 en el fenotipo depresivo y en la acción antidepresiva

El tercer estudio se diseñó con el objetivo de validar el interés de SIRT2 como potencial diana para el tratamiento de la depresión. Para ello, se investigó el efecto del inhibidor específico de SIRT2, 33i, en el modelo del CMS, validado como buen modelo de depresión.

El compuesto 33i es un análogo de la 3'-phenethyloxy-2-anilinobenzamida que representa una nueva clase de inhibidores selectivos de SIRT2 (Suzuki y col., 2012). En primer lugar, en nuestro estudio, confirmamos que el 33i es capaz de inhibir la actividad desacetilasa de SIRT2 en homogenados de PFC de ratón.

Posteriormente se estudió el efecto de una administración subcrónica de 33i (5 y 15 mg/kg), durante 10 días, sobre diferentes marcadores farmacodinámicos que nos pudieran dar evidencias de que el 33i alcanza con éxito el cerebro y ejerce una función inhibitoria de SIRT2. La primera evidencia fue la observación de un aumento de los niveles de AcH3 y AcH4, que son sustratos naturales de todas las Hdacs. Ensayos enzimáticos previos habían demostrado *in vitro* la capacidad de SIRT2 de desacetilar la histona 3 (H3) y, con mayor preferencia, la 4 en lisina 16 (H4K16Ac) (Vaquero y col., 2006). Nuestros resultados confirmaron esta preferencia de SIRT2 por H4 también *in vivo*, ya que sólo la dosis más alta de 33i administrada incrementó AcH3 de manera significativa.

La segunda evidencia se refiere a que los inhibidores selectivos de SIRT2 aumentan Abca1 (Taylor y col., 2011: Spire-Jones y col., 2012), un gen que codifica para el

transportador de colesterol del cerebro y un importante regulador de la homeostasis de colesterol (Zhang y col., 2005; Luthi-Carter col., 2010). De acuerdo con esto, observamos que los niveles de ARNm de Abca1 estaban aumentados por el tratamiento con 33i. Por tanto, tomados en conjunto, el aumento de la acetilación de las histonas y la abundancia del ARNm de Abca1, sugieren que el tratamiento con 33i ejerce una función inhibitoria de SIRT2 en la PFC de ratón.

3.1. La inhibición de SIRT2 modula los receptores NMDA y aumenta los niveles de serotonina

Posteriormente estudiamos cómo los cambios en la expresión génica inducidos por la inhibición de SIRT2 podrían modular el sistema de glutamato y serotonina en la PFC, un área en la que ambos sistemas interactúan desempeñando funciones importantes en los trastornos afectivos, en la memoria de trabajo y la atención. El tratamiento subcrónico con 33i aumentó la expresión de las subunidades del receptor NMDA, GluN2A y GluN2B así como del cociente GluN2A/GluN2B. En conjunto, estos resultados sugieren que la inhibición de SIRT2 podría aumentar la expresión de receptores NMDA facilitando la transmisión glutamatérgica (Paoletti et al., 2013). Por otra parte, los niveles de ARNm de GluN2B estaban, también, aumentados sugiriendo que la inhibición de SIRT2 estimula la síntesis de esta subunidad. Además, se observó que el tratamiento con 33i aumentó los niveles de serotonina.

En la PFC, los receptores de NMDA pueden estimular la liberación de serotonina a través de fibras glutamatérgicas que descienden hasta los núcleos del rafe (Fink y col., 1996), efecto que podría explicar el aumento de serotonina observado tras el tratamiento. Del mismo modo, la serotonina en la PFC también mejora la transmisión de glutamato a través de la activación del receptor 5-HT2A postsinápticos (Aghajanian y Marek, 1999). Sin embargo sería necesario realizar estudios dirigidos a dilucidar la posible interacción entre los receptores NMDA y los niveles de serotonina regulados por la inhibición de SIRT2.

3.2. La inhibición crónica de SIRT2 posee actividad antidepresiva

Seguidamente, nos propusimos investigar si la inhibición de SIRT2 presentaba acción antidepresiva. Para ello utilizamos el modelo del CMS, ya que, como se ha explicado anteriormente, se presenta como un modelo validado para estudiar la anhedonia y la acción antianhedonica de posibles antidepresivos (Monleón y col., 1995; Kubera y col., 2001; Harkin y col., 2002; Strekalova y col., 2006; Elizalde y col., 2008; Garcia-Garcia y col., 2009).

En primer lugar, por tanto, se analizó el efecto que la inhibición de SIRT2 tenía sobre la anhedonia. Curiosamente, el comportamiento anhedónico inducido por el CMS se revirtió totalmente por el tratamiento con 33i. A continuación, utilizando el test de la interacción social, observamos que el tratamiento repetido con 33i durante más de tres semanas revertió totalmente la disminución de la interacción social inducida por el CMS. En conjunto estos resultados indican que la inhibición selectiva de SIRT2 en la PFC induce una acción antidepresiva en este modelo de depresión en ratón.

3.3. Efecto del CMS y del 33i sobre los receptores NMDA y AMPA

El CMS aumentó la expresión de las subunidades del receptor NMDA, GluN2A y GluN2B en la fracción postsináptica. Sin embargo, se observó una disminución de GluN2B en la fracción citosólica, lo que sugiere que el estrés crónico podría promover el transporte de subunidades NMDA desde el citosol a la membrana celular. Un estudio previo ha descrito que la enzima SGK1 está involucrada en el desplazamiento intracelular del receptor NMDA inducido por el estrés (Yuen y col., 2011), sin embargo, en nuestro estudio, la expresión de la enzima no se vio modificada por CMS por lo que son necesarios más trabajos que estudien el efecto del estrés crónico en la actividad sináptica y el papel de los receptores NMDA en el mismo.

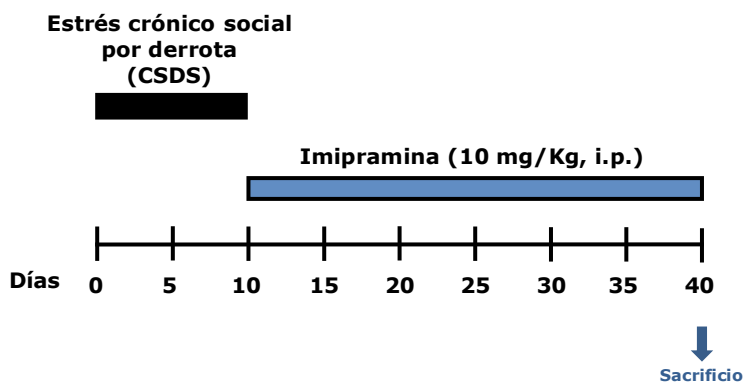
Mientras que la inhibición crónica de SIRT2 aumentó la expresión de la proteína GluN2B en la fracción postsináptica, no se observaron cambios en la fracción citosólica. Estos resultados sugieren que la inhibición de SIRT2 promueve la síntesis de esta subunidad, así como su localización en la membrana postsináptica. Con respecto a los receptores AMPA, aunque la subunidad GluA1 no cambió ni en la fracción postsináptica ni en la citosólica, pudimos ver un aumento de la forma fosforilada de esta proteína (P-GluA1) en la Ser831 en la fracción postsináptica de los ratones tratados con 33i, indicando que la inhibición de SIRT2 podría estimular la activación de receptores AMPA en la membrana.

Los receptores NMDA son mediadores moleculares de la plasticidad y, entre las diferentes subunidades, GluN2B es especialmente importante para la plasticidad sináptica (Shipton y Paulsen, 2014). Evidencias actuales sugieren que la presencia de receptores de NMDA con subunidades de GluN2B en la densidad postsináptica podría ser necesaria para el fortalecimiento de la plasticidad sináptica. Específicamente, GluN2B interactúa con la proteína dependiente de calcio/calmodulina kinasa II (CaMKII) y media la inserción de los receptores AMPA a través de la fosforilación de la Ser831 en la subunidad GluA1 (Shipton y Paulsen, 2014). Así, la inhibición de SIRT2 podría aumentar la actividad sináptica a través de un incremento en la transmisión del receptor AMPA mediado por el aumento en GluN2B

observado. En línea con nuestros resultados, un número creciente de estudios muestran que fármacos que tienen como diana los receptores de NMDA inducen una respuesta antidepresiva rápida en pacientes y que esto es debido a una activación transitoria de los receptores AMPA (Koike y col., 2011). En consonancia con esto, estudios recientes han demostrado que los ISRS y otros antidepresivos mejoran la transmisión de glutamato y la fuerza sináptica en la PFC (Koike y col, 2011; Wolak y col., 2013).

Tomados en conjunto, estos resultados sugieren que la inhibición SIRT2 en la PFC modula el sistema glutamato y serotonina e induce acción antidepresiva. Se destaca el potencial terapéutico de SIRT2 como una nueva diana farmacológica para el tratamiento de la depresión mayor y la necesidad de investigar más el papel de los inhibidores de SIRT2 como agentes antidepresivos.

A



B

Tratamientos (21 días)

Imipramina: 10 mg/Kg, i.p.
Fluoxetina: 15 mg/Kg, i.p.
Reboxetina: 10 mg/Kg, i.p.

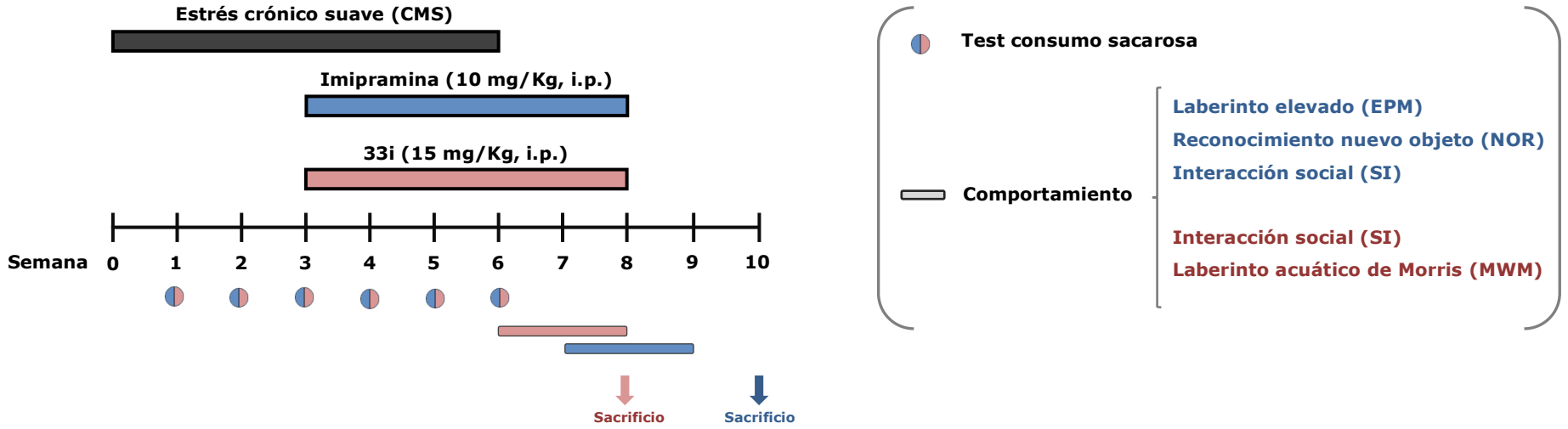
33i: 1 mg/Kg, i.p.
MC1568: 10 mg/Kg, i.p.

C

	WB							
	AcH3	**AcH4**	**CREB**	**Pro-BDNF**	**HDAC5**	**P-HDAC5**	**SIRT2**	**Ac. Tub.**
CSDS	↓	↓	↓	NC	↑	NC	↑	NC
CSDS + Imi	R	R	R	↑	NR	↑	R	NC
Imipramina	↑	↑	↑	↑	NC	↑	↓	↑
Fluoxetina					NC	NC	↓	NC
Reboxetina					NC	↑	↓	↑
33i	NC	↑	↑	↑				
MC1568	↑	↑	NC	↑				

Las flechas indican un aumento (↑) o descenso (↓) significativo respecto a los ratones control tratados con salino. NC, no cambio; NR, no reversión del efecto; R, reversión del efecto.

Fig. 1. (A) Resumen del protocolo experimental del modelo del CSDS y el tratamiento con imipramina (experimento1). **(B)** Resumen de los distintos tratamientos administrados en los experimentos 2 y 3. **(C)** Tabla resumen de los principales resultados obtenidos en los experimentos 1, 2 y 3 (capítulo 4).



	Behavior					WB					
	Anhedonia	**EPM**	**MWM**	**NOR**	**SI**	**GluN2B**		**GluN2A**	**GluN2A/GluN2B**	**GluA1**	**P-GluA1**
	Consumo sacarosa	Tiempo latencia	Latencia escape	Tiempo exploración	Tiempo interacción	FP	FC	FP	FP	FP	FP
CMS	↓	↑	NC	↓	↓	↑	↓	↑	↓	NC	NC
CMS + Imi	R	R		NR	R						
CMS + 33i	R		NC		R	↑	R	NR	R	NC	↑

Las flechas indican un aumento (↑) o descenso (↓) significativo respecto a los ratones control tratados con vehículo. NC, no cambio; NR, no reversión del efecto; R, reversión del efecto; FC, fracción citosólica; FP, fracción popstsináptica

Fig. 2. Resumen del protocolo experimental y de los principales resultados obtenidos con el modelo del CMS y el tratamiento con imipramina y 33i (capítulo 5).

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CAPÍTULO VII

CAPÍTULO 7

Conclusiones

1. El modelo del estrés crónico suave (CMS) induce un fenotipo molecular y conductual depresivo a largo plazo que es revertido, en parte, por el tratamiento antidepresivo, lo cual apoya la validez de este modelo para estudiar nuevos compuestos con potencial acción antidepresiva. Concretamente, el CMS induce anhedonia, síntoma central de la depresión, y evitación social, que son revertidas por el tratamiento crónico con imipramina.

2. El modelo de estrés crónico social por derrota (CSDS) y el tratamiento antidepresivo regulan opuestamente la expresión de histonas desacetilasas (Hdacs) en la corteza prefrontal, afectando a la neuroplasticidad. Específicamente, el estrés crónico aumenta las histonas desacetilasas HDAC5 y SIRT2 en la corteza prefrontal, induciendo fenómenos neuroadaptativos mantenidos. A su vez, los efectos beneficiosos de los antidepresivos sobre la plasticidad sináptica podrían deberse a una pérdida de función de estas enzimas, bien porque el aumento de noradrenalina en el espacio sináptico exporta HDAC5 del núcleo al citoplasma (P-HDAC5), o bien porque disminuyen la expresión de SIRT2.

3. El inhibidor selectivo de SIRT2, 33i, revierte el fenotipo conductual depresivo inducido por el CMS, acción que podría estar mediada por un aumento de la transmisión glutamatérgica en la corteza prefrontal, dependiente de los receptores AMPA y NMDA.

4. En este trabajo se han identificado dos enzimas desacetilasas que aparecen reguladas por el estrés y por el tratamiento antidepresivo. En concreto, se demuestra el potencial terapéutico de la enzima SIRT2 como una nueva diana farmacológica para el tratamiento de la depresión y la necesidad de estudiar con mayor profundidad el papel de distintos inhibidores de SIRT2 como fármacos antidepresivos. En su conjunto, este trabajo apoya la hipótesis que asocia la depresión mayor y la terapia antidepresiva a cambios epigenéticos que podrían explicar la enorme variabilidad interindividual de respuesta hacia la adversidad o al tratamiento.