VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

Doctoral Thesis

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2013

ALTERATIONS IN THE STRUCTURE OF NEURONAL INHIBITORY NETWORKS IN PSYCHIATRIC DISORDERS

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VNIVERSITAT DE VALÈNCIA

PhD program in Biotechnology

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A mi familia

Ante todo es necesario cuidar del alma si se quiere que la cabeza y el resto del cuerpo funcionen correctamente

Platón (427 a. C. – 347 a. C.)

AGRADECIMIENTOS

Si hay algo que vale la pena cuando uno hace una tesis, eso es sin duda la sensación tan placentera que te produce pensar que en algún momento de tu vida la acabarás y te la quitarás de encima para siempre. Pues bien, con este pensamiento voy a intentar escribir los agradecimientos de la susodicha.

Encontrar un buen director de tesis es algo realmente complicado en el mundo de la ciencia, encontrar dos buenos directores es harto difícil, encontrar a los dos mejores directores parece imposible, pues bien, ese ha sido mi primer logro en esta tesis. Juan y Loli, no puedo imaginar mejor desarrollo científico y humano de una tesis que el que yo he tenido con vosotros como guías. Os admiro, os quiero y os odio (a ratos), lo que os convierte en parte de mi familia. Gracias por todo.

Desde pequeño quería estudiar una carrera, como mi hermano, en el cole decidí que sería de ciencias, en el instituto gracias a Dolores González me decanté por la biología, en la facultad me fascinó la genética y Rosa de Frutos me acogió como colaborador en su laboratorio, cuando quise hacer el máster, Loli me introdujo en el estudio de la esquizofrenia y cuando no conseguía beca predoctoral y estaba a punto de tirar la toalla Juan me tendió la mano. Soy lo que soy por vosotros.

Otros de los grandes modeladores de la tesis son tus compañeros de laboratorio, de los que aprendes y con los que compartes las alegrías y las tristezas. En mi caso los compañeros del 4º y del 6º. Empezaré por orden cronológico. Cuando era un pollete y empecé de colaborador en genética, primero de Olga y luego de Jose Luis, me trataron muy bien y aprendí mucho de ellos, sobre todo a pasarlo bien en el laboratorio, fueron años de cenas, fiestas, bolos, karaokes… gracias. Con Jero y Noe he pasado unas noches de fiesta geniales, además de disfrutar el día a día en el laboratorio, no os pienso como colegas sino como amigos. Sirena ha sido una compañera perfecta, y la echo mucho de menos, necesitamos recuperar la Sirenocracia, y las cervezas en el ambit. Josep, el último de la vieja guardia, me alegro mucho de que hayas seguido en este laboratorio y poder disfrutar de este tiempo con un amigo como tú. Pablo con el que me encanta discutir de política, aunque él sabe que es desde el cariño. A vosotros y a todos los que nos precedieron en este laboratorio de genética molecular humana, Ivette, Juan Antonio, Amparo, Isabel… gracias.

Y mi gente del 4º, que ahora vivimos en barracones. Esther me ha ayudado siempre y ha sido quien me ha enseñado casi todas las técnicas celulares, nunca podré agradecérselo demasiado. Clara que siempre me da la visión sosegada de las cosas, que tanta falta me hace. Tere con la que me encanta hablar de todos los temas humanos y divinos. Laura que siempre tiene una sonrisa y está pendiente de todos. Marta, que es toda simpatía y estoy

seguro que llegará muy lejos en este mundo. Raúl, que me toma el relevo en el genotipado de ratones quitándome un peso de encima. A todos vosotros y a la gente que ya se fue como Sandra, Samuel, Mariángeles, David, Ulises, Ramón, muchas gracias.

Y no me puedo olvidar de mis colaboradores!! Ana Rosa, que siempre estuvo dispuesta a aprender cosas nuevas y puso mucho empeño, tal que así. Héctor, mi rasta-colaborator, que ya tiene un máster en cortar cerebros. María que me riñe tanto por mis despistes como yo le riño a ella por los suyos. Rocío, que es mi última adquisición y que en la fusión con Noelia han formado el dúo Nocio que apunta al premio Nobel. Y finalmente Ana y Lucía, que nos alegran el laboratorio, gomet azul para las dos. Y a tod@s l@s colaborador@s que formaron parte de nuestro grupo, gracias.

Además he contado con el apoyo del resto de miembros de ambos departamentos. En genética quiero dar las gracias a l@s GMDs, tanto las nuevas como las viejas generaciones, dais vida al departamento!! Las bioquímicas, y bioMols con las que he pasado ratos geniales y espero seguir pasándolos cuando organicemos nuestro viaje fin de tesis y más. Gloria, que aunque nos cueste quedar a tomar una cerveza siempre sirve de terapia y es absolutamente necesario de forma trimestral. A los profesores, a Maria José, que me ha sacado las más grandes carcajadas en las reuniones de laboratorio, a Lluis, que siempre tiene algo que polemizar conmigo, a Julio Sanjuan, con el que aprendo muchísimo de psiquiatría y me acerca siempre a la visión clínica, a Núria Paricio que fue durante un tiempo mi directora de tesis circunstancial y siempre me facilitó el trabajo. A Salva, que a veces me saca de mis casillas pero siempre me divierto con él. A Rosa de Frutos, que me introdujo en el mundo de la genética y a la que admiro, aprecio y siempre echo de menos. A Carmen Nájera, que siempre me deja con la boca abierta de sus viajes por el mundo. En biología celular a Emilio, que cuando no le da por putearme me ayuda muchísimo y me hace reir, igual que Jose Miguel, mi paisano, ya que Chiva es un barrio de Alborache, y que ahora han formado el dúo cómico Epi y Blas-co. A Carlos Crespo, que me ha hecho muy fácil la docencia y siempre está dispuesto a echar una mano. A Carlos López, Chonchi y Xavi, que son los familiares a los que vemos poco y con los que comemos un día en Navidades. Y al resto de miembros de ambos departamentos porque siempre que lo he pedido me han ayudado.

También quiero dar las gracias a los componentes del laboratorio del Dr. Herbert Hildebrandt en Alemania que me acogieron en su laboratorio y con los que compartí unos meses de mi tesis. A todos los amigos que me hicieron la vida más fácil en aquel frio país, Marcos, Wiebke, Santi…

A mis amigos de la facultad porque son el andamio en el que me sujeto cuando las cosas van mal, y con los que comparto las cosas que van bien. A Carmen, y su laboratorio de

Fashion Microbiology, porque siempre está cuando se la necesita y he compartido gran parte de mi tesis con ella. A Judit, que me encanta llevarle la contraria y que sus palmeros la apoyen, jajajaja. A Frustuck, perdón, Noelia, que la veo poco pero siempre está ahí cuando te hace falta. A Javi, compañero de viajes y batallas, que te puede hacer de guía en cualquier país del mundo. A Blanca, porque hacemos el equipo perfecto, pinky y cerebro (yo soy cerebro). A Isa, que junto al Fidel es la única que queda en Zaidía, ajajajaj. A todos los que quiero pero veo poco, Elena, Mar, Amparo, Jose, Deme, Ramón.

A los Alboracheros, a mi peña el Armueso y cols. A los minces, las musas, las reinas marujis, las gemes, los primos, la peña el furgolín, los gelatinos, las jinetas, la xaranga la rexabia, los trutis, los chuches, los cuervos, la AFW, en fin a todo "Alborache Shore" (me estoy ahogando de risa mientras escribo esto). Porque son el contrapunto a todo lo políticamente correcto, porque son el sur, porque son necesarios, imprescindibles, porque somos zorros y se nos nota.

Finalmente a mi familia. A mis padres que han luchado toda la vida para que yo lo tuviera todo y porque todavía lo hacen. A mi hermano que siempre ha sido el espejo donde me he querido ver. A mi cuñada, porque nunca fue mi cuñada, siempre mi hermana. A mis sobrinas, que son una razón para luchar, a veces la única.

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Resumen

Introducción

El trabajo de investigación de la presente Tesis doctoral realizado por Javier Gilabert Juan, ha estado centrado en el estudio de la plasticidad estructural neuronal de interneuronas en trastornos psiquiátricos, abordado desde diferentes estrategias de estudio: modelos animales, estudios en muestras de cerebro postmortem de pacientes con enfermedades psiquiátricas y estudios de asociación.

Durante los últimos años, diversos trabajos han puesto de manifiesto que ciertas neuronas del sistema nervioso central (SNC) adulto son capaces de remodelar su estructura y cambiar sus conexiones. Esta plasticidad estructural subyace a fenómenos cognitivos y se produce también como respuesta a experiencias aversivas, como el miedo o la ansiedad, en animales de experimentación y en modelos animales de esquizofrenia y de depresión. Más aún, se han detectado cambios estructurales similares en el cerebro de pacientes, y los antipsicóticos comunes y los fármacos que inducen psicosis también son capaces de modular la plasticidad estructural neuronal. Estos descubrimientos han dado lugar a una nueva hipótesis, denominada hipótesis neuroplástica, para explicar el origen de diferentes trastornos psiquiátricos como la esquizofrenia o la depresión.

Algunas de las regiones más afectadas en los modelos animales de esquizofrenia, depresión o estrés y en pacientes con estos trastornos son la corteza prefrontal y el sistema límbico: hay un gran número de evidencias de que estas regiones sufren remodelado dendrítico y de espinas. Estos cambios morfológicos están normalmente mediados por cambios en la expresión de proteínas de adhesión, como la molécula neural de adhesión celular (NCAM). Ésta es particularmente interesante porque es capaz de incorporar largas cadenas de ácido polisiálico (PSA) que le confiere propiedades anti-adhesivas y, por tanto, promueve la capacidad de remodelado estructural de las neuronas que la expresan o de aquellas que están en contacto con ellas. Consecuentemente, tanto la corteza prefrontal como la amígdala o el hipocampo muestran niveles elevados de expresión de PSA-NCAM en el cerebro de roedores adultos, estas estructuras son moduladas paralelamente al remodelado estructural sufrido tras la exposición a experiencias aversivas.

Los objetivos de este estudio han sido: (i) Estudio del efecto del estrés crónico sobre sobre la plasticidad estructural y sobre la transmisión inhibitoria en la amígdala y la corteza prefrontal de roedores. (ii) Estudio de la plasticidad estructural en interneuronas en modelos experimentales de esquizofrenia. (iii) Análisis de la posible alteración de marcadores de plasticidad y de transmisión inhibitoria en muestras de cerebro postmortem de pacientes con diferentes enfermedades mentales. (iv) Estudio de la asociación caso-control entre la esquizofrenia y el gen *ST8SIAII* que codifica para una de las enzimas responsables de la polisialización de la molécula NCAM.

Metodología y Resultados

ARTÍCULO 1. ANÁLISIS DE LA EXPRESIÓN DE PSA-NCAM Y LA ARBORIZACIÓN DE INTERNEURONAS EN LA AMÍGDALA DE UN MODELO ANIMAL DE ESTRÉS CRÓNICO.

El objetivo de este trabajo es estudiar si existen diferencias en la expresión de PSA-NCAM o de diferentes moléculas relacionadas con la neurotransmisión inhibitoria en la amígdala de ratones transgénicos portadores de la proteína verde fluorescente (EGFP) en una población de interneuronas (GIN) expuestos a estrés crónico, un modelo de depresión o ansiedad. RESULTADOS: Encontramos una disminución de las proteínas PSA-NCAM, GAD67 y sinaptofisina en la amígdala de estos ratones. Además, estas disminuciones en la expresión de estas moléculas van acompañadas de un descenso en la expresión de los genes de la polisialiltransferasa *St8SiaII* y de *GAD67*. En cuanto a la arborización dendrítica de las interneuronas, encontramos que existe una menor arborización en individuos estresados respecto a la de individuos control.

Publicación: Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amígdala of adult mice. Gilabert-Juan J, Castillo-Gomez E, Pérez-Rando M, Moltó MD, Nacher J. Experimental Neurology 2011 Nov;232(1):33-44.

ARTÍCULO 2. ANÁLISIS DE LA EXPRESIÓN DE PSA-NCAM Y LA ARBORIZACIÓN DE INTERNEURONAS EN LA CORTEZA PREFRONTAL DE UN MODELO ANIMAL DE ESTRÉS CRÓNICO.

En este trabajo se ha estudiado la arborización dendrítica de interneuronas de la corteza prefrontal. Además, se ha realizado una comparación de la expresión génica de diferentes marcadores de neurotransmisión inhibitoria y plasticidad neuronal, junto con recuentos de somas neuronales que expresan GAD67 y PSA-NCAM. RESULTADOS: Encontramos una disminución del número de somas que expresan GAD67, sin detectar cambios en el número de somas PSA-NCAM inmunoreactivos. Además, estas disminuciones en el número de somas van acompañadas de un aumento en la arborización dendrítica de interneuronas. En lo que respecta a la expresión génica, vemos un aumento de expresión de *NCAM, sinaptofisina* y la subunidad *alfa* del receptor A de GABA.

Publicación: Chronic stress alters inhibitory networks in the medial prefrontal cortex of adult mice. Gilabert-Juan J, Castillo-Gomez E, Guirado R, Moltó MD, Nacher J. Brain Structure and Function. 2012 Nov 21.

ARTÍCULO 3. ANÁLISIS DE MARCADORES RELACIONADOS CON LA NEUROTRANSMISIÓN INHIBITORIA Y LA EXPRESIÓN DE PSA-NCAM EN LA AMIGDALA DE UN MODELO ANIMAL DE ESQUIZOFRENIA: AISLAMIENTO SOCIAL PROLONGADO TRAS EL DESTETE.

El este caso, el objetivo general ha sido determinar las posibles alteraciones en la expresión de diversos genes relacionados con la neurotransmisión inhibitoria y la plasticidad estructural en la amígdala de ratas aisladas socialmente después del destete. RESULTADOS: En la amígdala de ratas sometidas a aislamiento encontramos un aumento de la expresión de las proteínas NCAM, PSA-NCAM y GAD67, sin cambios en expresión de ARNm.

Publicación: Post-weaning social isolation rearing influences the expression of molecules related to inhibitory neurotransmission and structural plasticity in

the amígdala of adult rats. Gilabert-Juan J, Moltó MD, Nacher J. Brain Research. 2012 Apr 11;1448:129-136.

ARTÍCULO 4. ANÁLISIS DE CAMBIOS ESTRUCTURALES Y FUNCIONALES EN UN MODELO ANIMAL "DE DOBLE IMPACTO" DE ESQUIZOFRENIA.

Este estudio ha consistido en la valoración de diferentes cambios estructurales y bioquímicos en la corteza prefrontal medial y el hipocampo de ratas sometidas a aislamiento social perinatal y a antagonistas de receptores NMDA durante el desarrollo. RESULTADOS: En el modelo doble observamos reducción del volumen de la corteza prefrontal y el hipocampo, diferencias de peso entre los individuos, alteraciones en la expresión génica de *calbindina*, *calretinina* y *ErbB4* en la corteza prefrontal y reducción del número de células parvalbumina positivas. También se detectan disminuciones en el número de células inmaduras que expresan la proteína doblecortina en el hipocampo y activación de neuronas excitadoras en la corteza prefrontal medial.

Publicación: A "double hit" murine model for schizophrenia shows alterations in the structure and neurochemistry of the medial prefrontal cortex and the hippocampus. Gilabert-Juan J, Belles M, Saez AR, Carceller H, Moltó MD, Nacher J. Neurobiology of disease. En revisión

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El objetivo de este estudio es analizar si existen diferencias en la expresión de la molécula PSA-NCAM en la corteza prefrontal de cerebros postmortem procedentes de pacientes psiquiátricos y controles cedidos por el *Stanley Neuropathology Consortium* de la Fundación Stanley. Este análisis implicó el estudio de la expresión y distribución de PSA-NCAM y otras moléculas relacionadas con la plasticidad neuronal, tanto en somas neuronales como en el neuropilo. RESULTADOS: Encontramos una disminución de la expresión de las

proteínas sinaptofisina y GAD67 en los pacientes de esquizofrenia, que es similar a la encontrada en los pacientes con desorden bipolar. En cuanto a los individuos que cursan con depresión mayor, hay una disminución en la expresión de PSA-NCAM, sinaptofisina y GAD67, junto con un aumento en la expresión del marcador de sinapsis excitadoras VGLUT1.

Publicación: Alterations in the expression of PSA-NCAM and synaptic proteins in the dorsolateral prefrontal cortex of psychiatric disorder patients. Gilabert-Juan J, Varea E, Guirado R, Blasco-Ibáñez JM, Crespo C, Nácher J. Neuroscience Letters. 2012 Nov 14;530(1):97-102.

ARTÍCULO 6. ESTUDIO DE ASOCIACIÓN CASO-CONTROL ENTRE LA ESQUIZOFRENIA Y EL GEN *ST8SIAII*.

Este trabajo de asociación caso-control pretende replicar las asociaciones encontradas previamente en dos poblaciones asiáticas entre la esquizofrenia y el gen *ST8SIAII*. Este gen codifica para una de las enzimas responsables de la polisialización de la molécula NCAM. Asimismo, otro objetivo del presente estudio ha sido identificar nuevos alelos, genotipos y haplotipos de *ST8SIAII* que puedan constituir factores de vulnerabilidad genética en la esquizofrenia. Se ha partido de una muestra de 508 pacientes diagnosticados de esquizofrenia según criterios DSMIV (36.4% mujeres y 63.6% hombres) y 428 controles sanos sin antecedentes familiares de enfermedad mental (30.8% mujeres y 69.2% hombres). Resultados: Se encuentra diferente asociación de polimorfismos de un solo nucleótido (Single Nucleotide Polimorphisms, SNPs), situados en el promotor del gen, en hombres y mujeres con esquizofrenia. Además se identifica un haplotipo de riesgo en hombres afectados por la enfermedad. Dicho haplotipo se construye a partir de varios polimorfismos del promotor y un SNP de la región codificante del gen.

Publicación: Sex-specific Association of the *ST8SIAII* Gene with Schizophrenia in a Spanish population. Gilabert-Juan J, Nacher J, Sanjuan J, Molto MD. American Journal of Medical Genetics part B. En revisión.

Discusión

De acuerdo a los resultados obtenidos en este trabajo, la expresión de la molécula PSA-NCAM se encuentra alterada en los diferentes modelos de enfermedades psiquiátricas estudiados y en las diferentes áreas del cerebro implicadas en la etiopatología de estos desordenes. Además, como hemos comprobado, esta molécula implicada en la plasticidad estructural neuronal se haya estrechamente ligada a las alteraciones en el sistema de neurotransmisión inhibitoria. Por lo tanto podemos afirmar que existe una estrecha relación entre el correcto funcionamiento de este sistema y su plasticidad. Asimismo, vemos que los resultados obtenidos en los modelos animales estudiados en esta tesis son en cierto modo similares a los que se obtienen en los estudios de muestras post-mortem de pacientes, lo cual apoya el uso de estos modelos en el estudio de las enfermedades psiquiátricas de las que pretenden reproducir algunos aspectos. Finalmente, se ha comprobado que en la población caucásica también se encuentra asociación entre el gen *ST8SIAII* y la esquizofrenia, en los polimorfismos situados en el promotor. Este resultado indica, por un lado que variaciones en la expresión del gen *ST8SIAII* pueden ser críticas para el buen funcionamiento del sistema nervioso, y por otra parte, que dicho gen puede presentar alelos que confieran vulnerabilidad a la esquizofrenia.

Conclusiones

1. Las células que expresan la proteína verde fluorescente (EGFP) en la corteza prefrontal medial (CPM) de ratones GIN adultos corresponden principalmente a una subpoblación de interneuronas Martinotti.

2. El estrés crónico en ratones GIN adultos induce hipertrofia dendrítica en interneuronas que expresan EGFP en la CPM.

3. El estrés crónico en ratones GIN adultos disminuye el número de interneuronas que expresan GAD67 y GAD-EGFP en la CPM.

4. El estrés crónico provoca hipotrofia dendrítica en las interneuronas que expresan EGFP en el núcleo basomedial de la amígdala de ratones GIN adultos.

5. El estrés crónico induce una disminución en la expresión de distintos marcadores moleculares de plasticidad estructural neuronal y de neurotransmisión inhibitoria (NCAM, sinaptofisina y receptor $GABA_A$ alfa) en la amígdala de ratones GIN adultos.

6. El aislamiento social prolongado tras el destete en ratas Lister Hooded induce aumentos en la expresión de las proteínas GAD67, PSA-NCAM y NCAM en los núcleos amigdalinos.

7. La combinación de una inyección perinatal del antagonista del receptor de NMDA, MK-801 y el aislamiento social prolongado tras el destete en ratas Lister Hooded, es un modelo animal de esquizofrenia "de doble impacto", que reproduce un espectro más amplio de alteraciones estructurales y moleculares que cualquiera de los modelos individuales por sí mismo.

8. El modelo animal de esquizofrenia "de doble impacto" desarrollado en esta tesis presenta reducciones en el volumen de la CPM y el hipocampo.

9. El modelo "de doble impacto" muestra una reducción en el número de interneuronas que expresan parvalbúmina, altera la expresión génica de *calbindina*, *calretinina* y *ErbB4 y* reduce la expresión de PSA-NCAM y GAD67 en la CPM. La expresión de PSA-NCAM también se encuentra reducida en el hipocampo en este modelo.

10. El modelo "de doble impacto" muestra un aumento en el número de neuronas granulares inmaduras que expresan la proteína doblecortina en el hipocampo.

11. El modelo "de doble impacto" presenta un aumento del número de neuronas que coexpresan el gen de expresión temprana "c-fos" y el marcador de neuronas excitadoras "CaMKII" en la CPM.

12. La expresión de distintos marcadores sinápticos y de plasticidad estructural neuronal se encuentra alterada en la corteza prefrontal dorsolateral de pacientes con trastornos psiquiátricos. Concretamente está reducida la expresión de PSA-NCAM se encuentra reducida en pacientes esquizofrénicos, la de sinaptofisina en pacientes de depresión mayor, la de VGLUT1 en pacientes con trastorno bipolares y en pacientes con depresión, y la de GAD67 en todos los grupos de pacientes estudiados.

13. El polimorfismo rs3759916 del gen *ST8SIAII*, situado en su región promotora, se encuentra asociado a esquizofrenia en la población femenina española, y un haplotipo de este gen también se asocia a la enfermedad en la población masculina española, sugiriendo que *ST8SIAII* podría representar un factor de riesgo genético en el desarrollo de la esquizofrenia, afectando de forma diferente dependiendo del sexo del individuo.

Introduction

1. NEURAL PLASTICITY AND INHIBITORY NEUROTRANSMISSION IN PSYCHIATRIC DISORDERS

Nowadays it is well known that brain plasticity is essential for the development of the Central Nervous System (CNS) or to its adaptation to new environments, for learning and for the basic survival of individuals. Some of these plastic changes imply new synaptic connections, cell migration, axon guidance and neurogenesis, which not only occur during development but also in the adulthood (Hensch, 2004; De Magalhaes and Sandberg, 2005).

The limbic system is one of the most plastic regions in the brain, with a high presence of molecules implicated in structural as well as molecular plasticity (Bonfanti, 2006). An important number of studies have been performed on the alterations in plasticity and excitatory neurotransmission that occur in psychiatric or mood disorders such as stress, anxiety, depression or schizophrenia, in which the limbic system and the prefrontal cortex (PFC) play a crucial role. However, there is an increasing interest concerning the changes occurring in the inhibitory neurotransmission in these disorders, in which fewer studies have been conducted until now.

In order to show the objectives of this doctoral thesis, this introduction is going to highlight the most important features of the limbic system and the mPFC, its inhibitory neurotransmission and its plasticity, and the psychiatric disorders studied: the chronic stress as a model of depression and schizophrenia.

2. THE LIMBIC SYSTEM AND THE PREFRONTAL CORTEX

The limbic system is composed by different brain areas, being some of the most important regions, the hypothalamus, the hippocampus and the amygdala. The hippocampus and the prefrontal cortex are portions of the cerebral cortex, while the hypothalamus and the amygdala are part of the subcortical portions of the limbic system. These four regions are functionally and anatomically interconnected and have several functions, necessary for self-

preservation and species preservation. They regulate the autonomic and endocrine functions, responding to environmental stimuli and regulating the emotions. This regulation affects the level of arousal and motivation and reinforces behaviors involved in some type of memory.

This system involving the prefrontal cortex and the limbic system has two differentiate parts, the input and processing side, composed by the prefrontal cortex, amygdala and hippocampus, in which this thesis is focused, and the output side composed by the hypothalamus and other nuclei.

2.1. Amygdala

The amygdala is a complex structure located in the anterior temporal lobe of the brain, within the uncus. The rodent amygdala is composed by many subnuclei, each one with a differentiate structure and composition, which receives or send projections to different brain areas. This complexity makes this region one of the hardest to study in the brain. We distinguish two different regions in the amygdala, the striatal amygdala (central and medial nuclei), with a high density of interneurons, and the rest of the amygdala (cortical amygdala) with more excitatory elements (Figure 1).

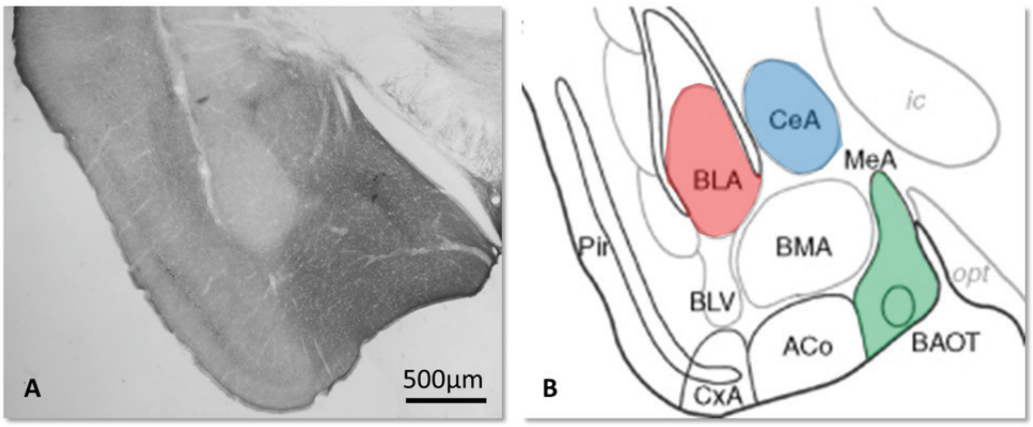

Figure 1. (A) Image of the amygdala stained using PSA-NCAM immunohistochemistry. **(B)** Schematic view of some of the subnuclei of the amygdala: marked in red the basal-lateral nucleus, in blue the central-medial nucleus, and in green the medial nucleus.

The regions, which receive connections from some nuclei of the amygdala, are the hypothalamus, thalamus, septal nuclei, frontal cortex,

cingulated gyrus, hippocampus, parahippocampal gyrus and brain stem. The amygdala also receives reciprocal connections from all these areas.

The main function of the amygdala is to coordinate behavioral, autonomic and endocrine responses to environmental stimuli, mainly fear and anxiety. Some of these stimuli are stressors, and, consequently, this region is very important for the survival of individuals and the appropriate and coordinated responses to these stressors. Lesions of the amygdala promote impaired responses to stress or anxiety with behavioral arousal and rage reactions (LaBar and LeDoux, 1996).

2.2. Hippocampus

The hippocampus is located in the medial region of the temporal lobe of the brain, forming the medial wall of the lateral ventricle in this area. The hippocampus in rodents has 3 layers: molecular, pyramidal and polymorphic; and several parts: the dentate gyrus and the Cornu Ammonis (CA), divided in four regions (CA1-CA4). The CA blends into the adjacent subiculum, which is connected to the entorhinal cortex (Figure 2).

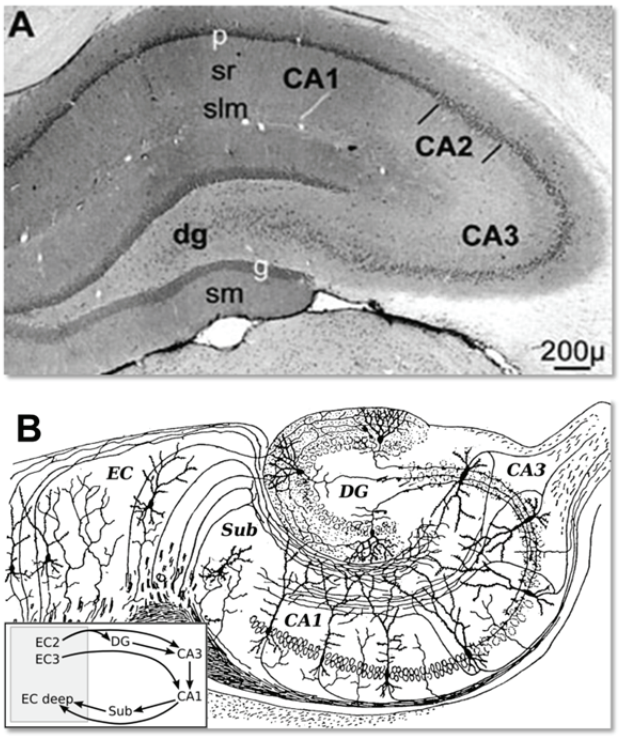

Figure 2. (A) Image of the ventral hippocampus with its different layers and most important regions, extracted from Kauselmann et al., 1999. CA1-3, hippocampal fields; dg, dentate gyrus; g, granular cell layer; p, pyramidal cell layer; slm, stratum lacunosum moleculare; sm, stratum moleculare; sr, stratum radiatum. **(B)** Schematic view of the hippocampus by Ramon y Cajal, 1911. EC, entorhinal cortex; Sub, subiculum.

The hippocampus receives inputs from the septum and the hypothalamus via the fornix and the entorhinal cortex, from the neocortex and the amygdala. All these connections are involved in memory formation, mainly involving long-term potentiation. The outputs of the hippocampus go via the fornix to the mamillary bodies, septal nuclei, preoptic nucleus of the hypothalamus, ventral striatum and to portions of the frontal lobe. Other projections go back to the entorhinal cortex.

The hippocampal functions are the control of corticosteroid production, the processing of spatial relationships within the environment and the storage of memories in the cerebral cortex (Eichenbaum, 2000).

2.3. Prefrontal Cortex

The prefrontal cortex (PFC) is located anterior to the motor cortex and posterior to the orbital frontal cortex, receiving inputs from other limbic cortex regions, the amygdala and the septal nuclei. The PFC also projects to all these areas and to the dorsomedial nucleus of the thalamus. The prefrontal cortex in rodents is divided in four areas, prelimbic, infralimbic, cingulate 1 and 2. In general, the prelimbic region is involved in attentional functions and visual working memory. The cingulate regions participate in the generation of rules associated with temporal ordering and motor sequencing of behavior. Finally, the infralimbic region is associated to autonomic control and the response and modulation of fear (Uylings and Van Eden, 1990; Paxinos and Watson, 2007; Seamans et al., 2008).

The cells and fibers in the rodent medial prefrontal cortex (mPFC) follow the architectural distribution of the neocortical regions in mammals, without the layer IV of internal granule cells (Fuster, 2008). Basically, the distribution of the rodent mPFC is: layer I, molecular layer; layer II, external granule cell layer; layer III, external pyramidal cell layer; layer V, internal pyramidal cell layer and layer VI, polymorphic layer (Figure 3).

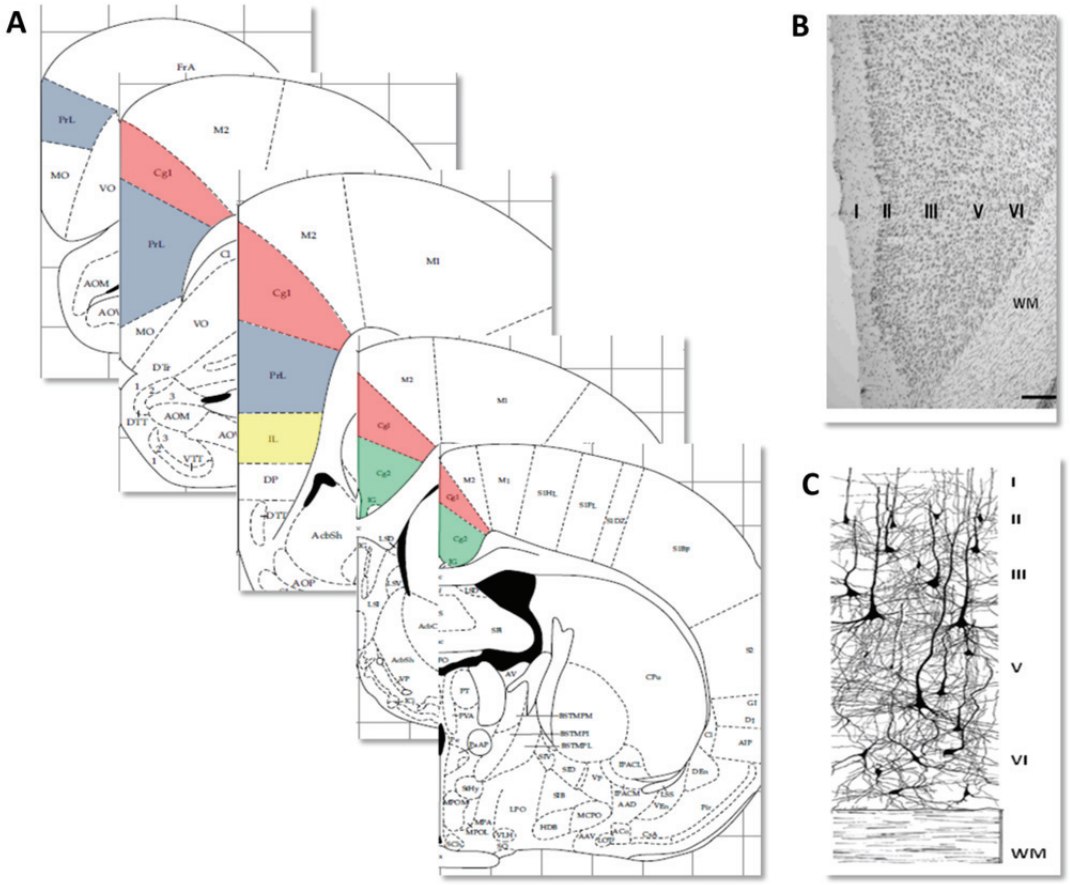

Figure 3. **(A)** An overview of the rat mPFC, with the regions (Prl, IL, Cg1 and Cg2) marked in different colors, adapted from the atlas of Paxinos and Watson, 2007. **(B)** Microscopic view of the rat mPFC layers and white matter (WM). Scale bar: 250µm. **(C)** Scheme of the neuronal types in the rat mPFC (modified from Fuster, 2008).

The functions of the prefrontal cortex are related with the judgment, insight, motivation and mood, explaining why this area is altered in mood disorders. For example, major depression is usually associated with an increased activity in portions of the frontal lobe and a decreased activity in the posterior cingulate gyrus (Fitzgerald et al., 2006). Some lesions of the mPFC affect emotional responses, such as fear, or engage sexual behavior (Blanco et al., 2009). Alcohol and drugs also modify this area and it is though that positive symptoms of psychosis (delusions and hallucinations) are also associated to this region (Gizewski et al., 2013).

3. INHIBITORY NEUROTRANSMISSION

The inhibitory neurotransmission system is based mainly on the transmission of the Gamma-aminobutyric acid (GABA). The GABAergic neurons synthesize GABA from glutamate by the action of the glutamic acid decarboxylase enzyme (GAD). This monomeric enzyme has two isoforms with different molecular weight called GAD65 (65 KDa) and GAD67 (67 KDa). The function of each isoform is not totally understood and each one plays a different role during development or in response to the environment. These enzymes are expressed in different synaptic terminals and during different periods of development (see Gonzalez-Burgos et al., 2011 for review).

The synthesis of GABA occurs in the cytosol and it is transported in vesicles to the synaptic cleft, by means of the vesicular transporter of GABA (vGAT). The GABA release is produced in a Ca^{2+} dependent manner after an action potential. Calcium binding proteins such as parvalbumin, calbindin or calretinin, present in different subpopulations of interneurons, bind Ca^{2+} after the activation of the Ca^{2+} cascade, acting as buffer molecules. After being released, the GABA molecule arrives to the postsynaptic density, where GABA receptors are located (Oláh et al., 2009).

3.1. Functions of the Inhibitory Neurotransmission

From the 1980s it is widely assumed that the basic building blocks of neuronal representations are provided by synchronously firing neurons. The GABAergic neurons, or interneurons, have been studied for a long time in different areas of the brain and it has been assumed that they provide stability to the principal neurons using feedback and feedforward inhibition by means of somato-dendritic, axo-somatic and dendro-dendritic connections with other interneurons and with pyramidal cells. Some of these neurons receive dopaminergic or serotoninergic inputs (Mrzljak et al., 1996; Jakab and Goldman-Rakic, 1998), indicating a tight relationship with the monoaminergic neurotransmission and its regulatory control.

The firing control regulated by interneurons is maintained by a network of cells interrelated with the $GABA_A$ receptors, by means of the GABA neurotransmitter (Buzsáki and Chrobak, 1995). This control is exerted by different integrated firing systems of oscillations in the theta (6-12 Hz), gamma (40-100 Hz) and ultrafast (200 Hz) frequency ranges (Bredkjaer, 1998). It has been demonstrated that the gamma frequency activity is implicated in the perception of auditory stimuli and the conscious somatic perception. The wide distribution of gamma frequency oscillations in all the cortical areas indicates a highly conserved GABAergic connections and the importance of the spatio-temporal synchrony of the pyramidal cells for their correct functioning (von der Malsburg, 1995). Consequently, if this tigh regulation is altered, it may provoke disintegration of reality and susceptibility to psychiatric and mood disorders (for review see Keverne, 1999).

3.2. Cell Subpopulations, Cytoarchitecture and Receptors

Interneurons were firstly described by Ramon y Cajal, who used the Golgi-impregnation technique, showing different morphologies in the cerebral cortex and hippocampus (Ramon y Cajal, 1893; 1911). The GABAergic neurons represent approximately 15-30% of the cortical neurons (Somogyi et al., 1998). These interneurons project internally to regions of the cortex, but in the amygdala, the connections of these neurons may project to other structures of the brain. The interneurons are mainly classified attending to their morphology, and the calcium binding proteins and the neuropeptides expressed (Figure 4) (see Benes and Berretta 2001 for review).

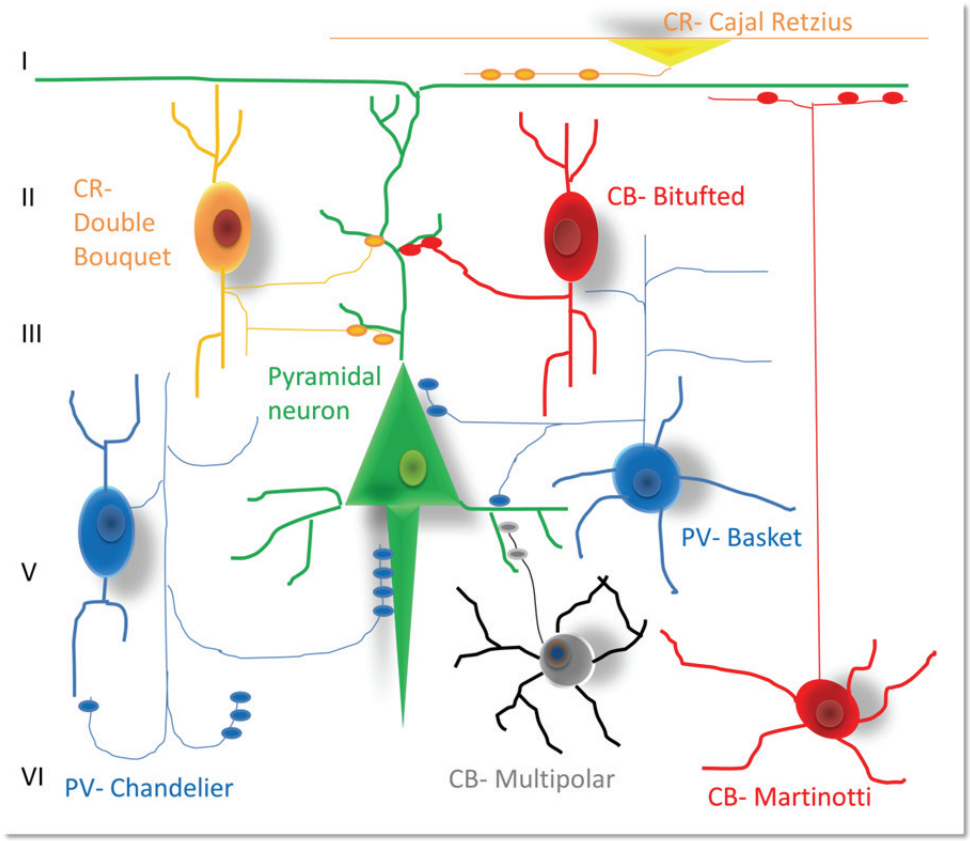

Figure 4. Schematic representation of the interneuronal subtypes around a pyramidal neuron of the mPFC attending to their morphology and calcium binding protein expression. Abbreviations: CB, calbindin; CR, calretinin; PV, parvalbumin. Adapted from Lewis and Gonzalez-Burgos, 2008.

Regarding their morphology, interneurons are classified as:

Basket cells. These cells are the most common interneurons. They are multipolar, with big somata and are involved in innervations that form a basket-like arrangement around a large proportion of the surface of pyramidal cell bodies (Hendry and Jones, 1983). These cells are situated in the cerebral cortex layers III to V.

Chandelier cells. These cells receive their name from their candle-like morphology, which shows the axonal branches extending at right angles from the somata. These cells form axo-axonic synapses with the initial segment of pyramidal cell axons (Somogyi, 1979). In the cortex, chandelier cells are predominantly situated in layers II and III.

Double bouquet. Double bouquet cells have axonal arborizations that distribute themselves within narrow, radially oriented columns of the cortical

mantle (de Felipe and Fairen, 1982). These cells contact mainly dendritic shafts and spines on the side branches of apical dendrites or basal dendrites of pyramidal neurons (Somogyi and Cowey, 1981) and are situated in the cerebral cortex layer III.

Other Interneurons. There are other minor represented cell subpopulations, such as the Cajal-Retzius cells, situated in the layer I of the cortex. These cells contact with the distal dendrites of the pyramidal neurons or other interneurons. Finally, there are the neurogliaform cells and Martinotti cells situated in deep layers of the cortex, which innervate proximal and distal dendrites respectively of pyramidal cells.

Attending to their neurochemical markers, interneurons are also classified as follows:

Parvalbumin (PV) expressing interneurons. PV is expressed mainly in chandelier and basket cells (Gabbott and Bacon, 1996). These neurons are characterized as "fast-spiking" neurons, connected to other neurons through chemical as well as electrical synapses. It has been proposed that PV expressing cells form networks regulating synchronization (Gibson et al., 1999). PV neurons receive synaptic contacts from calretinin neurons, which inhibit them. Some of the PV expressing interneurons also express calbindin, but no neuropeptides (Kubota et al., 1994).

Calbindin (CB) expressing interneurons. CB is mainly expressed by double bouquet cells (Gabbott and Bacon, 1996) and by some Cajal-Retzius cells. Cells expressing CB are classified as "low threshold spike" cells, connected to other cells by electrical synapses and are proposed to play a role in generating synchronous inhibitory activity in the cortex (Gibson et al., 1999). These neurons receive calretinin contacts and projections from the Broca band.

Calretinin (CR) expressing interneurons. CR is expressed in double bouquet and bipolar cells, as well as in Cajal-Retzius neurons (Gabbott and Bacon, 1996). The cells expressing CR are classified as "regular spiking" cells and they contact PV and CB neurons, suggesting a role for these neurons in the control of disinhibition .

Somatostatin (SOM) expressing interneurons. Cells expressing SOM co-express CB, neuropeptide Y as well as nitric oxide synthase (NOS) (Kubota et al., 1994; Smiley et al., 2000). Morphologically, they are multipolar or bitufted cells, some of which have been identified as Martinotti cells (Kawaguchi and Kubota, 1996). SOM cells are "regular spiking" cells found in layers II and III and sometimes in layer V. Some of them have also been classified as "burst spiking" cells and are located exclusively in layer V. Some of these SOM expressing cells also express calretinin and vasoactive intestinal polypeptide (VIP).

Vasoactive Intestinal Polypeptide (VIP) expressing interneurons. Cells expressing VIP co-express SOM and PV and are bipolar, double bouquet and basket cells. They are considered "regular spiking" or "burst spiking" cells.

Regarding the GABA receptors, we are going to focus our attention on the $GABA_A$ receptors, because of their main implication in the inhibitory neurotransmission in the cerebral cortex. The $GABA_A$ receptors are heteropentamers composed of subunits from 7 different families (α1 to α 6, β1 to β3, γ1 to γ3, δ, ε, θ, ρ1 to ρ3). The most common combination is 2α:2β:γ forming a GABA-activated chloride channel (Farrant and Kaila, 2007; Olsen and Sieghart, 2009). The composition of these receptors influences their activity and their functional properties: For example, receptors containing α1 subunit have much faster decay kinetics than those containing other α subunits (Farrant and Kaila, 2007). The effect of some drugs, such as benzodiazepines, also depends on the subunit composition of the $GABA_A$ receptors (for review see Mohler, 2011).

4. NEURONAL PLASTICITY

There are different levels of neuronal plasticity, ranging from molecular to structural changes. The molecular plasticity is based in complex mechanisms and pathways, being among the most important those involved in protein dynamics in synapses, such as the neurotransmitter receptors and their associated proteins (Renner et al., 2008). Changes in these proteins affect

directly the remodeling of neurites or synapses, mostly by means of changes in the expression or the organization of cytoskeletal proteins and/or adhesion molecules. One of the most studied cell adhesion molecules (CAMs) is the neural cell adhesion molecule (NCAM). It has been demonstrated that NCAM is necessary for some plastic processes, such as synaptogenesis, growth cone development, neurite outgrowth (Maness et al., 1996) and dendritic remodeling (Stewart et al., 2010).

Regarding the structural plasticity, there are several brain processes that usually fall under this definition, all of them referring to changes in the shape and structure of the adult CNS. One of these processes is neurogenesis, which occurs mainly in two regions of the adult brain, the subventricular zone of the lateral ventricle (SVZ) (Alvarez-Buylla et al., 2000), and the subgranular zone of the dentate gyrus (SGZ) (Gage et al., 1998). Other structural changes occurring in the adult CNS are the remodeling of neurites and the turnover of synapses. The first one involves the modification of the structure of dendrites, axons and spines in neurons. Synapse turnover can be described as a change in the efficacy of synaptic transmission due to the structural reorganization of synapses and it involves synaptic facilitation, synaptic depression and potentiation (Zucker and Regehr, 2002).

Traditionally neuronal plasticity and specifically structural plasticity have been studied in excitatory neurons. However, nowadays it is becoming essential to improve our knowledge about interneuronal plasticity, specially because of the evidence of the implication of interneurons in the regulation of the excitatory/inhibitory balance, which is proposed to be involved in the etiology of different psychiatric disorders (Sun and Zhan, 2011). Some studies have shown that interneuron remodeling also occurs in the adult brain, both in control conditions and after experimental perturbations, including changes in dendritic arborization, or the dynamics of dendritic branch tips (Lee et al., 2008; Chen et al., 2011). A recent study has described also a dynamic behavior of the spine turnover rate in interneurons of the visual cortex co-expressing VIP (Keck et al., 2011).

In the same line, studies on an animal model of epilepsy have demonstrated that interneurons show shrinkage of dendrites, decreased axon

length, and axonal buttons and inhibitory contacts with excitatory neurons (Prince et al., 2009). Reductions in soma size and dendritic arbour in interneurons, or in the total number of these inhibitory neurons have been observed in the brains of schizophrenic patients (Kalus et al., 2002; Benes and Berretta, 2001). All these structural changes may involve the participation of cell adhesion molecules. Among them, one of the most interesting regarding the plasticity of interneurons is NCAM and its polysialylated form (PSA-NCAM).

5. THE NEURAL CELL ADHESSION MOLECULE AND ITS POLYSIALYLATED FORM

NCAM protein is a surface glycoprotein with three isoforms, differing in their molecular weight (120, 140, 180 KDa), generated by alternative splicing in the *NCAM* gene (Gascon et al., 2007). The extracellular domain shared by the three isoforms is a globular structure, immunoglobulin-like, composed by around 70-110 amino acids. NCAM180 has a long cytoplasmic domain and it is predominantly expressed in mature neurons in cell contacts and postsynaptic densities (Persohn et al., 1989). NCAM140 has a shorter cytoplasmic domain and is mainly expressed in developing neurons. Finally, NCAM120 is expressed mainly in glial cells (Bhat and Silberberg, 1986; Walmod et al., 2004). NCAM has homophilic and heterophilic interactions with other adhesion molecules or with diverse signal transduction proteins (Walmod et al., 2004).

One of the main modifications suffered by the NCAM proteins is the addition of the polysialic acid (PSA) by the action of the polysialyltransferase enzymes, St8SiaII and St8SiaIV. The PSA is a linear homopolymer of α-2,8-linked N-acetylneuraminic acid, which can form chains ranging from 50 to 150 units. It is attached to NCAM at the fifth immunoglobulin-like domain (Kiss and Rougon, 1997; Acheson et al., 1991). This unique post-translational modification of NCAM is absolutely necessary for the correct development and function of the brain, including processes such as learning and memory (Figure 5) (Bork et al., 2007; Hildebrandt et al., 2007).

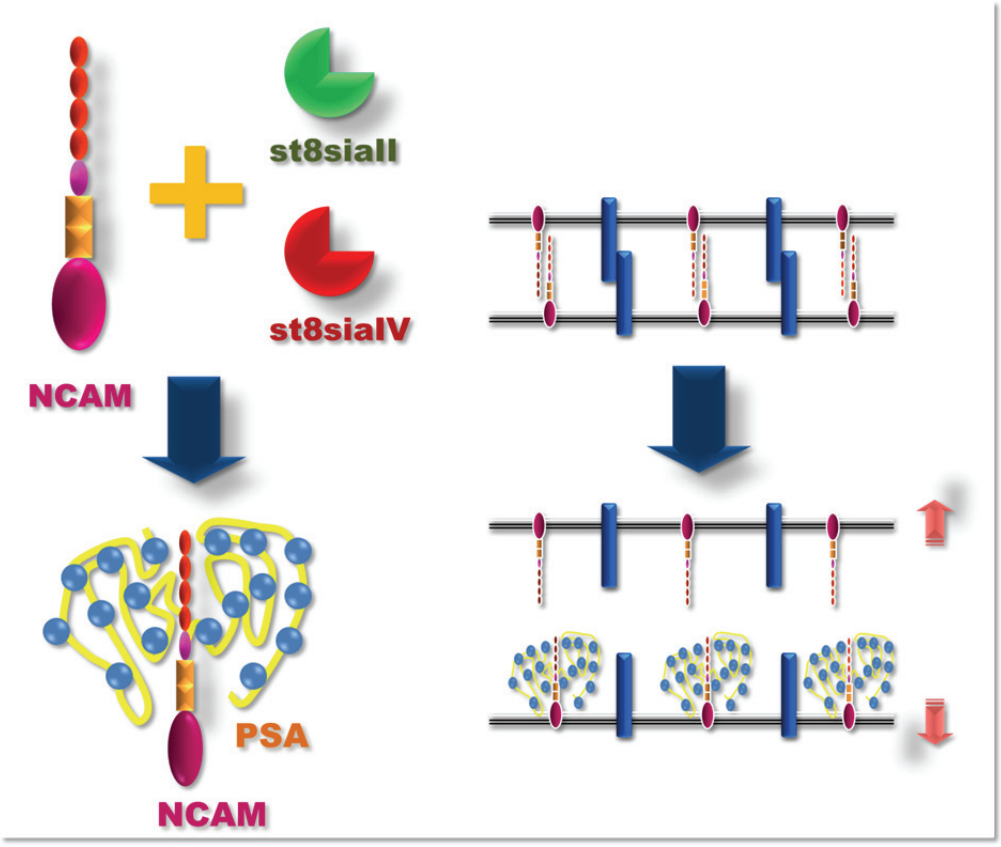

Figure 5. Representation of the the NCAM molecule and the two polysialyltransferases adding the PSA sugar to the molecule and conferring antiadhesive properties.

In the adult brain PSA-NCAM is expressed intensely in immature neurons of the hippocampus, the olfactory bulb/rostral migratory stream/subventricular zone axis or the paleocortex layer II (See Bonfanti and Nacher 2012 for review). Apart from these immature neurons, PSA-NCAM is also found in a population of larger cells that show multipolar morphology and are heterogeneously distributed in the brain. In the mPFC, there is a higher density of PSA-NCAM expressing cells in deep layers than in superficial layers (Varea et al., 2005). In the hippocampus, the highest density of these cells is located in ventral region (Nacher et al., 2002a) being more abundant in *stratum lucidum* or *lacunosum moleculare*. In the amygdala, there are more cells expressing PSA-NCAM in its medial division than in its basal region (Nacher et al., 2002b). In the hypothalamus, PSA-NCAM expression is detected in neurons and glial cells (Theodosis et al., 1991). There is also expression of PSA-NCAM in other regions of the adult nervous system such as the spinal cord, the optic nerve, different retinal layers and others.

PSA carries carboxyl groups conferring negative charge and attracting water and ionic molecules, which hydrate the molecule. This impedes cell-cell and cell-extracellular matrix adhesion, preventing both the homotypic and the heterotypic binding of NCAM (Yang et al., 1992). Such anti-adhesive properties are critical in cell migration, axophilic migration, axon guidance, synaptic modulation or in other kinds of plasticity (Rutishauser, 1996; 2008). In contrast, the removal of PSA from NCAM induces neuronal differentiation (Seidenfaden et al., 2003; Petridis et al., 2004). With these evidences, apart from the facilitation of plastic processes, it was formulated the "shielding" hypothesis of PSA: The addition of PSA to NCAM may difficult the accessibility to NCAM of some membrane receptors. The function of brain-derived neurotrophic factor (BDNF) and other neurotrophins are also affected, and, as a result, increase dendritic spine density (Tyler and Pozzo-Miller, 2003) in a process that is likely regulated by PSA-NCAM. Moreover, PSA-NCAM interacts with other receptors participating in synaptic plasticity processes, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors), *N*-methyl-D-aspartate (NMDA receptors) and others, that play important roles in mechanisms of memory and learning (Vaithianathan et al., 2004; Hoffman et al., 1998; Muller, 2000). Another evidence of the importance of PSA-NCAM in the correct brain function is the genetic association found between *NCAM* and schizophrenia (Vawter, 2000; Sullivan et al., 2007) and between the polysialyltransferase II (*ST8SIAII*) and different psychiatric disorders such as schizophrenia (Arai et al., 2006; Tao et al., 2007; Isomura et al., 2011), autism (Anney et al., 2010) and bipolar disorder (Lee et al., 2011). A reduction in the amount of PSA-NCAM in the hippocampus and the amygdala of schizophrenic patients has been demonstrated (Barbeau et al., 1995; Varea et al., 2012), as well as increases of the level of expression of this protein in the amygdala of depressed patients (Varea et al., 2012).

Previous studies in our laboratory indicate that the mature neurons expressing PSA-NCAM in the cerebral cortex of rodents are mainly interneurons (Gomez-Climent et al., 2011). Moreover, there are several subpopulation of interneurons expressing PSA-NCAM in the hippocampus, the amygdala and the mPFC of humans (Nacher et al., 2002a; Varea et al., 2007c; 2012).

6. STRESS AND DEPRESSION

Stress is an essential complex process necessary for survival in humans and other animals. There are several components defining the stress response, but is hard to measure the importance of the impact of each one (see McEwen, 2008b for review). In this way, we have focused our attention in one of these factors affecting the stress response, the time of the stressor exposition. It is well known the need of the stress hormone cortisol to survive, consequently, the acute stress response during a discrete time, mediated by this hormone, is essential and adaptative. Nevertheless, if the exposure to the stressor is maintained for a prolonged time, it turns out in chronic stress and may become deleterious for the organism, having effects on the structure and function of the brain.

Psychosocial research in the late 1970s emphasized the importance of the stressful events in the development of major depression (Brown and Harris, 1978). In addition, Carroll pointed out the presence of alterations in the hypothalamic-pituitary-adrenal axis (HPA), finding increased cortisol concentrations in depressed patients (Carroll, 1982). These two findings constituted the basis to relate stress with the appearance of a depression-like phenotype. Follow-up studies in depressed subjects with a history of childhood abuse have shown an enhanced HPA axis response to psychosocial stress; however, this does not occur in non-depressed individuals with a history of childhood abuse (Heim et al., 2000; Newport et al., 2004). These and other studies suggest a high relationship between stressful events during life and the development of depressive disorders.

6.1. Hypothesis on Etiology and Risk Factors

Depression is a multifaceted condition promoted by genetic and environmental factors, such as stress. The depressive disorder has been estimated by the World Health Organization as one of the 10 leading medical causes of disability in the world and the major cause of morbidity worldwide (World Health Organization, 2001). The risk of suffering depression for a human along the life span is about 20% (Kessler et al., 2005), and the patients

suffering from depressive disorder usually develop other health problems (Evans et al., 2005). The prevalence of the disease is more common in women (7%) than in men (3%).

Attending to the four edition of the Diagnostic and Statistical Manual of Mental Disorders or DSM-IV (American Psychiatric Association, 2002), the Major Depression symptoms are characterized by a depressive status during most of the day and a reduced interest for pleasure (anhedonia). It may affect appetite, weight and sleep. Other symptoms described are getting tired, slower, and feeling a loss of energy. Regarding the mental aspects of the disease, the depressive patient has blame thinking, sometimes delusions, less ability to think and concentrate, and suicide attempts in some severe cases.

Depression is usually a chronic disease, with a significant morbidity and mortality. The majority of the major-depression patients suffer more than one episode of depression and the risk of recurrence is higher if the patient is young or has a family history of depression, or with each new episode, or with the intensity of each episode (Hollon et al., 2006; Kendler et al., 2000). Long term depression (more than 2 years), has more clinical symptoms and worst prognosis with high comorbidity (respiratory and cardiac symptoms), being stress or anxiety high risk factors for chronicity in depression (Merikangas et al., 2003).

Similar to other complex psychiatric diseases, there is not a single hypothesis on the basis of depression, but in all of them it is assumed that individuals suffering from this disorder have emotional problems altering the stress-alarm signaling system, the executive functions/cognitive appraisal system and the mood regulation system (for review see Sjöberg and Blomstedt, 2011). From these facts, two main hypotheses on the neurobiological basis of depression have been postulated:

Monoamine hypothesis: This hypothesis is based on the mode of action of antidepressant drugs, which act over the monoaminergic pathways. Such drugs increase the monoamine transmission acutely, either by means of the inhibition of the reuptake or the degradation of monoamines. This hypothesis was postulated when the first antidepressant medication used was shown to act

inhibiting the monoamine oxidase enzyme (MAO)(Dick, 1959). Therefore, this hypothesis postulates that depression is due mainly to a decrease in catecholamine availability (Schildkraut, 1965).

Neuroplastic hypothesis: The neuroplastic hypothesis of depression is currently one of the most accepted hypotheses, because structural plasticity plays an important role in the response to stress or fear as indicated above. This hypothesis, formulated by Duman in 2002 and amplified by Castren in 2004 and 2005, suggests that depression is promoted by alterations in neuron structure, mainly by a reduction in neuronal connectivity (Figure 6). In agreement with this hypothesis, antidepressant may act increasing neuronal branching and connectivity (Hayley et al., 2005). This hypothesis is also supported by the fact that chronic stress induces dendritic atrophy and retraction in excitatory neurons in the hippocampus (McEwen, 1999) and in the medial prefrontal cortex (Radley et al., 2004; Cook and Wellman, 2004). However, it has been described that chronic stress promotes dendritic growth in some regions of the amygdala (Vyas et al., 2002).

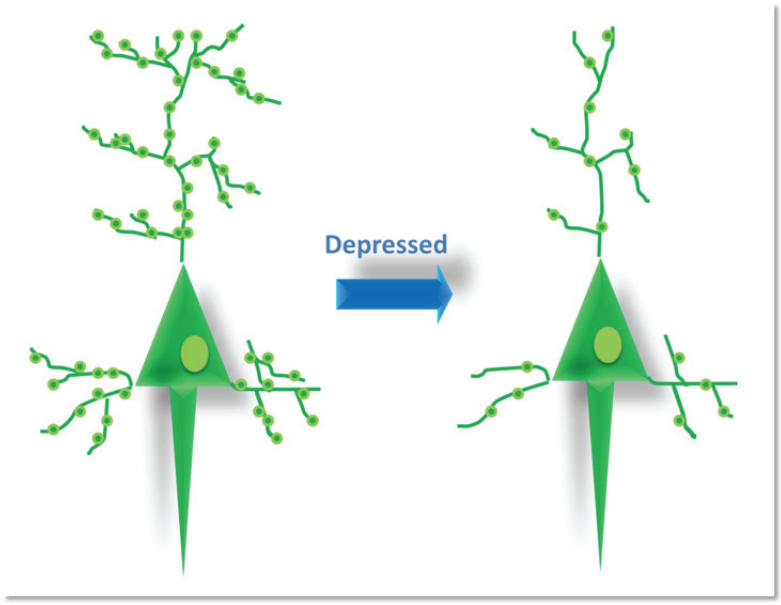

Figure 6. "Neuroplastic" hypothesis of depression, showing a retraction of the arborization in pyramidal neurons and a decreased number of synaptic buttons. Modified from Castren, 2005.

6.2. Physiological and Anatomical Alterations

As mentioned above, the limbic system and the mPFC, which are crucial in the experience of emotions and in memory storage, are among the most affected regions in depressive disorders. By means of the use of neuroimaging techniques in depressed patients, some changes in the structure and function of these regions have been described.

First, it has been observed that the volume of the PFC of patients is reduced, mainly the anterior cingulate and orbitofrontal cortex and other regions such as the hippocampus, putamen and caudate nuclei (Koolschijn et al., 2009). Other studies have shown reductions in the blood flow and glucose metabolism in some of these regions (see Drevets, 1992 for review). The white matter volume is also increased in the frontal lobe, impairing some of the functions dependent on this area (Tullberg et al., 2004).

The diverse symptomatology of depression and affective disorders has been recognized for decades. Nevertheless, the precise neurobiological basis of these disorders is not yet known. However, some of the post-mortem studies performed in patients with major depression have thrown some light on the systems that are altered in this disease.

Some of the changes found in post-mortem brains in major depression are reduced number of glial cells in the cortex and the hippocampus, reduced neuronal density, predominantly in layer II of orbitofrontal cortex or increased neuronal and glial density in the hippocampus with a decreased soma size and apoptosis. Furthermore, it has been shown a significant reduction in the number of pyramidal neurons and reduced pyramidal neuronal size in the orbitofrontal cortex, specially in layers III and V. The amygdala of depressed patients also presented a reduction in glial density (see Sacher et al., 2012 for review).

6.3. Animal Models

Due to the complex etiology of the disease, several animal models of depression have been generated (see Krishnan and Nestler, 2011 for review). Here we describe some of the most important animal models:

Models of Secondary Depression. Because overactivation or hypofunction of the HPA axis is a common feature of depressed patients, deregulation of the HPA axis is a strategy to develop models of depression. Several efforts have been done in this direction, being the most important achievement the knockout mouse of the glucocorticoid receptor (GR), which shows altered glucocorticoid function. These mice develop a number of both physiological and behavioral abnormalities that mimic major depressive symptoms in humans, including hyperactivity of the HPA axis, impaired negative feedback regulation of this axis and increased depression-like behavior. Alternatively, chemical treatments, such as the use of isotretinoin or proinflamatory cytokines, can generate similar models of depression.

Models of Acute Stress. These models are based on the effect produced by some stressors and are the most used models in the tests of antidepressant drugs. Two of the most widely used acute stressors are the forced swim test and the tail suspension test. In both cases, mice are submitted to adverse conditions during a short time period. Other model of acute stress is the learned helplessness model, in which the animal is exposed to an uncontrollable and inescapable stress such as different electric shocks. When the animal is re-exposed to the same stressor but has the possibility to escape, it will either display increased escape latency or completely fail to escape.

Models of Chronic Stress. These models are similar to the ones described above, but in this case the animals are exposed to the stressors for a longer time, promoting an anthropomorphized reaction of despair. The chronic mild stress is applied by means of different stressors or the same stressor intermittently during a period between 1 and 7 weeks. The stressors can be physical of psychosocial agents. The last ones are performed by the interaction of the rodent with natural wild predators or by promoting agonistic encounters between individuals of the same species. In the physical stressor paradigms,

rodents are exposed to damaging environments or extreme situations, such as strobe lights, swim, abrupt circadian disruptions and restraint or immobilization.

The chronic immobilization model of depression is a model of chronic stress based on the application of immobilization to the rodent during 1 to 6 hours a day in a period of 1 to 7 weeks. There are different ways to immobilize the animals, depending if the species is rat or mouse and the strategy also varies in the time of stress duration.

Alternatively, immobilization can be combined with other physical stressors. This model is termed Chronic Unpredictable Stress (CUS), and has been widely used but with poor reproducibility (Willner, 2005).

Table 1 shows the different phenotypes observed in the chronic immobilization model, parallel to human traits of depression.

Table 1. Phenotypes of the chronic immobilization rodent model.

Phenotypes Observed	Reference
Decreased sucrose preference (anhedonia)	Strekalova and Steinbusch, 2010
Reduced expression of pro-proliferative genes	Bergstrom et al.,2007
Reduction of the volume of hippocampus, amygdala and prefrontal cortex	Isgor et al., 2004; McEwen et al.,2008
Dendritic retraction in pyramidal neurons of CA3 and CA1 regions	McEwen et al., 1999; Sousa et al., 2000
Dendritic retraction and spine reduction in excitatory neurons of the mPFC	Radley et al., 2004; Perez-Cruz et al., 2009
Dendritic growth and spinogenesis with activation of excitatory neurons in the amygdala and altered expression of PSA-NCAM	Vyas et al., 2002; Mitra et al., 2005; Roozendaal et al., 2009; Cordero et al., 2005; Sandi, 2004 for review

7. SCHIZOPHRENIA

Schizophrenia is a complex psychiatric disease affecting 0.72% of the total world population (Saha et al., 2005), although this percentage varies in different populations. Men are more frequently affected than women in a 1,4:1 proportion (McGrath et al., 2008). The schizophrenic patients have a

misregulation of several mental processes, giving place to behavioral alterations. Usually the disease starts at adolescence or young adulthood and persists during the lifetime of the individual, with a chronic and deteriorating course. Due to the common onset in the early life stages, patients have in their infancy a prodromal stage with mild physical disturbances, altered social relationships and cognitive impairments (Figure 7) (Lewis and Lieberman, 2000).

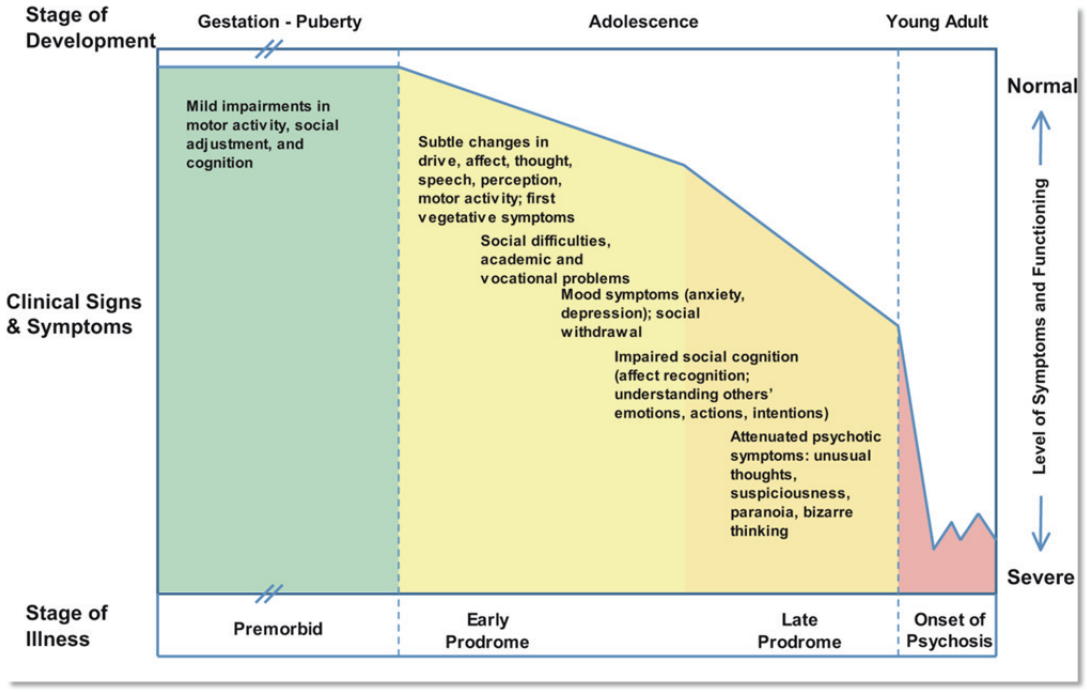

Figure 7. Clinical course of psychosis prodrome. Adapted from Lewis & Lieberman (2000) and extracted from Addington and Heinsser, 2012.

Schizophrenia was defined firstly by E. Kraepelin as *Dementia Praecox* (Kraepelin, 1971 for a translated version) to differentiate it from Manic-Depresive insanity (currently Bipolar Disorder). E. Bleuler in 1908 proposed the term *Schizophrenia* to describe a group of disorders altering cognition and having emotional and behavioral symptoms (Bleuler, 1911). From that moment, the knowledge and redefinition of the disease has been increasing to fit the best diagnosis of the disease.

The schizophrenic phenotype is very heterogeneous, with a high variety of symptoms and signs, which may vary in the same individual along his/her

life. For this reason, it may occur that two schizophrenic patients had few or none symptoms in common, complicating the study of this disease.

In general, the symptoms and signs of the disease are classified in 3 different groups included in the DSM-IV (American Psychiatric Association, 2002):

Positive Symptoms. Positive symptoms are the common denomination of the psychotic symptoms. They are an exacerbation of normal psychic elements not altered in healthy people, usually termed psychosis. Psychosis is an altered perception of the surrounding reality with hallucinations, delusions and thought disorder. *Hallucinations* consist of a false perception in a conscious state in the absence of external stimuli, which are felt as real by the person who suffers from them. *Delusions* of false beliefs are generally paranoid (persecution, grandiosity, personality substitution, thinking transmission…). *Thought disorder* consists in disorganization of thinking and behavior, affecting the development of ideas.

These are the most common positive symptoms and they appear also in other related diseases such as dementia or bipolar disorders, or in individuals with drug abuse or alcoholism (Carpenter, 2011). One of the most studied positive symptoms are the auditory hallucinations, which have been associated to several genetic risk factors and brain alterations (Sanjuan et al., 2004; 2006; Escartí et al., 2010)

Negative Symptoms: These symptoms are related to personality traits present in healthy people, which are altered in patients suffering from the disease. Some of the negative symptoms are altered social interaction, motivation, expression of affect or empathy, ability to experience pleasure and spontaneous speech, among many others. These symptoms promote deep social isolation of the individual, making very difficult the integration of the schizophrenic patients in society.

Cognitive Symptoms: They affect mainly the attention, information processing, executive function (mental processes involved in the environmental adaptation and response to some situations), working memory (short term

memory necessary to develop discrete tasks) and other processes such as associative impairment, inability to develop new ideas, learning difficulties, bad concentration… These are the most characteristic symptoms of schizophrenic patients, because they are present in almost all patients in all the disease course, appearing usually surrounding the adolescence or in the late infancy 6-14 years (Carpenter, 2011).

Other important symptoms or signs of the disease are grossly, disorganized and catatonic behavior, disorganized thoughts, limited speaking flow, unhopefulness or suicide attempts.

7.1. Hypothesis on Etiology and Risk Factors

The cause of schizophrenia is unknown yet. Current hypothesis postulate the existence of a genetic basis conferring susceptibility to the disease, together with environmental events that may act as precipitating factors of the psychotic processes.

The epidemiological studies, including studies of familiar aggregation, studies in monozygotic and dizygotic twins and studies of adoptions, have allowed the evaluation of the genetic and the environmental components of schizophrenia, suggesting an important participation of a genetic component in the development of the disease, with an heritability around 80% and indicating a modulating role, enhancing or silencing, of the environmental factors (Cardno et al., 1999; Sullivan et al., 2003).

As a complex psychiatric disease, the environmental risk is very important in the onset. Some studies have described diverse susceptibility environmental factors with different weight. For example, the prevalence is higher in males, developed countries, urban areas, immigrant population, drug abuse people and singles, perinatal complications and maternal depression, winter birth and elevated father age (see Vilain et al., 2012 for review). But none of these environmental factors has more influence in the development of the disease than having a schizophrenic first degree relative (Riley and Kendler, 2006), pointing to the genetic factors as the main risk factors in schizophrenia.

Once the involvement of a genetic component has been established in schizophrenia, it has been suggested that the disease model is in agreement with the complex multifactorial diseases. The common disease-common variant (often abbreviated CD-CV) hypothesis predicts that common disease-causing alleles are widespread in human populations and have small effect on the phenotype. The common disease-rare variant (CD-RV) hypothesis, on the contrary, argues that multiple rare DNA sequence variations, each with relatively high penetrance, are the major contributors to genetic susceptibility to common diseases (Schork, 2009). Both hypotheses are combined with some genetic risk factors conferring susceptibility to the disease and with vital events that unleash it. This fact difficults the study of schizophrenia, because not all patients have the same genetic risk factors and it one of the altered genes is not sufficient to set up the disease but it increases the likelihood. In addition, it is necessary to take into account the interaction between genes (G X G) and between genes and environment (G X E) to detect the risk combinations. These are the reasons why each risk factor in this disease has a limited power for diagnosis or prognosis (Kraft and Hunter, 2009).

Several genes have been associated with schizophrenia, first with the linkage studies in the 1980s and after that with the association studies of genetic variants, with the most common study case-control association test, associating insertions-deletions (Indels), variable number of tandem repeats (VNTRs) and more commonly, single nucleotide polymorphisms (SNPs). One of the most studied genes has been *DISC1* (*disrupted in schizophrenia 1*), a gene that is involved in neuronal generation and was described by Blackwood et al. in 2001 in a Scottish family with psychiatric disorders. Other genes participating in some neuronal developmental processes have been studied such as *DARPP32* (Reiner et al., 1998), *Neuregulin 1* (Stefansson et al., 2003), *Reelin* (Rice and Curran, 2001).

Several hypotheses on the origin of schizophrenia have been proposed and they can be organized in two groups: genetic hypotheses or neurodevelopmental hypotheses.

Genetic Hypotheses: This group of hypothesis includes those involved in neurotransmission, which implicate alterations in genes participating in the

pathway of the neurotransmitter. Here we found the *Dopamine hypothesis,* the *Serotonin hypothesis* and the *Glutamate hypothesis*. This group of hypotheses is based on the convincement that the alterations in some genes are sufficient (combined with the environmental effects) to promote the onset of the disease. Some of the studied genes have been traditionally genes involved in neurotransmission, or genes involved in brain connectivity. First, based on evidences on the mode of action of antipsychotics, the dopamine receptors were studied in relation to the disease (Seeman et al., 1975), focusing the attention on D_1 and D_2 receptors (Pani, 2002). Later, it was found that the antipsychotics of second generation, showed a high affinity for serotonin receptors, indicating that this neurotransmitter was a candidate for the susceptibility to the disease (Geyer and Vollenweider, 2008). Finally, the glutamate hypothesis was developed after studies analyzing the effect on the glutamatergic system of some propsychotic drugs (Coyle, 2006).

These hypotheses are interrelated, because there is a high regulation between all the neurotransmitter systems, indicating a functional association (Yao et al., 2008; Frigourg et al., 2011).

Neurodevelopmental Hypotheses: One of the most accepted theories for schizophrenia nowadays is the one involving genetic risk factors combined with damage during early development and the influence of an adverse environment (Murray and Lewis, 1987; Weinberger et al., 1986). It is based on the prodromical symptoms associated to the later onset of the disease (cognitive and negative symptoms…) and it is linked to the apparent lack of big neuro-pathological changes in the schizophrenic individuals (Weinberger and McClure, 2002). Consequently, it has been postulated that schizophrenia may be the result of early alterations in brain development, which appear to develop symptoms only late in the adolescence or in adulthood, resulting in alterations in the "social brain" and cognitive deficits (Owen et al., 2011). The reason why the disease onset occurs mainly during adolescence or early adulthood is unknown, but several studies suggest a deficit in the normal brain maturation, causing a deregulation in certain neuronal systems (Inta et al., 2010).

This hypothesis does not exclude those described above, because some of the genes participating in basic neural processes and neurotransmission are also implicated in the neurodevelopment.

7.2. Physiological and Anatomical Alterations

As mentioned above, there are few clear neurophysiological alterations in the patients suffering from schizophrenia. The complex etiology of the disease difficults the identification of such alterations, because schizophrenia shows a high diversity of symptoms and patients may not show the same clinical and pathological alterations. Some of the most robust alterations found in patients are described below.

Since the 1920s there is evidence of a reduction in the brain volume in schizophrenic patients. Subsequent studies, using magnetic resonance imaging (MRI), showed specifically reductions in the hippocampus, amygdala, superior temporal gyri (STG), prefrontal cortex, thalamus, anterior cingulate cortex, white matter structures such as the corpus callosum and an increase in ventricular volumes (for review see Jaaro-Peled et al., 2010).

Some other brain imaging studies have found lack of activation of the dorsolateral prefrontal cortex (DLPFC) in response to cognitive tasks mediated by this region, which is called hypofrontality (Meyer-Lindenberg et al., 2002; Davidson and Heinrichs, 2003). However, other studies have shown the opposite results (Manoach et al., 1999). This conflictive data on the prefrontal response to cognitive tests suggests an increased background noise leading to inefficient information processing (Winterer et al., 2004). Furthermore, there are signs of altered GABA concentrations in some brain areas of the patients (Yoon et al., 2010).

Regarding brain physiology, abnormalities in the P-50 and P-300 latencies and amplitudes of event-related potentials, including the mismatch negativity (MMN), have been found in schizophrenic patients (Umbricht and Krljes, 2005). In this way, sleep abnormalities, eye movement alterations (Monti and Monti, 2005) and impairments of neural synchrony, such as gamma

oscillations (Uhlhaas and Singer, 2010; Farzan et al., 2010) or theta frequencies (Winterer et al., 2000), are common in patients suffering from schizophrenia. Another remarkable trait is the increased amygdala and parahippocampal gyrus activation observed in patients (Escarti et al., 2010). Finally, alterations in the the pre-pulse inhibition (PPI) are also common in these patients. This shows that the reaction after a given stimuli is reduced in schizophrenia (Braff and Light, 2005). PPI is one of the most consistent alterations shown both by treated and naïve-medication patients and by their healthy relatives (Cadenhead, 2002). All these physiological changes must have neurochemical bases, probably involving glutamatergic, serotoninergic, cholinergic, dopaminergic and GABAergic systems (Javitt, 2008).

Postmortem studies of the brains of schizophrenic patients have replicated some of the observations found *in vivo* using neuroimaging: These include the reduction in gray matter volume (Harrison, 2004), or in those of the white matter (Gogtay et al., 2008) in some areas and the increase in the volume of the ventricles. Some other studies have studied the density of some neurotransmitters and their receptors at the mRNA and protein levels.

Some of the most interesting results are the alterations of some serotoninergic pathway genes in the prefrontal cortex of schizophrenic patients (Abi-Dargham, 2007), and the decrease in the expression of several markers of inhibitory transmission, such as GAD67 or parvalbumin expression (Lewis et al., 2012).

Table 2 shows the most interesting findings in genetic and post-mortem studies in pathways implicated in inhibitory neurotransmission and neural plasticity in schizophrenia.

Table 2. Findings in genetic and post-mortem studies in inhibitory neurotransmission and plasticity in schizophrenia.

Marker	Finding	Reference
GAD67	**Reduced GAD67 mRNA in prefrontal cortex** **Reduced GAD67 level in PV cells in the cortex** **Preserved number of PV cells in the cortex** **Associated polymorphysms of GAD67 promoter**	Volk et al., 2000 Akbarian et al.,1995 Woo et al.,1997 Hashimoto et al., 2003 Addington et al., 2004 Woo et al., 1998
Chandelier	**Decreased chandelier cartridges (GAT1+) in prefrontal cortex**	Volk et al., 2001
SST	**Decreased SST mRNA, cells in prefrontal cortex**	Hashimoto et al., 2007
NPY/CCK	**Decreased levels of NPY and CCK mRNAs**	Hashimoto et al., 2007
NRG1	**Association of the NRG1 gene**	Wang et al., 2009 Yang et al., 2003
ERB4	**Association of the ERB4 gene**	Silberberg et al., 2006
BDNF/Trkb	**Downregulation of BDNF in prefrontal cortex**	Wong et al., 2010 Takahashi et al., 2000
PSA-NCAM	**Downregulation of PSA-NCAM in hippocampus and amygdala**	Barbeau et al., 1995 Varea et al., 2012

7.3. Animal Models

Some of the most useful tools for the study of schizophrenia and other psychiatric disorders are the animal models of these diseases, which usually are developed in rodents. However, the heterogeneity of schizophrenia makes extremely difficult the development of a unique animal model and, consequently, researchers have to investigate different models to reproduce different hallmarks of the disease. Currently there is no animal model capable of reproduce exactly all the symptoms and alterations found in schizophrenic patients. Very few models show altered social behavior, learning or memory impairment, which are the negative and cognitive symptoms of schizophrenia and are resistant to the treatment with antipsychotics, even after remission of the positive symptoms. Consequently, the search for new animal models, which will be able to reproduce some of the negative and cognitive symptoms is absolutely necessary in order to understand the disease and evaluate possible new therapies (for review see Jones et al., 2011).

The animal models for schizophrenia used in experimental research have been classified in four types, althought some of them can be placed in more than one group:

Neuronal Lesion Models. The main strategy used for neuronal injury is the lesion of the ventral hippocampal areas in neonates. This model is based on the hypothesis that postulates the involvement of the hippocampus in the development of schizophrenia. It was in 1995 when Lipska and Weinberger developed this model in rats administrating ibotenic acid in the CA1 and subicular areas of the hippocampus at postnatal day 7 (P7). Other similar models result from the acute injection of the $GABA_A$ receptor antagonist, picrotoxin, into the ventral hippocampus (Bast et al., 2001).

Mutant Models. These models result from mutations in genes potentially implicated in the disease. Some examples are the heterozygote mice for neuroregulin 1 (NRG1), a protein implicated in glutamate neurotransmission (Stefansson et al., 2002) and also in the development of cortical inhibitory networks (Fazzari et al., 2010); the *DISC1* partially-deficient mouse (for review see Jaaro-Peled, 2009); the hypofunctional model for the NR1 subunit of the NMDA receptor (Mohn et al., 1999). Other models include the *reeler* mice (Impagnatiello et al., 1998), which shows a decreased expression of reelin, a molecule implicated in neuronal migration, and the mutant mouse for the *Dysbindin* gene (Papaleo et al., 2012), which is implicated in neurotransmitter release.

Behavioral or Neurodevelopmental Models. To generate these models animals are submitted during their embryonic development, infancy or early adolescence to stressors or adverse environmental insults. They are based on the hypothesis that the risk of developing schizophrenia is enhanced by alterations during early development. Administration of methylazoxymethanol (MAM), a DNA methylase with anti-proliferative properties, to pregnant rats has been used to affect the brain development of the litter (Moore et al., 2006). Other models are based on maternal exposure to bacterial or viral infection (Khandaker et al., 2012). The social isolation rearing model, in which we have based some of our research, is included in this type of models.

Drug Administration Models. Some models have been developed by means of the administration of toxic substances, which mimic some of the symptoms suffered by schizophrenic patients. The most common and one of the first models obtained is based on amphetamine administration (Robinson and Becker, 1986). Other recently used models, which we have also used in this thesis, result from the administration of non-competitive antagonists of NMDA receptors such as ketamine or phencyclidine and other derivates (MK801) in order to alter the glutamatergic system and the normal neurodevelopment.

7.3.1. Social Isolation Rearing Model

One of the simplest models used in the study of schizophrenia is the Social Isolation Rearing Model; this model consists in the social isolation of the rodent from their littermates after weaning. The isolated animal is able to see, smell and listen to the other animals, but is unable to have any physical contact. This situation promotes the development of psychotic-like behaviors.

Rats have a social structure resulting in a marked hierarchy. The establishment of this social system is very important for the correct development of the animal. Consequently, the isolation from the littermates causes severe deficits in the CNS and the development of altered behavior. The most relevant alterations found in this model are listed in table 3.

Table 3. Phenotypes of the social isolation rodent model.

Phenotypes Observed	Reference
Disrupted prepulse inhibition	Geyer et al., 1993
Reduced prefrontal cortex volume	Day-Wilson et al., 2006
Reduced expression of AMPA glutamate receptors in hippocampus	Sestito et al., 2011
Impaired spatial cognition with affected prefrontal cortical synaptic plasticity	Quan et al., 2010
Altered novel object recognition	McLean et al., 2010
Increased aggression	Ferdman et al., 2007

7.3.2. Perinatal MK-801 Administration Model

In accordance with the neurodevelopmental hypothesis of schizophrenia, some models that alter the regular development of the brain have been proposed. Adverse events during the prenatal, perinatal or postnatal period may affect brain development in a way that could culminate in the manifestation of symptoms in the adulthood (Weimberger, 1996; Lewis and Levitt, 2002).

The administration of NMDA receptor antagonists, such as MK-801, to young rodents during their early postnatal developmental period not only results in NMDA receptor hypofunction, but also alters neurodevelopmental components (Stefani and Moghaddam, 2005; Baier, 2009).

Different kinds of MK-801 administration regimes have been proposed by different authors, differing in the day or days of intraperitoneal administration and in the dose, althoug almost all the models suggest that the administration of the MK-801 should be done during the early infancy of the rodent: from the 3rd to 19th day after birth, being more common the procedure around the 7th day. The dosis used also varies in different studies: from 0.13 mg/Kg to 1mg/Kg in one or more doses (for review see Lim et al., 2012).

Some of the features seen in these models of schizophrenia involving perinatal MK-801 administration in rodents are listed in the recent review of Lin et al. in 2012: reduced weight during treatment, apoptosis in the 24h post injection, altered prepulse inhibition and locomotor activity in adulthood, impairments in novel object recognition, spatial memory, cognitive flexibility and increased anxiety with altered social behavior in adulthood, reduced brain volume, altered expression of NR1 subunit of NMDA receptor, augmented dopamine, serotonine and noradrenaline turnover, loss of prefrontal parvalbumin interneurons, changes in BDNF expression and altered number of glial cells.

7.3.3. Combined or "Double Hit" Model

Nowadays there is only one *double hit* model described combining social isolation with the administration of antagonists of NMDA receptors (MK-801). The researchers administered the MK-801 during adulthood in a subchronic (7 days) dose of 0.5 mg/Kg twice daily (Ashby et al., 2010; Hickey et al., 2012). In these studies, Beninger and col. show that the effects of isolation and the antagonists of NMDA receptors produce complementary, although not very robust, behavioral alterations, indicating that the two manipulations are acting through different pathways.

Some of the main features of this model are: increased locomotor activity, increased GABA transporter-1 (GAT-1) activity in frontal cortex and hippocampus, upregulation of $GABA_A$ receptor expression, increased long term potentiation in CA1.

Objectives

The main objective of this thesis is to study potential alterations in neuronal plasticity and inhibitory neurotransmission in the adult brain and their implication in psychiatric disorders. This objective is approached from the study of animal models of these disorders, the analysis of postmortem patient brains and genetic association studies. From this main objective, derive the subsequent specific objectives:

1. To evaluate the changes in the expression of PSA-NCAM induced by chronic stress in the amygdala and mPFC of adult mice, and to study the effects of this aversive experience on the structure of interneurons and molecules implicated in inhibitory neurotransmission.

2. To determine the existence of specific changes in the expression of different molecules involved in structural plasticity and inhibitory neurotransmission in the amygdala of rats reared in isolation.

3. To find whether the combination of a perinatal injection of MK-801 (P7), and a postweaning social isolation rearing reproduces some of the structural and molecular changes found in the mPFC and the hippocampus of schizophrenic patients, particularly in their inhibitory networks.

4. To compare changes in the expression of PSA-NCAM, synaptophysin, vesicular glutamate transporter type 1 (VGLUT1) and GAD67, in the dorsolateral PFC from post-mortem samples of patients of Major Depression, Bipolar Disorder and Schizophrenia.

5. To evaluate the involvement of *ST8SIAII* gene in the etiology of schizophrenia in the Spanish population, taking into account the gender of the individuals.

Articles

Article 1: Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice

Experimental Neurology 232 (2011) 33–40

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Regular Article

Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice ☆

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ARTICLE INFO

Article history:
Received 26 January 2011
Revised 17 June 2011
Accepted 19 July 2011
Available online 28 July 2011

Keywords:
Interneuron
Polysialyltransferase
Glutamic acid decarboxylase
Synaptophysin

ABSTRACT

Chronic stress in experimental animals, one of the most accepted models of chronic anxiety and depression, induces structural remodeling of principal neurons in the amygdala and increases its excitation by reducing inhibitory tone. These changes may be mediated by the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), a molecule related to neuronal structural plasticity and expressed by interneurons in the adult CNS, which is downregulated in the amygdala after chronic stress. We have analyzed the amygdala of adult mice after 21 days of restraint stress, studying with qRT-PCR the expression of genes related to general and inhibitory neurotransmission, and of PSA synthesizing enzymes. The expression of GAD67, synaptophysin and PSA-NCAM was also studied in specific amygdaloid nuclei using immunohistochemistry. We also analyzed dendritic arborization and spine density, and cell activity, monitoring c-Fos expression, in amygdaloid interneurons. At the mRNA level, the expression of GAD67 and of St8SiaII was significantly reduced. At the protein level there was an overall reduction in the expression of GAD67, synaptophysin and PSA-NCAM, but significant changes were only detected in specific amygdaloid regions. Chronic stress did not affect dendritic spine density, but reduced dendritic arborization in interneurons of the lateral and basolateral amygdala. These results indicate that chronic stress modulates inhibitory neurotransmission in the amygdala by regulating the expression of molecules involved in this process and by promoting the structural remodeling of interneurons. The addition of PSA to NCAM by St8SiaII may be involved in these changes.
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Introduction

Aversive experiences, such as stress or fear can induce neuronal structural plasticity, which may act as a neuroprotective mechanism (McEwen, 2000; McEwen, 2005; McEwen and Chattarji, 2004; Roozendaal, et al., 2009). In particular, chronic stress induces dendritic atrophy and decreases spine density in principal neurons of the medial prefrontal cortex (mPFC) (Cook and Wellman, 2004; Radley, et al., 2004; Radley, et al., 2005; Seib and Wellman, 2003) and the hippocampus (Sousa, et al., 2000; Watanabe, et al., 1992). By contrast, chronic stress induces opposite effects in principal neurons of the basolateral amygdala (Vyas, et al., 2002). However these structural effects of chronic stress in the amygdala are more complex, since the same paradigm induces loss of spines in the medial amygdaloid nucleus (Bennur, et al., 2007) and leaves the central nucleus unaffected (Vyas, et al., 2003).

In addition to these structural changes, different electrophysiological experiments indicate that stress or high levels of corticosteroids induce activation of the amygdala, leading to an increase in the excitability of principal neurons (Duvarci and Pare, 2007; Roozendaal, et al., 2009). This increase in excitability can be also the result of a stress-induced reduction in inhibitory neurotransmission, which has also been reported in the amygdala (see Davis, et al., 1994) for review. Although, to our knowledge, all the studies on the stress-induced neuronal structural plasticity have been focused on principal neurons, it is also possible that changes in the structure of interneurons may mediate the effects of stress on the amygdala, especially those affecting inhibitory networks. In fact, recent reports have found that,

☆ Grant Support: Spanish Ministry of Science and Innovation (MICINN-FEDER) BFU2009-12284/BFI, MICINN-PIM2010ERN-00577/NEUCONNECT in the frame of ERA-NET NEURON, Generalitat ValencianaCS2009-AP-127 and ACOMP2009/271 and the Stanley Medical Research Institute to JN. Javier Gilabert-Juan and Esther Castillo-Gómez have a FPU predoctoral fellowships from the Spanish Ministry of Education and Science (AP2008-00937 and AP2006-01953).
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0014-4886/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2011.07.009

as it occurs with principal neurons, interneurons can also remodel their structure in the adult cerebral cortex (Lee, et al., 2006; Lee, et al., 2008).

Different molecules have been studied in order to understand the molecular bases of these changes in the structure of amygdaloid neurons and in the physiology of inhibitory and excitatory circuits of the amygdala. The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is a very promising candidate to mediate these changes, because it is intensely expressed in the different nuclei of the amygdala of rodents (Nacher et al., 2002b) and its expression is regulated by chronic stress in the amygdala (Cordero, et al., 2005) and other cerebral regions (see Sandi, 2004, for review). The addition of PSA to NCAM confers it anti-adhesive properties and consequently, facilitates plastic processes, such as neurite and spine remodeling or synaptogenesis (see Rutishauser, 2008 for review). Moreover, in different regions of the cerebral cortex this molecule is expressed exclusively in interneurons (Gomez-Climent et al., 2011; Nacher et al., 2002a; Varea, et al., 2005) and, consequently, changes in its expression may influence inhibitory neurotransmission. In fact, we have recently reported that PSA-NCAM expressing interneurons have reduced synaptic input and less dendritic arborization and spine density than interneurons lacking PSA-NCAM (Gomez-Climent et al., 2011). Changes in PSA-NCAM expression induced by different experimental manipulations are accompanied by changes in the expression of markers of synaptic density in the amygdala and different cortical regions (Varea et al., 2007a, 2007b). Moreover, consequent with its presence in interneurons, at least in the mPFC, the modulation of PSA-NCAM expression is also accompanied by changes in the expression of molecules related to inhibitory neurotransmission (Castillo-Gomez, et al., 2008).

In order to understand the role of PSA-NCAM in the response of the amygdala to chronic stress and to study the effects of this aversive experience on the structure of interneurons and molecules implicated in inhibitory neurotransmission, we have subjected mice to 21 days of chronic restrain stress. These mice belong to a strain in which the expression of the enhanced green fluorescent protein (GFP) is under the control of the glutamic acid decarboxylase gene and thus the complete morphology of these inhibitory neurons can be observed. We have studied the effects of stress on the dendritic arborization and spine density of these cells using confocal microscopy and we have also analyzed the expression of different molecules related to inhibitory neurotransmission and PSA synthesis using immunohistochemistry and quantitative RT-PCR.

Material and methods

Animals

Male GIN mice (3 months-old; GFP-expressing Inhibitory Neurons, (Tg(GadGFP)45704Swn)) were purchased from Jackson laboratories (Bar Harbor, Maine, USA) and bred in our animal facility. Animals were housed in groups of 5 to 7 per cage, at a room temperature of 25 °C and on a 12-h light/dark cycle with food and water available ad libitum, and were assigned randomly to control or stress group. Animals were weighed at day 1, 2, 6, 9, 13, 16, 20 from the start of stress procedure. All efforts were made to minimize the number and suffering of animals used. All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València.

Chronic immobilization stress procedure

Fourteen mice were subjected to chronic immobilization stress similar to a published procedure (Patel, et al., 2004), but with some modifications. Briefly, mice were immobilized for 1 h per day for 21 days (from 11 to 12 am) in modified, transparent 50 ml plastic conical tubes with many air holes to allow ventilation. Animals inside the tube were placed on the bench top, close to a sonicator bath. Control animals (n = 11) were handled daily, but were left undisturbed in their cages after less than 1 min. Mice were euthanized 24 h after the last stress session, in a different room than the one in which restraints were carried out in a random order.

Quantitative retrotranscription-polymerase chain reaction

The mice used for qRT-PCR were sacrificed by decapitation using a guillotine. After that, brains were removed from the skull and the whole amygdalae of 5 control mice and 7 stressed mice were extracted. Each amygdala was individually analyzed in this experiment. Total mRNA from amygdala was extracted using TriPure reagent (Roche Applied Science, Indianapolis, IN) following manufacturer's instructions. The concentration and purity of total RNA for each sample were determined by Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). cDNA synthesis was performed using the Expand reverse transcriptase (Roche Applied Science).

For qRT-PCR analyses, each sample was run in triplicates. qPCR was carried out with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems), specific primers for all genes (Table 1) at a concentration of 240 nM, and cDNA of each sample. TATA binding protein gene was used as a reference gene. Following a 95 °C denaturation for 10 min, the reactions were cycled 40 times with a 95 °C denaturation for 15 s, and a 60 °C annealing step for 1 min. After this, a melt curve was performed to assess the specificity of primers.

Relative quantification was performed using the comparative threshold (Ct) method according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Changes in gene expression were reported as fold changes relative to controls. ANOVA study followed by Student–Newman–Keuls post-hoc test was performed to analyze the statistical significance of results.

Immunohistochemistry

Six control mice and 7 stressed mice were perfused transcardially with a 4% paraformaldehyde solution in phosphate buffer (PB, 0.1 M, pH 7.4). Brains were removed from the cranium and the hemispheres were separated. The right hemisphere was cryoprotected in a 30% sucrose solution in PB and cut in a sliding microtome at 50 μm. These

Table 1
Sequences of gene specific primers and associated amplicon lengths for qRT-PCR.

Target gene	Primers	Sequence (5′→3′)	Amplicon size(1)
ST8SiaII	Forward	GGCTGTGGCCAGGAGATTG	72
	Reverse	GGCATACTCCTGAACTGGAGCC	
ST8SiaIV	Forward	GCACCAAGAGACGCAACTCATC	68
	Reverse	CAGAGCTGTTGACAAGTGATCTGC	
NCAM	Forward	GGATGCCTCCATCCACCTC	67
	Reverse	GGCCGTCTGATTCTCTACATAGG	
GAD67	Forward	GGGTTCCAGATAGCCCTGAGCGA	120
	Reverse	TGGCCTTGTCCCCTTGAGGCT	
GAD65	Forward	AGCCTCAACACACAAATGTCTGCTTCT	135
	Reverse	TGGTCCCATACTCCATCATTCTGGCT	
SYN	Forward	TCTTTGTCACCGTGGCTGTGTT	268
	Reverse	TCCCTCAGTTCCTTGCATGTGT	
GABAAα1	Forward	GCCATGGACTGGTTTATTGC	99
	Reverse	CCACGCATACCCTCTCTTGGTG	
GAT	Forward	TCTGCCCGCCTTGGCTCTGA	134
	Reverse	TGGGGGTGGGTCCTGGAAGC	
RELN	Forward	CGGAAGGAAGGCGTGCTGCT	125
	Reverse	GCCCCCTCAGGCAGGAGGAT	
TATABP	Forward	CACTTCGTGCAAGAAATGCTG	89
	Reverse	AATCAACGCAGTTGTCCGTG	

(1) Amplicon length in base pairs.

sections were destined for immunohistochemical analyses. The contralateral hemisphere was cut in 100 μm sections with a vibratome and the resulting sections were used to analyze dendritic spine density on GFP expressing interneurons.

The immunohistochemistry protocol was performed as follows: Briefly, floating sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6 at 100 °C). After cooling down the sections to room temperature, they were incubated with 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 10% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton-X-100 (Sigma-Aldrich) and they were incubated overnight at room temperature with one of these antibodies: anti-PSA-NCAM (AbCys, 1:700), anti-GAD67 (Chemicon, 1:500), anti-SYN (Sigma, 1:200) with PBS containing 0.2% Triton-X-100 and 3% NDS. The second day sections were incubated for 1 h with either donkey anti-mouse IgM or IgG biotinylated antibodies (1:200; Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton-X-100 and 5% NDS. Then, sections were incubated in an avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min in PBS. Color development was achieved by incubating with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.033% hydrogen peroxide in PB for 4 min. Finally, sections were mounted on slides, dried for 1 day at room temperature, dehydrated with ascending alcohols and rinsed in xilol. After this, sections were coverslipped using Eukkit mounting medium.

In order to amplify the GFP fluorescent signal in interneurons, a fluorescent immunohistochemistry against GFP was performed. Briefly, floating sections were blocked with NDS in buffer as above and incubated overnight with chicken anti-GFP antibody (Millipore, 1:500). The day after, the sections were incubated for 1 h with an Alexa Fluor 488 conjugated antibody against chicken (Molecular Probes,1:500). Finally, sections were mounted on slides using DakoCytomation fluorescent mounting medium (Dako North America, Inc., Carpinteria, CA).

In order to characterize neurochemically the somata expressing PSA-NCAM in the amygdala, we have performed double fluorescence immunohistochemistry and confocal analysis. The first day, sections were incubated overnight at room temperature with mouse monoclonal IgM anti-PSA antibody and either mouse monoclonal IgG2a anti-GAD67 or mouse monoclonal IgG1 anti-Ca(2+)/CaM dependent protein kinase II (CAMKII). The second day sections were washed and incubated for 1 h with donkey anti-mouse IgM in combination with either donkey anti-mouse IgG1, or donkey anti-mouse IgG2a secondary antibodies conjugated with Alexa 555 or Alexa 647 (1:200; Molecular Probes, Eugene, OR) in PBS containing 0.2% Triton-X-100 and 3% NDS. All sections were processed as described above and then observed under a confocal microscope (Leica TCS SPE) using a 63X oil objective. Z-series of optical sections (1 μm apart) were obtained using the sequential scanning mode. These stacks were processed with LSM 5 image software. One in 10 series of coronal telencephalic sections from 3 control animals was double-labeled as described. Fifty randomly-selected immunoreactive cells were analyzed in each case to determine the co-expression of PSA and the markers described above.

To identify c-Fos expression in GFP labeled interneurons and to compare the percentages of these c-Fos/GFP expressing cells between stressed and control mice, a fluorescent immunohistochemistry against c-Fos was performed as described above, using a polyclonal IgG antibody generated in rabbit (1:2000, Santa Cruz Biotechnology Inc.) and an Alexa Fluor 555 conjugated secondary antibody against rabbit IgG (Molecular Probes,1:500).

Quantification of neuropil immunoreactivity

From each immunostaining (PSA-NCAM, GAD67, SYN), 3 amygdaloid nuclei were selected in order to measure immunoreactivity as previously described (Varea et al., 2007a, 2007b): Centromedial, Basolateral and Medial nuclei (CeM, BLa and Me). Sections were examined with an Olympus CX41 microscope under bright-field illumination, homogeneously lighted and digitalized using a CCD camera. Photographs to the different areas and layers were taken at 20× magnification. Gray levels were measured using Image J software (NIH). Means were determined for each experimental group and data were analyzed by means of ANOVA followed by Student–Newman–Keuls post-hoc test.

Analysis of dendritic arborization and spine density

Dendritic arborization and spine density were studied using confocal microscopy (Leica TCS SPE). Z-series of optical sections (0.2 μm apart for spine density and 1 μm for dendritic arborization) covering the dendritic tree of selected interneurons were obtained using the sequential scanning mode and a 63× objective. From each animal, an average of 4 GAD-GFP expressing neurons were selected from the basolateral and lateral amygdaloid nuclei from control and stress groups. The analysis was focused in these 2 amygdaloid nuclei because most GAD-GFP expressing neurons in GIN mice are located in them and are almost absent from the rest of amygdaloid regions. A total of 40 neurons were analyzed from the 2 groups. In order to be analyzed, GFP-expressing cells had to fulfill the following features: (1) the cell must not show any truncated dendrites, (2) the dendritic arbor of the cell must show at least a process with a length greater than 120 μm and (3) the soma must be located at least 30 μm deep from the surface of the tissue. The stacks obtained were then processed using ImageJ software (NIH) in order to render 2D reconstructions, in which the exact distance of the branching and terminal points of the dendrites of a given interneuron were analyzed. The degree of dendritic arborization was analyzed using a procedure for deriving the Sholl profile. The Sholl analysis consists on the measure of the number of intersections of the dendrites with circles of increasing radius centered in the soma (Sholl, 1953). Spines were quantified in 3 successive segments of 50 μm distances up to a total length of 150 μm. Overall spine density values or densities per segment were expressed as number of spines/μm. For each experimental group, mean ± S.E.M. was determined and the resulting values was analyzed by one-way ANOVA (followed by Student-Newman-Keuls post-hoc test) with the number of neurons as the "n" (Magarinos et al., 1999; Guirado et al., 2009).

Quantification of GFP/cFos expressing cells

In order to compare the number of GFP cells expressing c-Fos, 25 GFP-expressing interneurons per animal were randomly selected in all amygdaloidal nuclei. Then, the number of c-Fos co-expressing cells was counted, and means were obtained and compared using unpaired Student's *t*-test analysis.

Results

Chronic stress does not induce changes in body weight gain

Body weight changes were measured at 0, 1, 2, 6, 9, 13, 16 and 20 days from the start of the stress procedure. This chronic immobilization stress paradigm did not induce any significant change in body weight gain when groups were compared after ANOVA analysis of all the monitored days of the stress-induction protocol ($p \geq 0.05$).

qRT-PCR analysis reveals a decrease in the mRNA expression of ST8SiaII and GAD67 genes in the amygdala of stressed mice

Polysialyltransferase II (*ST8SiaII*) gene expression in the amygdala was significantly decreased ($p = 0.024$) by chronic stress when

compared with that of control mice (Table 2; Fig. 1). In the same direction, *glutamic acid decarboxylase 67* (*GAD67*) gene expression was also significantly repressed in stressed individuals ($p = 0.042$; Table 2; Fig. 1). The remaining genes: *polysialyltransferase IV* (*ST8SiaIV*), *neural cell adhesion molecule* (*NCAM*), *glutamic acid decarboxylase 65* (*GAD65*), *synaptophysin* (*SYN*), *GABA vesicular transporter* (*GAT*), *reelin* (*RELN*) and the *GABA(A)α1 receptor* (*GABAAα1*) did not show any significant change between stressed and control mice.

The expression of PSA-NCAM, GAD67 and synaptophysin is altered by chronic stress in some of the amygdaloid nuclei

In consonance with previous results using chronically stressed rats (Cordero et al., 2005), in GIN mice chronic stress induced a decrease in the expression of PSA-NCAM, and also in that of GAD67 and SYN in all the 3 amygdaloid nuclei studied (Fig. 2). However, this down-regulation was not significant for all these 3 nuclei. For PSA-NCAM expression, we found a significant decrease in CeM nuclei ($p = 0.0041$), but only a tendency toward a decrease in the other 2 remaining nuclei ($p = 0.25$; 0.19 for Me and BLa respectively). GAD67 expression was significantly reduced in the Me nuclei ($p = 0.021$), but not in CeM or BLa nuclei ($p = 0.32$; 0.36, respectively). Finally, the reduction in SYN expression was significant in the Me nuclei ($p = 0.012$) but not in the CeM ($p = 0.34$) or BLa ($p = 0.32$) nuclei.

PSA-NCAM expressing cells in the amygdala express markers of interneurons and lack markers of principal cells

Many PSA-NCAM expressing cells in the amygdala co-expressed GAD67 (38% ± 1.73, Fig. 3A) but none of them co-expressed Ca(2+)/CaM dependent protein kinase II (CAMKII) a protein exclusively found in principal neurons (Fig. 3B).

Chronic stress reduces dendritic arborization but does not change spine density in GAD-GFP expressing interneurons of the amygdala

Dendritic arborization and spine density were analyzed in interneurons expressing GFP in the lateral and basolateral nuclei. Sholl analysis revealed reduced dendritic arborization in the GFP-labeled interneurons of stressed mice (Figs. 3C & D). These differences were significant in the 40–60 μm segment ($p = 0.013$) of distance from the soma. Selected dendrites were divided for analysis into three segments of 50 μm from the soma. We did not find significant differences in dendritic spine density between control and stressed groups in the whole 150 μm dendrite segment or in any of the three subsegments ($p = 0.71$; 0.09; 0.33 respectively, Figs. 3E–G).

Table 2
qRT-PCR results for tested genes in stress mice vs. control.

	Total amygdala	
	Δ	p-value
ST8SiaII	0.27	**0.024**
ST8SiaIV	0.73	0.54
NCAM	1.26	0.39
GAD67	0.57	**0.042**
GAD65	0.91	0.88
Synaptophysin	1.19	0.98
GABAAα1	0.77	0.24
GAT	1.08	0.59
RELN	0.83	0.29

Δ, change in gene relative to normalize. Information in bold represents significant changes.

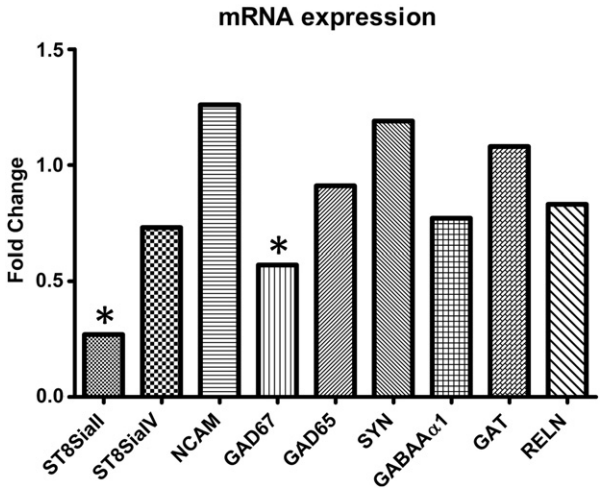

Fig. 1. qRT-PCR mRNA fold change data shown as stressed mice group versus control mice gene expression. All genes expression were normalized using TATA binding protein as a control gene.

Chronic stress does not induce changes in the number of c-Fos expressing interneurons of the amygdala

The number of c-Fos positive interneurons expressing GFP and the total number of interneurons expressing GFP were quantified in all the extension of the amygdala. Stressed mice did not differ significantly neither in the number of interneurons expressing GFP ($p = 0.947$) nor in the number of c-Fos positive interneurons expressing GFP ($p = 0.405$).

Discussion

The present results in mice confirm previous studies on the effects of chronic stress on amygdaloid PSA-NCAM expression in rats and expand them studying the expression of the enzymes responsible of its polysialylation. These effects on PSA-NCAM expression are paralleled by changes in molecules related to inhibitory neurotransmission, but not by structural changes in amygdaloid interneurons.

PSA-NCAM a putative mediator of the effects of chronic stress in the adult amygdala

Given its anti-adhesive properties, PSA-NCAM was one of the first molecules related to structural plasticity studied after chronic stress in experimental animals (Sandi, et al., 2001) and has remained the focus of several studies in different cerebral regions since then (Sandi, 2004). The distribution of PSA-NCAM expression in the amygdala of GIN mice is similar to that described previously in a different mouse strain (Nacher, et al., 2010) and in rats (Nacher et al., 2002b). As it has been demonstrated for many PSA-NCAM expressing structures in the cerebral cortex (excluding those of immature neurons) (Gomez-Climent et al., 2011; Nacher et al., 2002a; Varea, et al., 2005), many PSA-NCAM expressing neurons in the amygdala (present results) express markers of interneurons and lack expression of molecules exclusively found in principal neurons. Consequently, changes in PSA-NCAM expression should primarily affect the structure of interneurons, rather than that of principal neurons. In this line, we have recently reported that PSA-NCAM expressing cortical interneurons have reduced synaptic input and decreased dendritic arborization and spine density when compared with neighboring interneurons lacking PSA-NCAM (Gomez-Climent et al., 2011). It is possible then, that the stress-induced reductions in PSA-NCAM expression observed in the present study affect the connectivity of certain amygdaloid interneurons, leaving more plasma membrane extension free for the establishment of new synaptic contacts. Another non-excluding

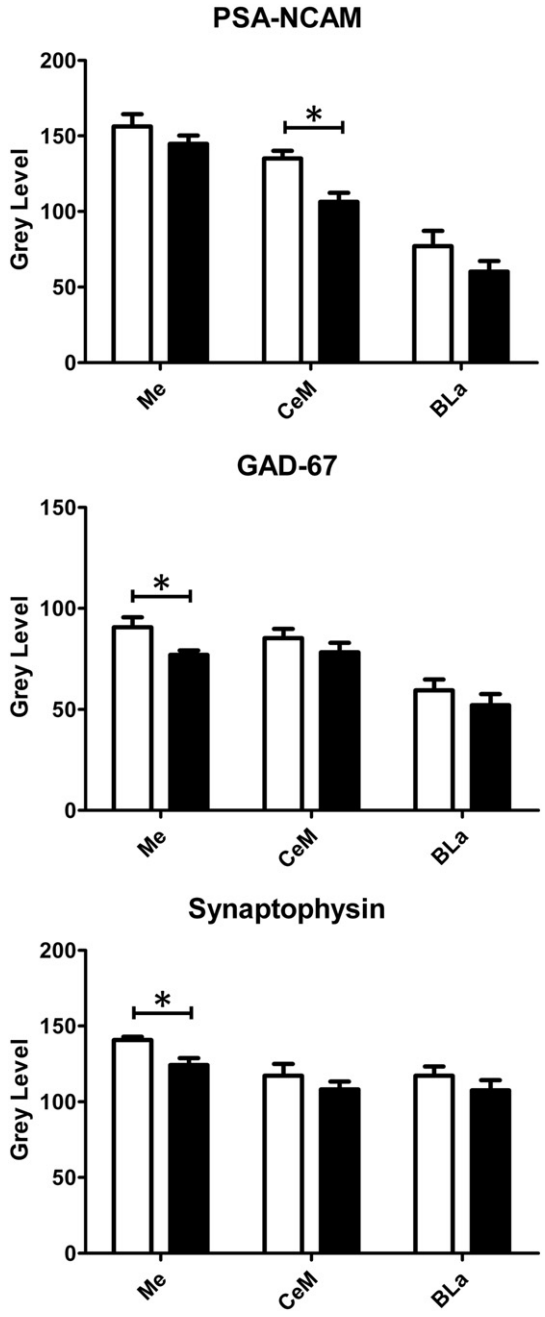

Fig. 2. Neuropil immunoreactivity of PSA-NCAM, GAD67 and SYN in the amygdala. Histogram bars show the gray level measured in amygdaloid nuclei (Me, CeM, BLa) of control (white bar) and stressed mice (black bar) groups. Data are the mean ± S.E.M. from 6 control mice and 7 stressed mice in each group. * $p<0.05$ vs the control group.

possibility is that, given its anti-adhesive properties, the reduction in PSA-NCAM expression may limit the ability of certain interneurons to remodel their structure in response to different stimuli. However, it is possible that these structural changes occur in an earlier time window during the stress procedure, in which changes in PSA-NCAM expression occur in a different direction, or only in certain amygdaloid nuclei and, consequently, we may have missed them studying the amygdala as a whole. The influence of PSA-NCAM on amygdaloid interneurons may also occur by the interference of the PSA in certain signaling cascades mediated by NCAM, especially those affecting inhibitory circuits (see (Maness and Schachner, 2007; Rutishauser, 2008) for review). However, future experiments manipulating PSA-NCAM expression are needed to understand whether this molecule plays a role in the remodeling of amygdaloid interneurons and how its direct effects on these inhibitory cells translate into structural changes in principal neurons.

Our present results are similar to those reported by Cordero et al. (2005), who described reductions in the expression of PSA-NCAM in the amygdala of chronically stressed rats, especially in their central and medial nuclei. However, although there is a general reduction in PSA-NCAM expression, we have only found significant changes in the central but not in the medial amygdaloid nucleus. This discrepancy may be due to species differences in the response to stress or to the fact that the chronic stress paradigm used in our mice is less intense (in terms of the duration of the stressor) than that used in rats. It is interesting to note that chronic stress induces dendritic spine loss in principal neurons of the medial amygdala of rats and mice and this structural plasticity appears to be mediated by tissue plasminogen activator (TPA) (Bennur, et al., 2007; Pawlak, et al., 2005). This extracellular matrix protease is increased in this amygdaloid region after stress (Pawlak, et al., 2005) and may be responsible of the stress-induced reduction in PSA-NCAM expression (Endo, et al., 1998).

Our results suggest that the decrease in polysialylation detected in the amygdala after chronic stress may be caused by the observed downregulation of polysialyltransferase St8SiaII expression, since St8SiaIV mRNA levels are not affected. These results are interesting, because the analysis of single polysialyltransferase knockout mice revealed that most PSA-NCAM expressing structures in the amygdala of adult control mice are polysialylated by St8SiaIV (Nacher, et al., 2010). However, it is possible that, although St8SiaIV may function as the "main" polysialyltransferase in the amygdala during normal circumstances, St8SiaII may take care of the addition of PSA to NCAM when the system is challenged, for instance by stress. In this regard, it is very interesting to note that St8SiaII, but not St8SiaIV, is a candidate susceptibility gene for schizophrenia and bipolar disorder (Arai, et al., 2004; Tao, et al., 2007). These two mental disorders share abnormalities in amygdaloid structure and function, and stress is considered a precipitating factor in both of them. The observed reductions in PSA-NCAM expression in the amygdala after chronic stress must have consequences on behaviors dependent on this cerebral region. In fact, previous studies in naive rodents with reduced PSA-NCAM expression indicate a potentiation of amygdala-dependent behaviors: PSA depletion in the amygdala of rats results in enhanced fear extinction (Markram et al., 2007) and *St8SiaII* knockout mice displayed increased agression (Calandreau et al., 2010).

Chronic stress may affect synapses and inhibitory neurotransmission in the amygdala

There are no previous reports showing changes in synaptophysin expression or in synaptic density in the amygdala after chronic stress. Our finding of a reduction in synaptophysin expression in the medial amygdala may indicate a reduction in active synapses, because the expression of this synaptic vesicle membrane protein is linked to synaptic remodeling (Greengard, et al., 1993) and it is considered a reliable index of synaptic density (Eastwood and Harrison, 2001; Masliah, et al., 1990). Since this decrease coincides with the previously reported reduction in dendritic spine density in principal neurons of the medial amygdala (Bennur, et al., 2007), it is possible that some of the lost synapses corresponded to those established on the lost spines. However, we have not observed increases in synaptophysin expression in the basolateral amygdala, where the density of spines in principal neurons is increased after chronic stress (Mitra, et al., 2005). Another possibility is that the synapses that disappear after stress were inhibitory. This would be in agreement with the parallel reductions observed in GAD67 expressing elements in the medial amygdala. In fact, different studies have demonstrated that stress can induce amygdala activation, affecting inhibitory neurotransmission, which in turn has an important role in stress-induced synaptic plasticity (Davis, et al., 1994). Moreover, an inverse

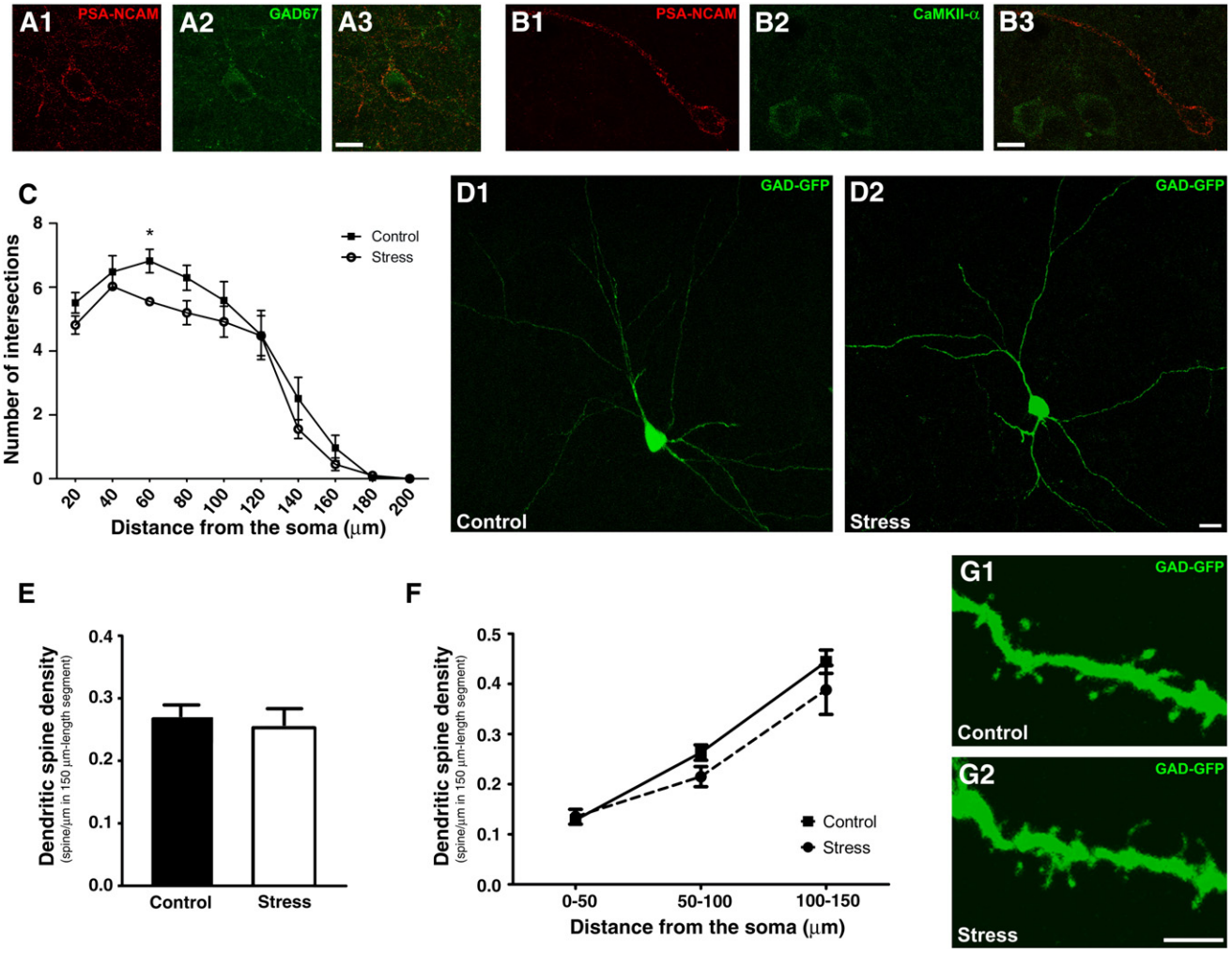

Fig. 3. A & B: Confocal microscopic analysis of the phenotype of PSA expressing cells in the amygdala. A: PSA-NCAM expressing interneuron coexpressing GAD67. B: PSA-NCAM expressing interneuron lacking CAMKII expression; note the presence of CAMKII expressing somata in the bottom left of the picture. C: Sholl analysis of GFP expressing interneurons, showing intersection number per 20 μm dendritic radial unit distance from the soma. D: 2D reconstructions of GFP expressing interneurons in the lateral and basolateral amygdala of control (D1) and chronically stressed animals (D2). E–G: Confocal microscopic analysis of dendritic spine number in GAD-GFP expressing interneurons from the amygdala. E & F: Histograms of the differences in the total density of dendritic spines (E) and the dendritic spine density in segments at different distances from the soma (F). Spines were counted in three 50 μm-length segments located 0–50, 50–100 and 100–150 μm from the interneuron soma, respectively. Unpaired Student *t*-test showed no statistically significant differences in any of the segments analyzed. G: Compositions, using fragments of different confocal planes, of spinous dendrites of GAD-GFP expressing interneurons in the amygdala of control (G1) and stressed animals (G2). Scale bar: 10 μm for A, B & D; 5 μm for G. Confocal images are 2D projections of 8 (A & B) and 20 (D),consecutive confocal planes located 1 μm apart; images in G are 2D projection of 25 confocal planes located 0.2 μm apart.

relationship has been found between inhibitory tone and behavioral anxiety in the basolateral nucleus of the amygdala (Roozendaal, et al., 2009). In fact, electrophysiological experiments in amygdala slices have shown that stress levels of corticosterone can reduce inhibitory neurotransmission and increase the excitability of principal amygdaloid neurons (Duvarci and Pare, 2007). However, these results and our findings on the expression of molecules related to inhibitory neurotransmission are apparently in contrast with reports of a reduced response of the amygdala to corticotropin releasing factor (CRF) after chronic stress (Sandi et al., 2008). CRF increases the excitability of principal neurons in the BLA (Rainnie et al., 1992) and, consequently, a reduced response to this factor may result in decreased excitability. Further analysis evaluating the expression of CRF and their receptors in relation to inhibitory neurotransmission during stress are necessary to understand this complex interactions.

The changes in the expression of molecules implicated in GABAergic neurotransmission described in our study are only restricted to GAD67 expression and other molecules, such as GAD65, GAT or GABAA(1) receptor do not show significant changes, at least at the mRNA level. Experiments evaluating different time points during the stress procedure will be necessary to discard any effect on the expression of these genes.

The effects observed in GAD67 expression may be mediated by the monoaminergic system, since it is known that stress enhances the release of monoamines in the amygdala (Goto, et al., 2007; Maier and Watkins, 2005) and these monoamines, in turn, affect amygdaloid inhibitory circuits (Braga, et al., 2004; Marowsky, et al., 2005). The stress-induced changes in the expression of GAD67 in the amygdala occur in parallel to the downregulation of PSA-NCAM expression and similar concomitant decreases in the expression of both molecules have been observed in the mPFC after dopaminergic depletion or chronic treatment with a dopamine D2 receptor antagonist (Castillo-Gomez, et al., 2008).

Changes in PSA-NCAM expression may promote remodeling of inhibitory circuits, which may lead to the observed decrease in GAD67 expression. We have observed a significant reduction in dendritic arborization in interneurons of the basolateral and lateral amygdala. This is, to our knowledge, the first report describing dendritic remodeling in interneurons after chronic stress. All the previous studies have been focused on the structure of principal neurons.

Particularly, in the basolateral amygdala chronic stress induces dendritic hypertrophy of stellate and pyramidal neurons (Vyas, et al., 2002). This dendritic growth of principal neurons, has been interpreted as a structural strengthening of excitatory neurotransmission in the basolateral amygdala, which may represent a cellular substrate for enhanced anxiety (Roozendaal et al., 2009). In the same way, the retraction of the dendrites of interneurons, which may also reduce inhibition on principal cells, can also contribute to this strengthening of excitatory neurotransmission. Whether this remodeling is due to increased corticosterone levels, as it has been demonstrated for the hypertrophy of principal neurons (Mitra and Sapolsky, 2008), still remains to be studied.

Unfortunately, we have not been able to study interneuron structure in the centromedial nucleus, where significant changes in PSA-NCAM expression have been detected, because very few interneurons express GFP in this nucleus in GIN mice. Future studies using different transgenic strains in which more amygdaloid interneurons appear labeled should shed light in this matter.

Our results on the amygdala of mice subjected to chronic stress may increase our understanding of the molecular and structural plasticity associated to the development of anxiety and mood disorders, specially that involving amygdaloid inhibitory circuits. This plasticity may be a substrate for the increases in anxiety-like behaviors, cognitive changes and mood alterations observed in this animal model and in these psychiatric disorders. In fact, although there are no studies on the expression of molecules related to inhibitory neurotransmission in these disorders, patients show overactivity in the amygdala, probably due to a reduction in inhibitory tone (Bremner, 2002; Phillips, et al., 2003).

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Article 2: Chronic stress alters inhibitory networks in the medial prefrontal cortex of adult mice

Brain Struct Funct
DOI 10.1007/s00429-012-0479-1

ORIGINAL ARTICLE

Chronic stress alters inhibitory networks in the medial prefrontal cortex of adult mice

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Received: 9 July 2012 / Accepted: 31 October 2012
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Abstract Chronic stress in experimental animals induces dendritic atrophy and decreases spine density in principal neurons of the medial prefrontal cortex (mPFC). This structural plasticity may play a neuroprotective role and underlie stress-induced behavioral changes. Different evidences indicate that the prefrontocortical GABA system is also altered by stress and in major depression patients. In the amygdala, chronic stress induces dendritic remodeling both in principal neurons and in interneurons. However, it is not known whether similar structural changes occur in mPFC interneurons. The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) may mediate these changes, because it is known to influence the dendritic organization of adult cortical interneurons. We have analyzed the dendritic arborization and spine density of mPFC interneurons in adult mice after 21 days of restraint stress and have found dendritic hypertrophy in a subpopulation of interneurons identified mainly as Martinotti cells. This aversive experience also decreases the number of glutamate decarboxylase enzyme, 67 kDa isoform (GAD67) expressing somata, without affecting different parameters related to apoptosis, but does not alter the number of interneurons expressing PSA-NCAM. Quantitative retrotranscription-polymerase chain reaction (qRT-PCR) analysis of genes related to general and inhibitory neurotransmission and of PSA synthesizing enzymes reveals increases in the expression of *NCAM, synaptophysin* and *GABA(A)α1*. Together these results show that mPFC inhibitory networks are affected by chronic stress and suggest that structural plasticity may be an important feature of stress-related psychiatric disorders where this cortical region, specially their GABAergic system, is altered.

Keywords Interneuron · Glutamic acid decarboxylase · Synaptophysin · Structural plasticity · Dendritic spines · Somatostatin · Calretinin

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Introduction

Aversive experiences, such as stress or fear can induce neuronal structural plasticity, which may act as a neuroprotective mechanism (McEwen 2000, 2008; Shansky and Morrison 2009). In particular, chronic stress induces dendritic atrophy and decreases spine density in principal neurons of the medial prefrontal cortex (mPFC) (Cook and Wellman 2004; Radley et al. 2004; Radley and Morrison 2005; Seib and Wellman 2003) and the hippocampus (Sousa et al. 2000; Watanabe et al. 1992) of adult male rats. By contrast, the opposite effects have been observed in principal neurons of the orbitofrontal cortex (Liston et al. 2006) or the basolateral amygdala (Vyas et al. 2002).

Published online: 21 November 2012

Most of the studies on the stress-induced neuronal structural plasticity have been focused on principal neurons, but a recent study from our laboratory has shown that chronic stress induces decreases in dendritic arborization in interneurons of the lateral and basolateral amygdala (Gilabert-Juan et al. 2011), the opposite effect to that observed in principal neurons of this region (Vyas et al. 2002). In fact, there are also recent reports that have found that, as it occurs with principal neurons, cortical interneurons can also remodel their structure in the adult cerebral cortex in normal circumstances (Lee et al. 2006, 2008; Chen et al. 2011) and after loss of sensory input (Keck et al. 2011). Chronic psychosocial stress also affects the density of interneurons in adult tree shrews, at least in the hippocampus, reducing the number of parvalbumin expressing neurons (Czeh et al. 2005; Hu et al. 2010). An observed increase in apoptosis in the hilus has been suggested as one possible cause of this decrease in interneurons and a similar increase in apoptosis has been reported in the neocortex (Lucassen et al. 2001).

Different molecules have been studied to understand the molecular bases of changes in the structure of mPFC neurons; the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is a very promising candidate to mediate these changes, because it is intensely expressed in interneurons of the mPFC of rodents (Gomez-Climent et al. 2011; Varea et al. 2005, 2007a) and humans (Varea et al. 2007b) and its expression is regulated by chronic stress in the hippocampus (Pham et al. 2003; Sandi et al. 2001), the amygdala, the piriform cortex (Nacher et al. 2004) and other cerebral regions (see Sandi 2004, for review). However, despite the implication of the mPFC in the response to chronic stress, PSA-NCAM expression has not been studied yet in this cortical region after this aversive experience.

In order to understand the effects of chronic stress on the structure of mPFC interneurons and in molecules implicated in inhibitory neurotransmission, as well as the role of PSA-NCAM in the response to this aversive experience, we have subjected mice to 21 days of chronic restraint stress. These mice belong to a strain in which the expression of the enhanced green fluorescent protein (EGFP) is under the control of the glutamic acid decarboxylase 67 (GAD67) gene (Oliva et al. 2000) and thus the complete morphology of these inhibitory neurons can be observed. After determining the neurochemical phenotype of these GAD67-EGFP expressing interneurons, we have studied the effects of stress on their dendritic arborization and spine density using confocal microscopy. We have also quantified changes in the total number of interneurons and of those expressing PSA-NCAM in the mPFC, and have determined with different methodologies the presence of interneuronal degeneration and apoptosis in this cortical region. Finally, we have analyzed the expression of different molecules related to inhibitory neurotransmission and PSA synthesis using quantitative RT-PCR.

Materials and methods

Animals

Male GIN mice (3-month-old; EGFP-expressing inhibitory neurons, Tg(GadGFP)45704Swn), in which EGFP expression is under the GAD67 promoter, were purchased from Jackson laboratories (Bar Harbor, Maine, USA) and bred in our animal facility. Twenty-five mice were used for the chronic restraint stress experiment and four animals were used to study the neurochemical phenotype of GAD67-EGFP-expressing neurons in the mPFC. Animals were housed in groups of 5–7 per cage, at a room temperature of 25 °C and on a 12-h light/dark cycle with food and water available ad libitum, and were assigned randomly to control or stress group. All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València.

Chronic restraint stress procedure

Fourteen mice were subjected to chronic restraint stress similar to a published procedure (Patel et al. 2004), but with some modifications as described before (Gilabert-Juan et al. 2011). Briefly, mice were immobilized for 1 h per day for 21 days (from 11 to 12 am) in transparent 50-ml plastic conical tubes with many air holes to allow ventilation. Animals inside the tube were placed on the bench top, close to a sonicator bath. Control animals ($n = 11$) were handled daily, but were left undisturbed in their cages after less than 1 min. Mice were euthanized 24 h after the last stress session in a random order, in a different room than the one in which restraints were carried out.

Histological procedures

Fresh tissue extraction and dissection of mPFC

Mice used for gene expression analysis (5 control and 7 stressed mice) were killed by decapitation using a guillotine. Brains were immediately removed from the skull and placed on Petri dishes filled with cold sterile phosphate buffer (PB). Under a stereo microscope (SZX7; Olympus), the overlying pia was removed and coronal cuts were made to remove portions of the rostral and caudal poles.

The mPFC from the left and right cortices were dissected with a microscalpel in sterile conditions and then stored on separated microcentrifuge tubes. Tissue was frozen in liquid nitrogen and kept at −80 °C until used. The whole procedure was performed at cold temperature and under RNAse-free conditions to prevent RNA degradation.

Perfusion and microtomy techniques

Thirteen mice from the chronic restrain stress experiment (six control and seven stressed mice) and four mice for the neurochemical phenotype study were perfused transcardially under deep chloral hydrate anesthesia, first for 1 min with NaCl 0.9 % and then for 30 min with 4 % paraformaldehyde in PB 0.1 M, pH 7.4. Thirty minutes after perfusion, brains were extracted from the skull and their hemispheres were separated.

One hemisphere was cryoprotected with 30 % sucrose in cold PB 0.1 M (4 °C) for 48 h and then cut in 50-µm thick coronal sections with a freezing-sliding microtome (Leica SM2000R, Leica, Nussloch, Germany). Slices were collected in 10 subseries and stored at −20 °C in a cryoprotective solution until used (30 % glycerol, 30 % ethylene glycol in PB 0.1 M). These sections were destined to immunohistochemical analyses. The other hemisphere was cut into 100 µm sections with a vibratome and the resulting sections were used to analyze dendritic spine density and dendritic arborization on EGFP-expressing interneurons.

Immunohistochemistry for conventional light microscopy

Four subseries (50-µm thick sections) from each animal from the chronic restraint stress experiment were processed "free-floating" for immunohistochemistry using the avidin–biotin-peroxidase complex (ABC) method as follows. Sections were first incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100 °C. After cooling down the sections to room temperature, they were incubated with 3 % H_2O_2 in phosphate buffered saline (PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 10 % normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 0.2 % Triton X-100 (Sigma-Aldrich, St. Louis, MO) and were incubated for 24 or 48 h (Table 1) at 4 °C in primary antibody (anti-PSA-NCAM or anti-GAD67). After washing, sections were incubated for 2 h (room temperature) with the proper biotinylated secondary antibody (anti-mouse IgM, or anti-mouse IgG), followed by ABC (Vector Laboratories, Peterborough, UK) for 1 h in PBS. Color development was achieved by incubating with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and 0.033 % H_2O_2 for 4 min. PBS containing 0.2 % Triton X-100 and 3 % NDS was used for primary and secondary antibodies dilution. Please, see Table 1 for further information about antibodies.

All the studied sections passed through all procedures simultaneously to minimize any difference from immunohistochemical staining itself. To avoid any bias in the analysis, all slides were coded prior to analysis and the codes were not broken until the experiment was finished.

Immunohistochemistry for confocal microscopy

In general, tissue was processed "free-floating" for immunohistochemistry as described above but omitting the endogenous peroxidase block. Sections were incubated for 24 or 48 h at 4 °C with proper primary antibody cocktails (see text below and Table 1). After been washed, sections were incubated at room temperature and light-protected with proper fluorescent secondary antibody cocktails (see Table 1) for 2 h.

In order to amplify the EGFP fluorescent signal in interneurons destined to morphological analysis (dendritic spine density and dendritic arborization), a simple fluorescent immunohistochemistry against EGFP was performed (see Table 1).

In order to characterize neurochemically the somata expressing GAD67-EGFP in the mPFC, we have performed seven different double immunostainings using a tissue from non-treated mice: anti-GFP primary antibody in combination with anti- (1) calbindin (anti-CB), (2) calretinin (anti-CR), (3) parvalbumin (anti-PV), (4) somatostatin (anti-SOM28), (5) neuropeptide Y (anti-NPY), (6) vasointestinal peptide (anti-VIP) or (7) anti-Cholecystokinin (anti-CCK) primary antibodies (see Table 1).

All sections processed for fluorescent immunohistochemistry were mounted on slides and coverslipped using DakoCytomation fluorescent mounting medium (Dako North America Inc., Carpinteria, CA, USA).

Analysis of the neurochemical phenotype of GAD67-EGFP expressing neurons in non-treated animals

Sections double labeled for GFP and interneuronal subpopulation markers [parvalbumin (PV), calbindin (CB), calretinin (CR), somatostatin (SOM), neuropeptide Y (NPY), cholecystokinin (CCK) and vasointestinal peptide (VIP)] or PSA-NCAM were observed under a confocal microscope (Leica TCS-SPE) using a 63× oil objective. Z-series of optical sections (0.5 µm apart) were obtained using sequential scanning mode and stacks were then processed with Zeiss LSM 5 image software. Fifty GAD67-EGFP-expressing neurons within the mPFC were randomly selected from each animal (non-treated mice) and each

Table 1 Primary and secondary antibodies

	Host	Isotype	Dilution	Incubation	Company
Primary antibodies (abbreviated names)					
Anti-CB	Rabbit	IgG	1:2000	O/N, 25 °C	Swant
Anti-CCK	Mouse	IgG	1:500	O/N, 25 °C	CURE
Anti-CR	Rabbit	IgG	1:2000	O/N, 25 °C	Swant
Anti-GAD67	Mouse	IgG	1:500	O/N, 25 °C	DSHB
Anti-GFP	Chicken	IgY	1:1000	O/N, 25 °C	Chemicon-Millipore
Anti-NPY	Rabbit	IgG	1:500	O/N, 25 °C	Provided by Dr. T.J. Görcs
Anti-PSA-NCAM	Mouse	IgM	1:700	36 h, 4 °C	Abcys
Anti-PV	Rabbit	IgG	1:2000	O/N, 25 °C	Swant
Anti-SOM28	Rabbit	IgG	1:500	O/N, 25 °C	Abcam
Anti-VIP	Rabbit	IgG	1:200	O/N, 25 °C	CURE
Secondary antibodies					
Anti-chicken IgY	Donkey	DyLightTM488	1:400	1 h, 25 °C	Jackson ImmunoResearch
Anti-mouse IgM	Donkey	Biotin-SP	1:400	1 h, 25 °C	Jackson ImmunoResearch
Anti-mouse IgG	Donkey	Biotin-SP	1:400	1 h, 25 °C	Jackson ImmunoResearch
Anti-mouse IgM	Donkey	Alexa Fluor® 555	1:400	1 h, 25 °C	Molecular Probes
Anti-mouse IgG	Donkey	Alexa Fluor® 555	1:400	1 h, 25 °C	Molecular Probes
Anti-rabbit IgG	Donkey	Alexa Fluor® 555	1:400	1 h, 25 °C	Molecular Probes

CB calbindin-D28 k, *CCK* cholecystokinin, *CR* calretinin, *GAD67* 67 kDa isoform of the glutamate decarboxilase enzime, *GFP* green fluorescent protein, *NCAM* neural cell adhesion molecules, *NPY* neuropeptide Y, *PSA-NCAM* polysialylated form of the NCAM, *PV* parvalbumin, *SOM28* somatostatin, *SYN* synaptophysin, *VIP* vasointestinal peptide

immunostaining to determine the co-expression of GAD67-EGFP and each marker. Percentages of co-localization were determined for each animal and mean ± SEM were calculated.

Analysis of dendritic arborization and spine density

Dendritic arborization and spine density were studied using confocal microscopy (Leica TCS-SPE) as previously described (Gilabert-Juan et al. 2011, Gomez-Climent et al. 2011). Z-series of optical sections (0.2 µm apart) covering the dendritic tree of selected interneurons were obtained using the sequential scanning mode and a 63× objective. From each animal, six GAD67-GFP expressing neurons were selected from the whole mPFC; no distinction was made to choose the neurons between layers and regions. In order to be analyzed, GFP-expressing cells had to fulfill the following features: (1) the cell must not show any truncated dendrites, (2) the dendritic arbor of the cell must show at least a process with a length greater than 150 µm and (3) the soma must be located at least 30 µm deep from the surface of the tissue. The stacks obtained were then processed using ImageJ software (NIH) to obtain 2D projections, in which the distance of the branching and terminal points of the dendrites of a given interneuron was analyzed. The degree of dendritic arborization was analyzed using a procedure for deriving the Sholl profile (Gutierrez and Davies 2007). The Sholl analysis consists of the measure of the number of intersections of the dendrites with circles of increasing radius centered in the soma (Sholl 1953). Spines were defined as any kind of protrusion found in a dendrite and were quantified in three successive segments of 50 µm distances up to a total length of 150 µm. Overall spine density values or densities per segment were expressed as number of spines/µm. For each experimental group, mean ± SEM was determined and the resulting values were then subjected to unpaired Student's *t* test statistical analysis with the number of animals as the "*n*", using the IBM SPSS statistics software (version 19).

Estimation of the total number of neuronal somata expressing PSA-NCAM, GAD67 or GAD67-EGFP

The number of neuronal somata expressing PSA-NCAM, GAD67 or GAD67-EGFP covering 100 % of the sample area (mPFC) was estimated using a modified version of the fractionator method (West et al. 1991), as described before (Castillo-Gomez et al. 2011; Varea et al. 2007a, b). That is, all labeled cells found in all mPFC within each 50-µm thick section. The fractionator sampling scheme refers to the methodology of examining one out of every six brain sections. One from six systematic-random series of sections covering the whole rostral to caudal extension of mPFC was viewed on an Olympus CX41 microscope for

PSA-NCAM and GAD67 and on an Olympus BX61 fluorescent microscope for GAD67-EGFP cells. Cell somata were identified and counted with a 40× objective. Cells appearing in the upper focal plane were omitted to prevent counting cell caps.

Means were determined for each experimental group and data were then subjected to unpaired Student's *t* test statistical analysis using the IBM SPSS statistics software (version 19).

Identification and quantification of pyknotic nuclei

A set of sections was stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO) in a concentration of 2 µg/ml during 10 min and used for the detection of pyknotic cells in the mPFC. These degenerated cells are characterized by intensely stained condensed chromatin, which often appears in different small spherical pyknotic bodies (Gould et al. 1991). The number of pyknotic nuclei covering 100 % of the sample area (mPFC) was estimated using the same methodology described for the quantification of neuronal somata described above.

Quantitative retrotranscription-polymerase chain reaction

Total mRNA from mPFC was extracted using TriPure reagent (Roche Applied Science, Indianapolis, IN) by means of one-step sample homogenization/lysis procedure. TriPure Isolation Reagent disrupted cells and denatured endogenous nucleases. Then, chloroform was added to the sample and the mixture was centrifuged. This step separates the sample into three phases: a colorless aqueous (upper) phase, a white interphase and a red organic (lower) phase. The upper phase was placed in a separate tube and RNA was recovered from it by isopropanol precipitation. Finally, RNA was isolated by alcohol precipitation steps. Purified total RNA was eluted in RNase-free water and stored at −80 °C. RNA concentration and purity was measured in a spectrophotometer at 260 nm and 260/280 nm, respectively (Eppendorf BioPhotometer plus; Eppendorf AG, Hamburg, Germany).

Reverse transcription (RT) reactions were performed as follows: 2 µl oligo dTplus (10 pmol) were hybridized to 1 µl of total RNA (100 ng/µl) in 10.5 µl volume by heating up to 65 °C for 10 min. First strand cDNA was then synthesized by incubating the hybridized RNA at 43 °C for 60 min with dGTP, dTTP, dCTP, dATP (1 mM each),1 µl expand reverse transcriptase (50 U/µl), 0,5 µl Protector RNAse inhibitor (40 U/µl), 2 µl 1,4-dithio-DL-threitol (DTT, 100 mM) in 20 µl Buffer for Expand reverse transcriptase. All products were purchased from Roche Applied Science (Indianapolis). cDNA reactions were then diluted fivefold in nuclease-free water. The quality of cDNA was checked by agarose gel electrophoresis after PCR amplification. Only the former small amplicon but not the latter larger amplicon was detected in all samples on the agarose gel electrophoresis, demonstrating no contamination of samples with genomic DNA.

For quantitative retrotranscription-polymerase chain reaction (qRT-PCR) analyses, each sample was run in triplicates. qPCR was carried out with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems), specific primers for all genes (Table 2) at a concentration of 240 nm, and 4 µl cDNA (50 ng) of each sample. These genes included those related to inhibitory neurotransmission, *GAD67,* Glutamic acid decarboxylase 2 (*GAD65*), *GABA* vesicular transporter (*GAT1*), Reelin (*RELN*), Cannabinoid receptor 1 (*CB1*), *N*-methyl-D-aspartic acid receptor 1 (*NMDAR1*), GABAA receptors subunits, Synaptophysin (*SYN*) and those of the polysialyltransferases *ST8SiaII* and *ST8SiaIV* and *NCAM*. In order to evaluate the presence of stress-induced apoptosis in the mPFC we also analyzed the expression of the apoptosis related genes, *BCL2*-associated X protein (*Bax*) and B cell leukemia/lymphoma 2 (*Bcl2*). *TATA* box-binding protein gene (*TBP*) was used as a reference gene. Following a 95 °C denaturation for 10 min, the reactions were cycled 40 times with a 95 °C denaturation for 15 s, and a 60 °C annealing step for 1 min. After that, a melt curve was performed to assess the specificity of primers. Primers were designed by Primer-Blast free software, between exons to avoid genomic DNA amplification, using Ensembl data sequences. All DNA oligonucleotide primers were custom synthesized by Metabion international AG (Martinsried, Germany).

Relative quantification was performed using the comparative threshold (CT) method according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001), where, $\Delta\Delta CT$ = (CT, target gene − CT, reference gene) exp. group − (CT, target gene − CT, reference gene) control group. Changes in gene expression were reported as fold changes relative to controls. Unpaired Student's *t* test was performed to analyze the statistical significance of results.

Results

Characterization of GAD67-EGFP expressing neurons in the mPFC

In the mPFC of GIN mice, most GAD67-EGFP expressing neurons were located in layers II, III and upper V, similar to what has been found in a previous study of the somatosensory cortex of this transgenic mice strain (Oliva et al. 2000; Ma et al. 2006). Most of these

Table 2 Sequences of gene specific primers and associated amplicon lengths for qRT-PCR

Target gene	Primers	Sequence (5′ → 3′)	Amplicon size[a]
ST8SiaII	Forward	GGCTGTGGCCAGGAGATTG	72
	Reverse	GGCATACTCCTGAACTGGAGCC	
ST8SiaIV	Forward	GCACCAAGAGACGCAACTCATC	68
	Reverse	CAGAGCTGTTGACAAGTGATCTGC	
NCAM	Forward	GGATGCCTCCATCCACCTC	67
	Reverse	GGCCGTCTGATTCTCTACATAGG	
GAD67	Forward	GGGTTCCAGATAGCCCTGAGCGA	120
	Reverse	TGGCCTTGTCCCCTTGAGGCT	
GAD65	Forward	AGCCTCAACACACAAATGTCTGCTTCT	135
	Reverse	TGGTCCCATACTCCATCATTCTGGCT	
SYN	Forward	TCTTTGTCACCGTGGCTGTGTT	268
	Reverse	TCCCTCAGTTCCTTGCATGTGT	
GAT1	Forward	TCTGCCCGCCTTGGCTCTGA	134
	Reverse	TGGGGGTGGGTCCTGGAAGC	
RELN	Forward	CGGAAGGAAGGCGTGCTGCT	125
	Reverse	GCCCCCTCAGGCAGGAGGAT	
CB1	Forward	TGTCCCTCACCCTGGGCACC	134
	Reverse	TCCCAGGAGATCGGCCACCG	
NMDAR1	Forward	GAGGCCATCCAGGCTGTGCG	133
	Reverse	TGCCAAAGCCGGAGCGGAAG	
GABAAα1	Forward	GCCATGGACTGGTTTATTGC	99
	Reverse	CCACGCATACCCTCTCTTGGTG	
GABAAα2	Forward	AAGAGGATGGGCTTGGGACGGG	100
	Reverse	GGCAACAGCTACCGCATAGGCG	
GABAAα3	Forward	AACAGCCTCAGCCACTTTGGATCTG	122
	Reverse	AGCCTTGCTCAGTGAGTGGGC	
GABAAα4	Forward	CTGGGCCCTGGAGAGCCTAACA	120
	Reverse	AAGCAGACAAAAGGCCTGTGCAGA	
GABAAγ2	Forward	TTGGATGGCAAGGACTGTGCCAG	131
	Reverse	GCGGTAGGGAAGAAGATCCGAGCAT	
BAX	Forward	AAACTGGTGCTCAAGGCCCT	92
	Reverse	AGCAGCCGCTCACGGAG	
BCL2	Forward	CCGGGAGAACAGGGTATGATAA	81
	Reverse	CCCACTCGTAGCCCCTCTG	
TATABP	Forward	CACTTCGTGCAAGAAATGCTG	89
	Reverse	AATCAACGCAGTTGTCCGTG	

[a] Amplicon length in base pairs

neurons had multipolar or bipolar morphology and all of them displayed dendritic spines. After careful observation of these EGFP expressing interneurons in the mPFC, we have found that none of them displayed degenerative symptoms such as swollen dendrites or axons or the presence of abnormal nuclei. In order to study to which subpopulation of interneurons pertained the GAD67-EGFP expressing neurons in the mPFC, we performed double immunostainings against EGFP and different calcium binding proteins or neuropeptides. Regarding the expression of calcium binding proteins, GAD67-EGFP expressing neurons in the mPFC mainly co-expressed CR (64.8 ± 7.1 %; Fig. 1a1–a3) and less frequently CB (16.8 ± 1.1 %; Fig. 1b1–b3), but none of them was found to co-express PV (0 ± 0 %; Fig. 1c1–c3). Regarding the expression of neuropeptides, a high percentage of GAD67-EGFP expressing neurons co-expressed SOM (75.0 ± 0.5 %; Fig. 1d1–d3), but no co-localization was found when studying all the other neuropeptides (CCK, NPY, VIP; Fig. 1e–g).

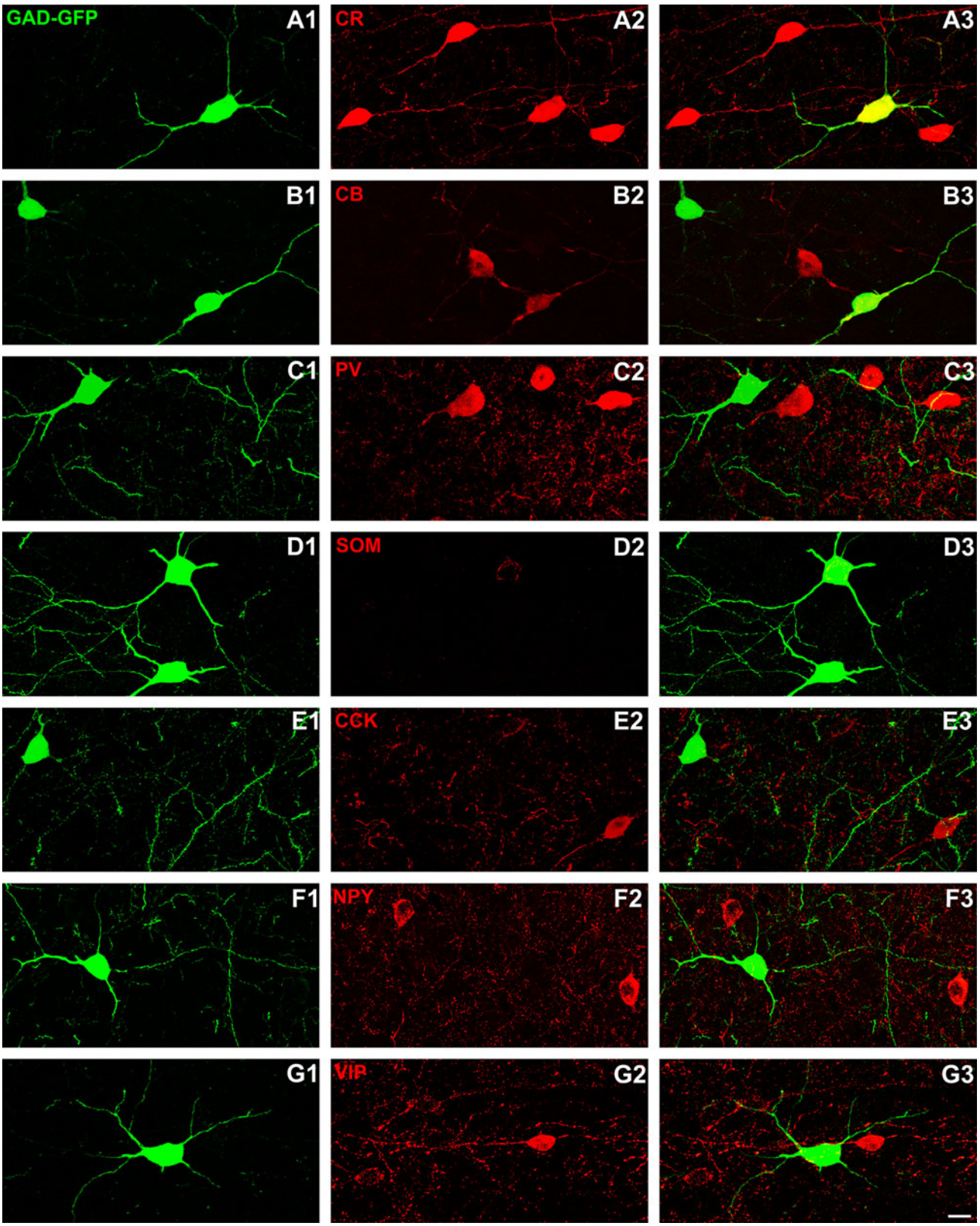

Fig. 1 Confocal microscopic analysis of the phenotype of GAD67-EGFP expressing cells in the mPFC. GAD67-EGFP expressing interneurons coexpressing calretinin (**a**), calbindin (**b**) or somatostatin (**d**). GAD67-EGFP expressing interneurons lacking parvalbumin (**c**), cholecystokinin (**e**), neuropeptide Y (**f**) or vasointestinal peptide (**g**) expression. *Scale bar* 10 µm. Confocal images are 2D projections of eight consecutive confocal planes located 1 µm apart

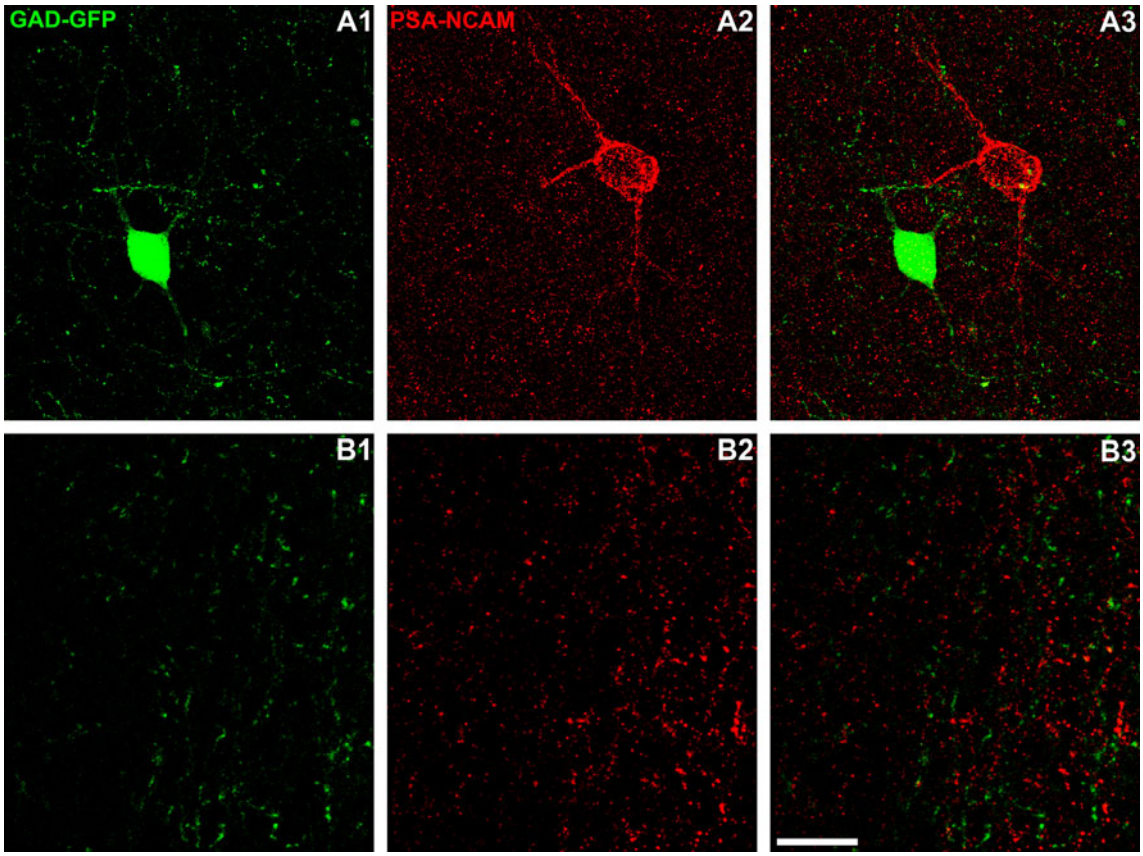

Fig. 2 Somata expressing PSA-NCAM do not show colocalization with GAD67-EGFP expressing somata (**a**). PSA-NCAM expressing puncta in mPFC layer II do not show GAD67-EGFP expression (**b**). *Scale bar* 5 μm for **a** and 10 μm for **b**. Confocal images are 2D projections of eight consecutive confocal planes located 1 μm apart (**a**) and four consecutive confocal planes located 0.5 μm apart

In order to know whether the GAD67-EGFP expressing interneurons in the mPFC expressed PSA-NCAM in their soma or their neurites we performed double PSA-NCAM/EGFP immunohistochemistry. None of these cells showed PSA-NCAM immunoreactivity, neither in their somata nor in their dendritic or axonal processes. Moreover, analysis of the projection field of these neurons in layers I and II also revealed lack of PSA-NCAM expression in the GAD67-EGFP expressing puncta (Fig. 2).

Chronic stress increases dendritic arborization but does not change spine density in GAD67-EGFP expressing interneurons in the mPFC

Sholl analysis revealed increased dendritic arborization in GAD67-EGFP expressing neurons in the mPFC of stressed mice (Fig. 3a, b). These differences were statistically significant in five of the 20-μm length segments of distance from the soma that were analyzed (Fig. 3b): 60–80 μm segment ($t_{11} = -2.33$, $p = 0.04$), 80–100 μm segment ($t_{11} = -3.11$, $p = 0.011$), 100–120 μm segment ($t_{11} = -3.24$, $p = 0.0088$), 120–140 μm segment ($t_{11} = -2.44$, $p = 0.035$), 140–160 μm segment ($t_{11} = -3.24$; $p = 0.0089$).

For the spine density study, selected dendrites were divided into three segments of 50-μm length of distance from the soma, and the number of total spines was counted (insets in Fig. 3a). We did not find statistically significant differences in dendritic spine density between control and stressed groups neither in any of the three 50-μm length segments ($t_{11} = -0.74$; 1.05; 0.67, $p = 0.47$; 0.31; 0.52 each segment, respectively; Fig. 3c), or when the whole dendrite length (150 μm) was taken into account ($t_{11} = 0.75$, $p = 0.46$).

Chronic stress reduces the number of GAD67 and GAD67-EGFP expressing neurons in the mPFC but not the number of those expressing PSA-NCAM

In order to compare the number of neuronal somata expressing PSA-NCAM, GAD67 or GAD67-EGFP in the mPFC of control versus stressed mice, somata expressing these molecules were quantified in all regions and layers of the mPFC. Results indicate that GAD67 and GAD67-EGFP

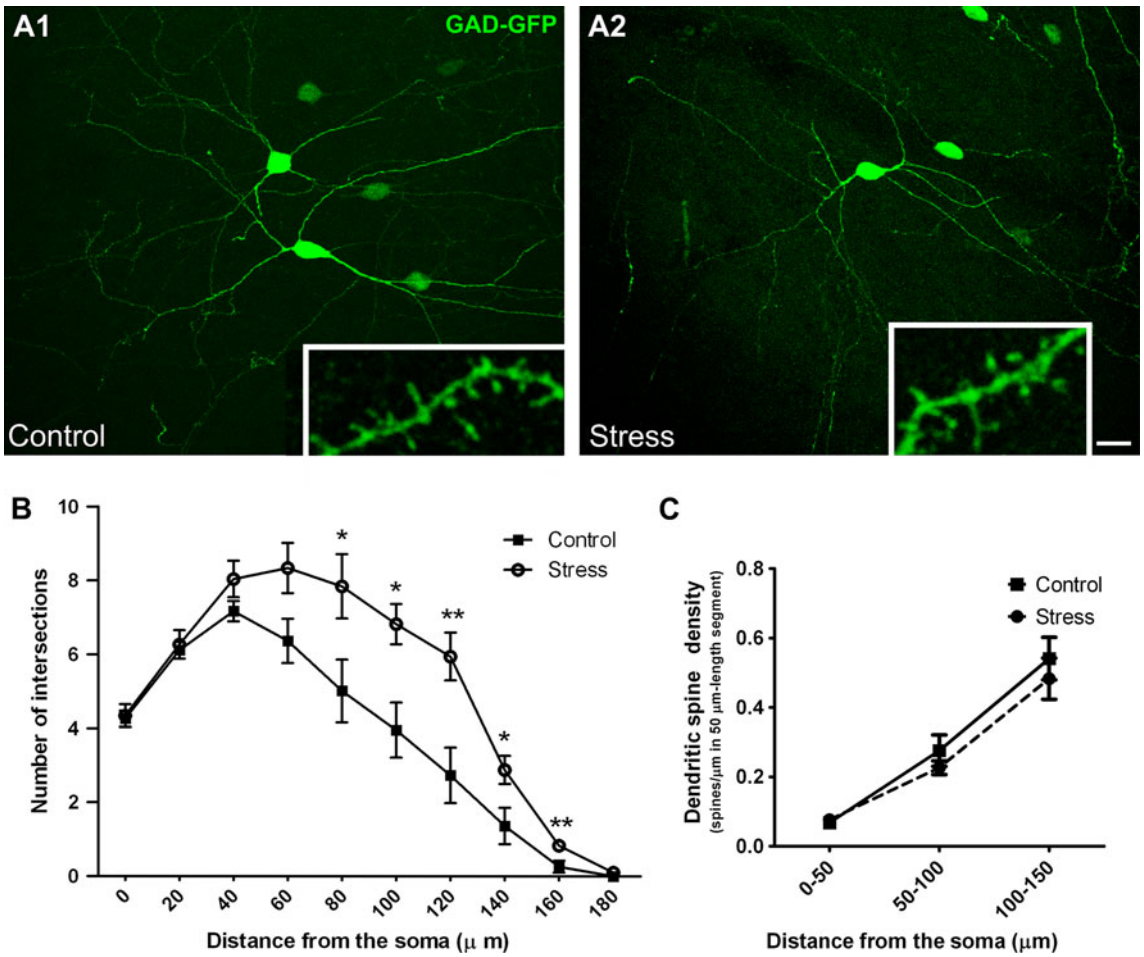

Fig. 3 **a** Sholl analysis of GAD67-EGFP expressing interneurons, showing the number of intersections per 20 μm dendritic radial unit distance from the soma. *Insets* in A1 and A2 show high magnification views of dendritic spines of GAD67-EGFP expressing interneurons. **b** 2D reconstructions of GFP-expressing interneurons in the mPFC of control (**b1**) and chronically stressed animals (**b2**). **e**–**g** Confocal microscopic analysis of dendritic spine number in GAD67-EGFP expressing interneurons from the mPFC. Histograms of the differences in the total density of dendritic spines (**e**) and the dendritic spine density in segments at different distances from the soma (**f**). Spines were counted in three 50-μm length segments located 0–50, 50–100 and 100–150 μm from the interneuron soma, respectively. Unpaired Student *t* test showed no statistically significant differences in any of the segments analyzed. **g** Compositions, using fragments of different confocal planes, of spinous dendrites of GAD67-EGFP expressing interneurons in the mPFC of control (**g1**) and stressed animals (**g2**). *Scale bar* 10 μm for **a**, **b,d**; 2 μm for *insets* in **a**; 5 μm for **g**. Confocal images are 2D projections of eight (**a**, **b**) and 20 (**d**), consecutive confocal planes located 1 μm apart; images in **g** are 2D projections of 25 confocal planes located 0.2 μm apart

expressing somata were significantly reduced in the mPFC of the stressed individuals ($t_{11} = 3.17$, $p = 0.0089$; $t_{11} = 2.265$, $p = 0.045$, respectively). The number of PSA-NCAM expressing somata in the mPFC of stressed mice did not differ significantly from those of the control mice ($t_{11} = -0.54$, $p = 0.6$; Fig. 4).

Analysis of pyknotic nuclei and the *Bax*/*Bcl2* ratio indicates the absence of apoptosis in the mPFC after chronic stress

The observation of DAPI stained sections revealed a complete absence of pyknotic nuclei in all the regions of the mPFC. In accordance with this result, we also failed to detect changes in the *Bax*/*Bcl2* genes ratio, which represents a critical balance of regulatory pro-apoptotic and anti-apoptotic proteins in normal living cells: The increase in *Bax*/*Bcl2* ratio leads to the release of Cytochrome *c* from the mitochondria, a decisive event in the apoptotic pathway. As measured by qRT-PCR, the fold-change in stressed mice with respect to control of *Bax* and *Bcl2* gene expression showed no significant changes with values of 1.3 and 1.4, respectively (Table 3). A non-significant fold-change of 0.92 ($t_{11} = 1.11$, $p = 0.29$) was achieved for the *Bax*/*Bcl2* ratio when stress mice were compared to control.

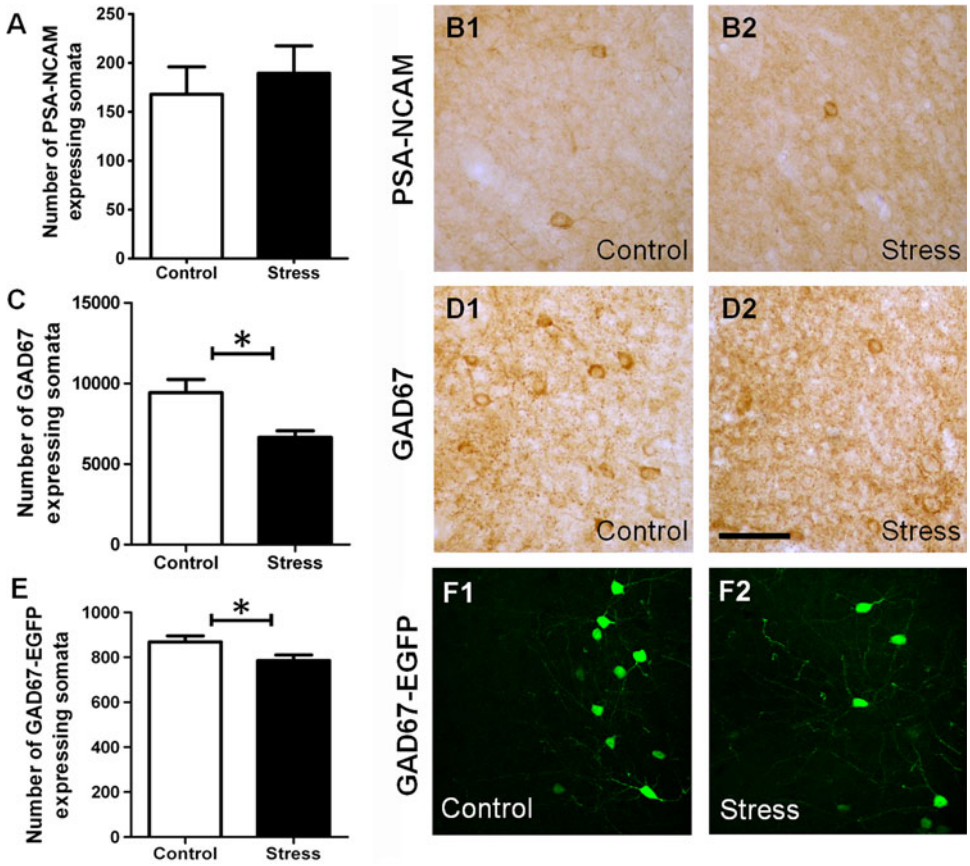

Fig. 4 Somata expressing PSA-NCAM, GAD67 and GAD67-EGFP in the mPFC. Histograms showing the differences in the total number of cells expressing PSA-NCAM (**a**), GAD67 (**c**) or GAD67-EGFP (**e**) in their somata. *Asterisks* indicate statistically significant differences (*$p < 0.05$) and values represent mean ± standard error of the mean. PSA-NCAM expressing somata in control (**b1**) and stressed (**b2**) individuals, GAD67 expressing somata in control (**d1**) and stressed (**d2**) individuals and GAD67-EGFP expressing somata in control (**f1**) and stressed individuals (**f2**). *Scale bar* 50 μm. Confocal images are 2D projections of 30 consecutive confocal planes located 0.2 μm apart

Table 3 qRT-PCR results for tested genes in stress mice vs. control

	Medial prefrontal cortex	
	Δ stress/control	*p* value
ST8SiaII	0.75	0.32
ST8SiaIV	1.02	0.68
NCAM	**1.19**	**0.039**
GAD67	1.55	0.13
GAD65	1.57	0.1
Synaptophysin	**1.92**	**0.027**
GAT1	1.5	0.19
RELN	1.27	0.62
CB1	1.39	0.47
NMDAR1	1.35	0.82
GABAAα1	**2.03**	**0.04**
GABAAα2	1.87	0.24
GABAAα3	2.01	0.14
GABAAα4	1.81	0.25
GABAAγ2	1.56	0.26
BAX	1.3	0.32
BCL2	1.4	0.25

Δ, change in gene relative to normalize

Information in bold represents significant changes

NCAM, *SYN* and *GABAAα1* gene expression in the mPFC is increased after chronic stress

In order to know whether chronic stress induces changes in the expression level of several genes related to neuronal plasticity and inhibitory neurotransmission, we performed qRT-PCR of the encoding transcripts (see Table 2) in the whole mPFC, using as a control the *TBP* gene. qRT-PCR analysis revealed a significant mild increase in mRNA expression (fold change = 1.19; $t_{10} = -2.36$, $p = 0.039$) of *NCAM* but no changes were observed in the expression of the polysialyltransferase genes (*ST8SiaII* and *ST8SiaIV*). In the same direction, the mRNA of the synaptic protein synaptophysin was significantly increased in the mPFC of the stressed mice showing an increase of 1.92 fold change when compared to control group ($t_{10} = -2.53$, $p = 0.027$). The third overexpressed gene was the *GABAAα1* receptor (*GABAAα1*), with a 2.03 fold increase when compared to non stressed individuals ($t_{10} = -2.34$, $p = 0.04$). No significant changes were detected neither in the other subunits of this receptor (α2, α3, α4 and γ2) nor in the genes of the glutamic acid decarboxylase (*GAD65* and *GAD67*) or the *GABA* vesicular transporter (*GAT1*). The rest of the molecules related to inhibitory neurotransmission, reelin

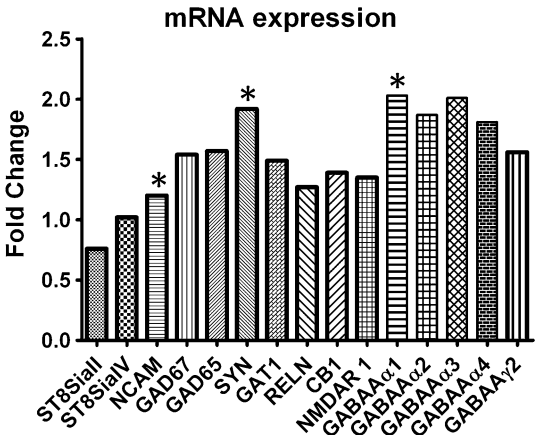

Fig. 5 qRT-PCR mRNA fold change in gene expression showed as stressed mice group versus control. All genes expression was normalize using TATA binding protein as a housekeeping gene

(*RELN*), cannabinoid receptor 1 (*CB1*) and *N*-methyl-D-aspartic acid receptor 1 (*NMDAR1*), did not show any statistical change (Fig. 5; Table 3).

Discussion

The present results show for the first time that interneurons in the mPFC of adult mice undergo dendritic remodeling after chronic stress. This remodeling is accompanied by significant changes in the number of neurons expressing GAD67 and in the expression of different molecules related to inhibitory neurotransmission and neuronal plasticity. Apparently, the decrease in GAD67 expressing neurons is not due to apoptosis but due to changes in the expression of this GABA synthesizing enzyme. Therefore, our study supports the idea that inhibitory networks in the mPFC are also targets of chronic stress and that their alteration may also contribute to the behavioral and cognitive impairments induced by this aversive experience.

Chronic stress induces dendritic hypertrophy in a subpopulation of prefrontocortical interneurons

We have observed a significant increase in the dendritic arborization of mPFC interneurons and this is, to our knowledge, the first report describing dendritic remodeling in inhibitory neurons after chronic stress in this cortical region. A chronic stress paradigm similar to that used in our study induces dendritic atrophy and reductions in spine density in pyramidal neurons of rats (Radley et al. 2004). However, we have not found differences in spine density in the subpopulation of interneurons studied in the present report. Similar results have been found by our group when studying the effects of chronic stress on spine density in the amygdala of GIN mice (Gilabert-Juan et al. 2011).

The atrophy of pyramidal neurons has been interpreted as a structural weakening of excitatory neurotransmission in the mPFC, which may represent an adaptive cellular substrate for responding to the increase in excitatory neurotransmission elicited during the first phases of stress (Lowy et al. 1995). It is tempting to interpret the hypertrophy of the dendrites of mPFC interneurons as another attempt of mPFC circuitry to minimize this overexcitation. An increased dendritic surface may favor the formation of synaptic contacts on mPFC interneurons. However, we do not know yet whether new synapses are established on this expanded dendritic surface and, if so, whether they are excitatory or inhibitory. Solving these questions is essential to understand what is the role of the stress-induced interneuronal remodeling. It is also very important to determine the sequence of the events that lead to the scenario that we observe after 21 days of stress: Is the interneuronal hypertrophy subsequent to the decrease in inhibitory neurotransmission suggested by the reduction in the number of GAD67 and GAD67-GFP expressing somata or viceversa? Are these two independent phenomena? Do changes in interneuron structure in the mPFC occur before, simultaneously or after the changes described in pyramidal neuron structure? Although, obviously, further experiments analyzing different time points along the chronic stress are necessary to elucidate these questions, it is interesting to note that a recent study by Keck et al. (2011) suggests that structural changes in inhibitory neurons may precede structural changes in excitatory circuitry in the visual cortex following sensory deprivation.

The effects of chronic stress in the mPFC interneurons are opposite to those found in the basolateral amygdala (Gilabert-Juan et al. 2011), in fact this aversive experience also induces opposite effects on the structure of principal neurons in these two regions (Radley et al. 2004; Vyas et al. 2002). The dendritic growth of principal neurons and the atrophy of interneurons in the basolateral amygdala have been interpreted as a structural strengthening of excitatory neurotransmission, which may represent a cellular substrate for enhanced anxiety (Gilabert-Juan et al. 2011; Roozendaal et al. 2009).

Our analysis of the phenotype of EGFP-expressing neurons in the mPFC of GIN mice reveals that they belong exclusively to those expressing somatostatin. According to a recent report (Xu et al. 2010), the interneurons analyzed structurally in our study cannot be chandelier or basket cells, which always express parvalbumin. Since most of the neurons in the mPFC of GIN mice are located in layers II, III and upper V, and their axons arborize profusely in superficial layers, we are confident that most of them have to be Martinotti cells, as it has previously suggested in the somatosensory cortex of this strain of transgenic mice (Ma et al. 2006). Martinotti cells can also express calbindin and

calretinin in addition to somatostatin and they never express NPY or parvalbumin (Xu et al. 2010). Martinotti cells are interneurons whose axons mainly target the apical dendritic tree of pyramidal neurons (Markram et al. 2004) and, interestingly, this apical region is the one that shows the dendritic retraction after chronic stress (Radley et al. 2004). Consequently, the dendritic hypertrophy that we observe in these interneurons may be related to the shrinkage of the apical region of pyramidal neurons. However, studies directed to evaluate structural remodeling in the axonal projection of EGFP-expressing interneurons in superficial layers are needed to understand their relationship to the dendritic atrophy of principal neurons.

Chronic stress alters the expression of molecules related to synapses and inhibitory neurotransmission and the number of GAD67 expressing cells in the medial prefrontal cortex

The changes we have observed in the structure of mPFC interneurons, together with those described before in the structure of pyramidal neurons, should be reflected in alterations in the number of synapses or their reorganization. Although a previous report has failed to find changes in the levels of synaptophysin, a protein linked to synaptic remodeling (Greengard et al. 1993) and considered a reliable index of synaptic density (Masliah et al. 1990), in the PFC after chronic stress (Carvalho-Netto et al. 2011), we have found significant increased expression of the *SYN* gene in the total mPFC. This difference may be due to the fact that Carvalho-Netto et al. (2011) studied the whole PFC while we only focused in its medial region. It has to be noted that synaptophysin is expressed both in excitatory and inhibitory synapses and, consequently, detailed studies on the synaptic input of pyramidal and inhibitory neurons must be performed to shed light on this intricate matter.

Our present results showing a decrease in the number of GAD67 and GAD67-EGFP expressing somata strongly suggest a downregulation in the expression of this GABA synthesizing enzyme. A previous report has described increases of GAD65 or GAD67 mRNAs in the hippocampus after chronic immobilization stress (Bowers et al. 1998), but we have not found differences in the expression of these mRNAs in the present study. It is possible that changes in mRNA expression occur before 21 days, since the study of Bowers et al., used a 15 days paradigm. On the other hand, previous studies found decreased GABA levels measured with HPLC in the PFC after 3 weeks of chronic mild stress (Shalaby and Kamal 2009) and a decrease in the number of parvalbumin cells in the hippocampus of tree shrews (Czeh et al. 2005). Another possible explanation for the decrease in GAD67 expressing interneurons found in our study may be cell death. In fact, a previous study found that the number of apoptotic cells was increased in the cerebral cortex and the hilus of adult tree shrews after chronic psychosocial stress (Lucassen et al. 2001) and this has been suggested as an explanation for the loss of hippocampal parvalbumin expressing cells (Czeh et al. 2005). However, we find this possibility unlikely, unless it occurred sooner during the stress procedure, since we have not found evidences of apoptosis or of degenerated interneurons in our material.

The analysis of the molecules related to inhibitory neurotransmission by qRT-PCR has only found a significant increase in the expression of the *GABAAα1* receptor. The function of this receptor appears to be necessary to mediate the effects of chronic stress in the structural remodeling of principal neurons, at least in the hippocampus, because treatment with specific agonists prevents dendritic atrophy in CA3 pyramidal neurons (Magarinos et al. 1999). It may be possible that the increase in GABAA receptor expression constitutes an adaptive response directed to augment the function of these receptors and to counteract deleterious effects of stress on mPFC circuitry. This response may also counteract the decreased binding to GABAA receptors described after chronic stress (Gruen et al. 1995).

Although previous reports have described changes in PSA-NCAM expression after chronic stress in the hippocampus and the amygdala (Cordero et al. 2005; Pham et al. 2003; Sandi et al. 2001), the present results suggest that this molecule is not directly implicated in the structural changes we have described in the mPFC. First, the GAD67-EGFP expressing interneurons in which the structural features have been analyzed (both in control and in chronically stressed mice) do not show PSA-NCAM expression in their somata, neurites or in the puncta located in their projection fields in layers I and II. Second, no changes in the number of PSA-NCAM expressing cells or in the expression of the mRNA of polysialyltransferases have been observed after chronic stress. It is, however, possible that changes in PSA-NCAM expression in the mPFC occur before 21 days of chronic stress in interneuronal populations different from the one studied in the present report.

Our study has found an increase in the expression of *NCAM* mRNA in the mPFC after chronic stress. A similar study did not find changes in this parameter in the PFC using in situ hybridization, although it described a reduction in the hippocampus (Venero et al. 2002), a region where decreases in NCAM protein expression have also been reported after chronic stress (Sandi et al. 2001). However, it has to be taken into account that these measures were obtained in the whole PFC and not only in the mPFC. The increase in NCAM expression after chronic stress may lead to increased cell adhesion and can influence

the different intracellular signaling cascades mediated by this protein (Maness and Schachner 2007). This increase in NCAM expression may have a neuroprotective role against the effects of stress, since reduced levels of this protein have been found to increase the vulnerability to behavioral alterations induced by this aversive experience: NCAM heterozygous mice (Jurgenson et al. 2012) and conditional NCAM-CAMKII mice (Bisaz and Sandi 2012) display increased immobility in the tail suspension test.

Implications of mPFC plasticity in anxiety and mood disorders

Animal models involving chronic stress produce brain changes that are relevant to human psychiatric conditions such as anxiety and depression (McEwen 2000) and there is a clear link between prefrontal cortex dysfunction and mood disorders such as major depression (Brody et al. 2001) or posttraumatic stress disorder (Bremner 2005). Consequently, the present results may increase our understanding of the molecular and structural plasticity associated to the development of anxiety and mood disorders, especially those involving prefrontocortical inhibitory circuits. In fact, several lines of evidence indicate the involvement of the GABAergic system in the pathophysiology of major depression (Krystal et al. 2002; Sanacora et al. 1999). Neuroimaging studies have reported reductions in GABA levels in the prefrontal cortex (Hasler et al. 2007; Sanacora et al. 1999), reduced GABA concentrations were also demonstrated in the plasma and cerebrospinal fluid in depression (Brambilla et al. 2003) and GAD-67 protein expression was significantly reduced in depressed subjects (Karolewicz et al. 2010). Moreover, similar to what we have found in our chronically stressed mice, post-mortem morphometric analyses in major depression patients have found reductions in the density and size of GABAergic interneurons immunoreactive for calbindin (Rajkowska et al. 2007) and calretinin (Oh et al. 2012) in the PFC.

Acknowledgments Spanish Ministry of Science and Innovation (MICINN-FEDER) BFU2009-12284/BFI, MICINN-PIM2010ERN-00577/NEUCONNECT in the frame of "ERA-NET NEURON", Generalitat Valenciana ACOMP/2012/229 to JN. Javier Gilabert-Juan has a FPU predoctoral fellowship from the Spanish Ministry of Education and Science (AP2008-00937).

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Article 3: Post-weaning social isolation rearing influences the expression of molecules related to inhibitory neurotransmission and structural plasticity in the amygdala of adult rats

BRAIN RESEARCH 1448 (2012) 129–136

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Research Report

Post-weaning social isolation rearing influences the expression of molecules related to inhibitory neurotransmission and structural plasticity in the amygdala of adult rats

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ARTICLE INFO

Article history:
Accepted 28 January 2012
Available online 4 February 2012

Keywords:
Schizophrenia
Animal model
Inhibitory neurotransmission
Interneuron

ABSTRACT

Several lines of evidence indicate that alterations in the structure of neural circuits and inhibitory neurotransmission underlie the physiopathogenesis of schizophrenia. Most of the studies on these parameters have been focused on cortical regions and, despite the crucial role of the amygdala in this psychiatric disorder, there is less information on this region. In order to expand this knowledge, we have studied the expression of molecules related to inhibitory neurotransmission and structural plasticity in rats subjected to post-weaning isolation rearing, an animal model that reproduces several core symptoms of schizophrenia. We have analyzed, using qRT-PCR and immunohistochemistry, the expression of synaptophysin, GAD65, GAD67, the neural cell adhesion molecule (NCAM), its polysialylated form (PSA-NCAM) and its synthesizing enzymes (St8siaII and St8SiaIV). Isolation-reared rats showed significant increases in the expression of GAD67 protein in the centromedial, medial and basolateral amygdaloid nuclei, but no significant changes in GAD65 or synaptophysin expression were found in these regions. The expression of PSA-NCAM and NCAM was significantly increased in the basolateral and medial nuclei respectively. Our results indicate that isolation-rearing influences positively inhibitory neurotransmission and neuronal structural plasticity in the amygdala, probably through PSA-NCAM. These findings are in contrast to reports describing decreased expression of molecules related to inhibitory neurotransmission in the amygdala of schizophrenic patients. Consequently, although the social isolation rearing model can reproduce some of the behavioral traits of schizophrenics it may fail to reproduce some of the neurobiological features of this disorder, particularly in the amygdala.

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1. Introduction

Schizophrenia is a complex disease, affecting approximately 1% of the population worldwide. The heritability of this disease is around 80% (Owen, 2005), highlighting the importance of altered genetic pathways in their development. Current pathophysiological theories of schizophrenia are pointing to the GABAergic system as responsible for some of the alterations in

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doi:10.1016/j.brainres.2012.01.073

schizophrenic brains, because of its implication in the regulation of other neurotransmitter systems, such as dopaminergic or serotoninergic, and its tight relationship with the excitatory transmission (Benes and Berretta, 2001). GABA is the principal inhibitory neurotransmitter in the central nervous system (CNS) and genes implicated in its metabolism have been associated with schizophrenia in different human populations (Straub et al., 2007; Zai et al., 2009) and in animal models of this psychiatric disorder (Peleg-Raibstein et al., 2008). Almost all of these studies have found important alterations in the isoenzymes responsible for GABA synthesis (GAD65 or GAD67) and in their coding genes, showing that alterations in the synthesis of GABA may be one of the crucial facts in schizophrenia.

In addition to these changes in inhibitory neurotransmission, structural changes have also been found in the brains of schizophrenic patients, as well as in animal models of this disorder (Phillips et al., 2003). This structural remodeling involves changes in synaptic density, which are frequently estimated analyzing the expression of synaptophysin, a synaptic vesicle membrane protein, whose expression is linked to synaptic remodeling (Eastwood and Harrison, 2001; Greengard et al., 1993). One of the mechanisms involved in this structural plasticity is the addition of polysialic (PSA) acid to the Neural Cell Adhesion Molecule (NCAM): This process, mediated by the two polysialyltransferases (St8SiaII and St8SiaIV), facilitates the formation of new synapses, the remodeling of neurites (see Bonfanti, 2006; Rutishauser, 2008 for review), or the partial isolation of neuronal elements (Gomez-Climent et al., 2011), because, when polysialylated, NCAM becomes anti-adhesive. Both *NCAM* and *ST8SIAII* genes have been associated with schizophrenia and alterations in the expression of NCAM and PSA-NCAM have been found in postmortem brain studies of this disorder (Brennaman and Maness, 2010; Sullivan et al., 2007; Tao et al., 2007).

Most of these data on the expression of molecules related to inhibitory neurotransmission or structural plasticity have been obtained from the prefrontal cortex, the nucleus accumbens and the hippocampus, regions specially affected in schizophrenia. However, despite the fact that the amygdala is also severely affected in schizophrenic patients, this knowledge is still scarcer in this region. The amygdaloid complex plays a critical role in the recognition and the response to emotional stimuli, including fear and anxiety (Adolphs et al., 1995; Cahill and McGaugh, 1998; Davis et al., 1994; LeDoux, 2000), which are frequently abnormal in schizophrenic patients. Recent brain imaging studies have shown that abnormalities in limbic lobe regions including the amygdala may be responsible of the inadequate affective responses in schizophrenics (Phillips et al., 2003). Several studies have reported changes in the volume of the amygdala in psychiatric patients, which probably reflect structural changes in amygdaloid neurons (reviewed in Drevets et al., 2008; Sheline et al., 1998; Tebartz van Elst et al., 2000). In light of these set of evidence pointing to the amygdala, we have decided to study the expression of molecules related to structural plasticity and inhibitory neurotransmission using an animal model, which reproduces some of the core symptoms of schizophrenia.

The post-weaning social isolation-paradigm offers a well established and characterized animal model to study schizophrenic symptoms in a rodent. Some of the behavioral and neurochemical changes induced by the isolation-rearing are disrupted prepulse inhibition (Geyer et al., 1993), reduced expression of AMPA glutamate receptors in hippocampus (Sestito et al., 2011), impaired spatial cognition with affected prefrontal cortical synaptic plasticity (Quan et al., 2010), impaired novel object recognition (McLean et al., 2010), increased aggression (Ferdman et al., 2007) and reduced prefrontal cortex volume (Day-Wilson et al., 2006). Similar environmental interventions during early-life in humans may contribute to the development of common psychiatric disorders, such as depression and schizophrenia in genetically predisposed individuals (see Fone and Porkess, 2008 for review).

The main objective of this study is to determine specific changes in the amygdaloid expression of different molecules involved in structural plasticity and inhibitory neurotransmission. For this purpose we have analyzed the expression of GAD67, GAD65, synaptophysin, NCAM and PSA-NCAM by means of immunohistochemistry and optical densitometry in the centromedial (CeM), medial (Me) and basolateral (BLa) amygdala. We have also studied the expression of mRNAs for GAD67, GAD65, synaptophysin, NCAM and the polysialyltransferases (St8SiaII and St8SiaIV) using quantitative real-time PCR (qRT-PCR) of total amygdala.

2. Results

2.1. *Immunohistochemical analysis reveals that the expression of GAD67, but not of GAD65 or synaptophysin, is altered in the amygdala of isolation-reared rats*

No changes were observed in the expression of GAD65 in any nucleus of amygdala when comparing social versus isolation-reared rats. By contrast, the expression of GAD67 was significantly increased in all the three amygdaloid nuclei of the isolation reared rats respect to the socially housed animals: Me, CeM and BLa ($t_9=-2.822$, $p=0.02$; $t_9=-3.572$ $p=0.006$ and $t_9=-2.835$ $p=0.0196$ respectively; Figs. 1 and 2). The expression of synaptophysin, which is a general marker of synapses (Greengard et al., 1993), showed no differences between the 2 groups of study in the three nuclei of the amygdala analyzed.

2.2. *Immunohistochemical analysis reveals increases in the expression of PSA-NCAM and NCAM in different amygdaloid nuclei of the isolation-reared rats*

An increase in PSA-NCAM expression was observed in all the 3 nuclei analyzed in the amygdala of isolated rats, being only significant for the BLa nucleus ($t_9=-2.775$, $p=0.0216$). NCAM expression was significantly increased in the Me nucleus ($t_9=-3.14$, $p=0.0119$) of the isolated animals and did not show significant changes in the other two nuclei (Figs. 1 and 2).

2.3. *qRT-PCR analysis does not reveal any significant change in the expression of the studied genes in the amygdala of isolation-reared rats*

No significant changes were found in the expression of the genes analyzed in the total amygdala when comparing the socially housed rats with the isolation-reared rats. The mRNA

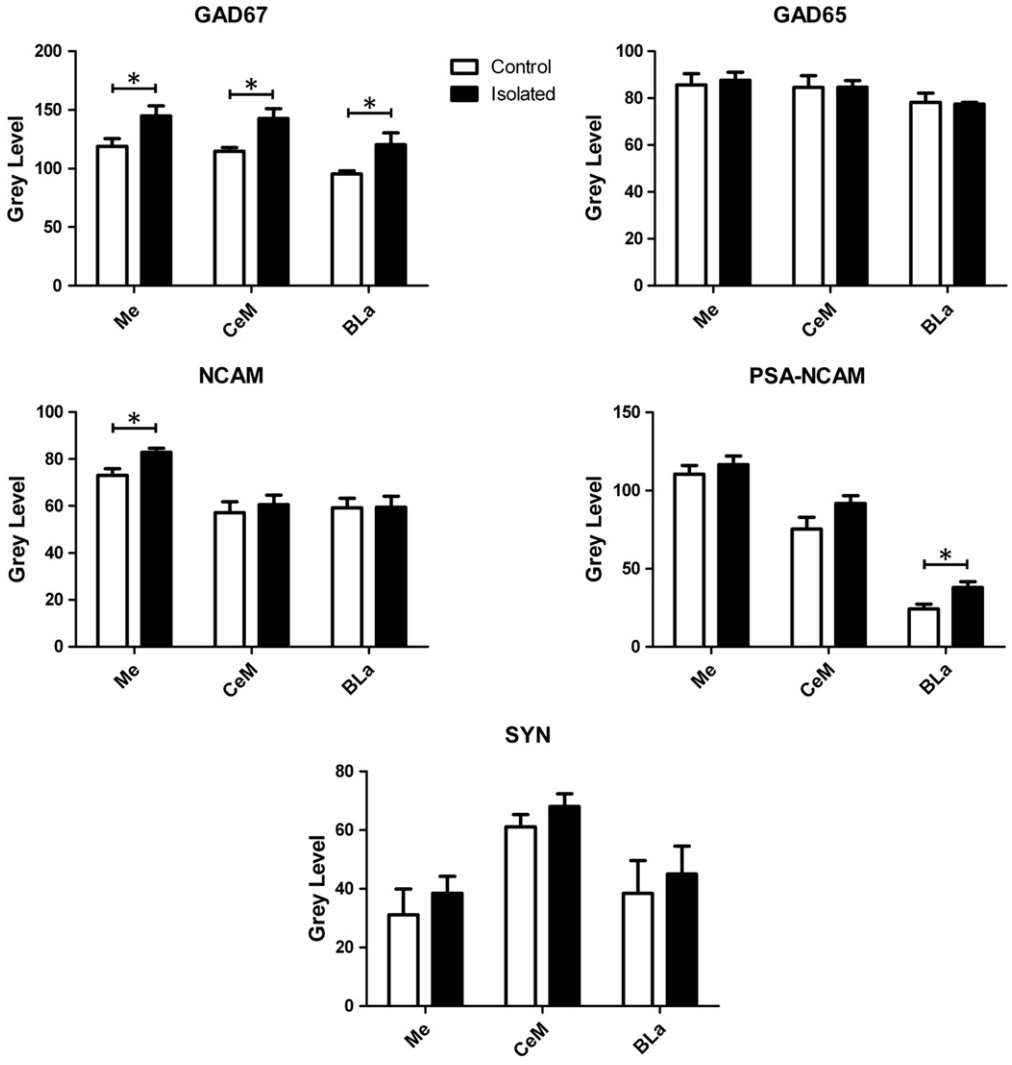

Fig. 1 – Neuropil immunoreactivity of GAD67, GAD65, NCAM, PSA-NCAM and SYN in the amygdala. Histogram bars show the gray level measured in amygdaloid nuclei (Me, CeM, BLa) of control (white bar) and isolated-rearing (black bar) groups. Data are the mean ± S.E.M. from 6 control rats and 5 isolated rats in each group. *$p < 0.05$ vs the control group.

levels of *GAD* genes (*GAD65* and *GAD67*) were both increased around 1-fold in the isolated rats and similar results were obtained with *synaptophysin* gene. *ST8SIAII* and *ST8SIAIV* expressions were reduced (−1.38 and −2.43 fold respectively) in these animals (Table 2; Fig. 3).

3. Discussion

Early-life adverse events markedly influence the development of the nervous system and may facilitate, in genetically predisposed individuals, the development of psychiatric disorders such as schizophrenia or major depression. Exposing rodents to postweaning social isolation affects brain development and leads to behavioral, morphological and neurochemical alterations during adulthood, which resemble core symptoms of schizophrenic patients. The behavioral alterations include neophobia, disrupted prepulse inhibition (Geyer et al., 1993), impairments in sensorimotor gating, spatial cognition (Quan et al., 2010) and novel object recognition (McLean et al., 2010). Rats reared in social isolation also show reduced prefrontal cortex volume (Day-Wilson et al., 2006). At the molecular level, these animals show reduced expression of AMPA glutamate receptors in the hippocampus (Sestito et al., 2011) and alterations in serotoninergic and dopaminergic systems. However, despite the fact that both brain imaging and postmortem studies have suggested a central role of the amygdala in the pathophysiology of schizophrenia (Benes, 2010; Phillips et al., 2003), most of the studies conducted in post-weaning socially-isolated rodents have been focused in cortical regions and very few of them have analyzed the amygdala.

This is particularly puzzling, because rodents reared in social isolation show alterations in behaviors in which the amygdala plays an important role, indicating that this limbic region must be affected in this model. These animals show increased aggression (Ferdman et al., 2007; Vale and Montgomery, 1997; Valzelli, 1973), deficits in contextual fear conditioning (Weiss et al., 2004) and appear more emotional than rodents reared in groups (see Fone and Porkess, 2008 for review).

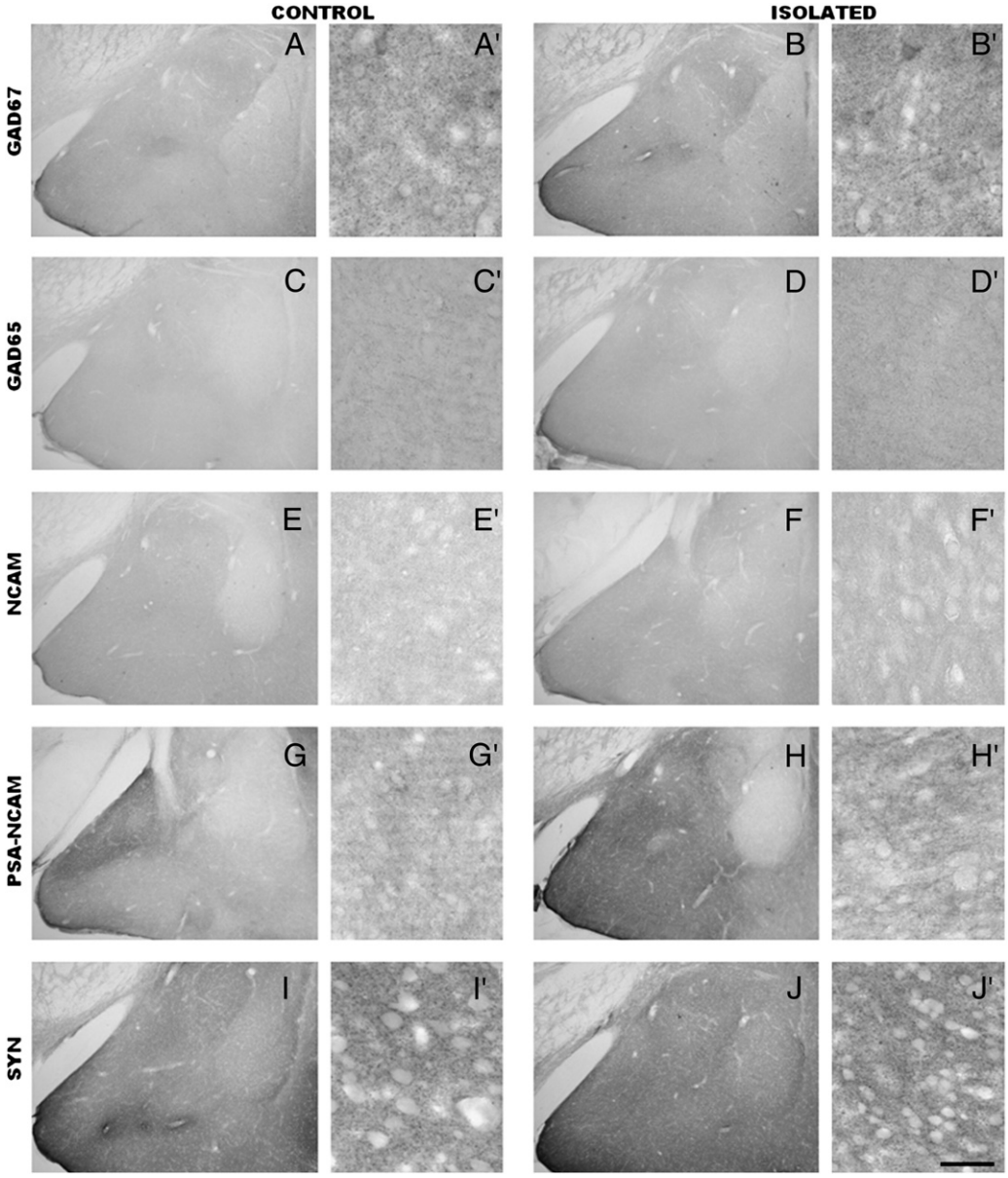

Fig. 2 – Panoramic and high magnification microphotographs showing immunohistochemistry for GAD67 (A, A′, B and B′), GAD65 (C, C′, D and D′), NCAM (E, E′, F and F′), PSA-NCAM (G, G′, H and H′) and SYN (I, I′, J and J′) in the amygdala of control and isolated rats. Scale bar: 500 μM for A–J and 50 μM for A′–J′.

Very few studies have analyzed the expression of molecules related to synaptic transmission in the amygdala of isolation-reared rodents. Despite of the behavioral alterations showed by these animals as described above, all of them have reported only minor changes. Our study has found that the expression of GAD67, but not GAD65, protein was increased in different amygdaloid nuclei of isolation-reared rats. By contrast, no differences were found in mRNA expression, which may be due to the masking effect of total GAD67 mRNA when using whole amygdala extracts. Our findings are in agreement with those of a recent study, which did not find differences in GAD65 protein expression in the amygdala of isolation-reared Sprague–Dawley rats (Lim et al., 2011). These authors did not find differences in amygdaloid GABA(B)R1 expression either. Unfortunately, there are no more previous studies exploring the expression of molecules related to inhibitory neurotransmission in the amygdala of isolation-reared rodents. Our results indicating an increase in GAD67 expression are in contrast with those reported in the amygdala of human schizophrenic patients. These postmortem studies showed reduced GAD activity (Bird et al., 1977), GABA concentration (Spokes et al., 1980) and GAD67 expression (Varea et al., 2012), which are consistent with an increased activation of the amygdala in schizophrenia. Consequently, we should be cautious when using isolation-reared rats as an animal model of schizophrenia, because while some of its features may correspond to those observed in human patients, some of them may be substantially different.

Table 1 – Sequences of gene specific primers and associated amplicon lengths for qRT-PCR.

Target gene	Primers	Sequence (5′→3′)	Amplicon size [a]
ST8SiaII	Forward	GGCAACTCAGGAGTCTTGCT	123
	Reverse	GTCAGTCTTGAGGCCCACAT	
ST8SiaIV	Forward	CCTTCATGGTCAAAGGAGGA	125
	Reverse	CCAGTAACCTCTGACCGCAT	
NCAM	Forward	AACGGACTCCAAACCATGAC	123
	Reverse	CTGGCTTTGCTTCTGACTCC	
GAD67	Forward	CTGGAGCTGGCTGAATACCT	120
	Reverse	TCGGAGGCTTTGTGGTATGT	
GAD65	Forward	CTGCTTCTGGTTTGTACCTCCT	122
	Reverse	CCATTGTGGTCCCATACTCC	
SYN	Forward	CTATGGGCAGCAAGGCTATG	120
	Reverse	CAGGCCTTCTCTTGAGCTCTT	
Ywhaz	Forward	TTGAGCAGAAGACGGAAGGT	136
	Reverse	GAAGCATTGGGGATCAAGAA	

[a] Amplicon length in base pairs.

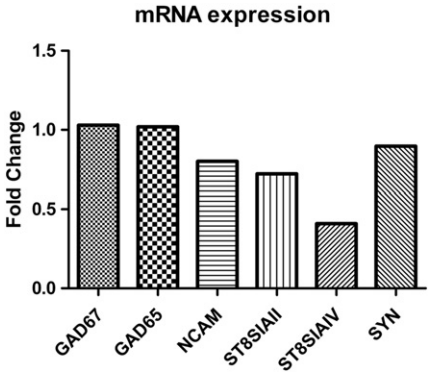

Fig. 3 – qRT-PCR mRNA fold change data shown as isolated-rearing group versus control group gene expression. All gene expression was normalized using *Ywhaz* as a control gene.

Also in agreement with Lim et al. (2011) we have failed to find differences in the expression of synaptophysin in the amygdala of isolation-reared animals. These results are also consistent with previous studies describing no differences in the number of synapses in the medial amygdala (Ichikawa et al., 1993).

The differences in PSA-NCAM expression observed in our study may be related to structural changes in neurons, given the anti-adhesive properties of this molecule (Rutishauser, 2008; Sandi, 2004). However, this structural plasticity should be limited initially to interneurons, because, as it has been demonstrated for many PSA-NCAM expressing structures in the cerebral cortex (excluding those of immature neurons) (Gomez-Climent et al., 2011; Nacher et al., 2002; Varea et al., 2005), many PSA-NCAM expressing neurons in the amygdala express markers of interneurons and lack expression of molecules exclusively found in principal neurons (Gilabert-Juan et al., 2011). Consequently, changes in PSA-NCAM expression should primarily affect the structure of interneurons, rather than that of principal neurons. In this line, using a transgenic mice strain expressing GFP mainly in somatostatin expressing interneurons (Oliva et al., 2000), we have recently reported that PSA-NCAM expressing cortical interneurons have reduced synaptic input and decreased dendritic arborization and spine density when compared with neighboring interneurons lacking PSA-NCAM (Gomez-Climent et al., 2011). It is possible then, that the increases in PSA-NCAM expression observed in the present study affect the connectivity of certain amygdaloid interneurons, leaving less plasma membrane extension free for the establishment of synaptic contacts. Whether these changes in PSA-NCAM expression are related to the increase in GAD67 expression still remains to be explored. Another non-excluding possibility is that, given its anti-adhesive properties, the increase in PSA-NCAM expression may facilitate the structural remodeling of certain interneurons in response to different stimuli.

Table 2 – qRT-PCR results for tested genes in isolated rats vs. control.

	Amygdala	
	Δ	*p*-value
Gad67	1.03	0.877
Gad65	1.02	0.915
NCAM	−1.25	0.375
St8siaII	−1.38	0.437
St8siaIV	−2.43	0.292
Synaptophysin	−1.11	0.582

Δ, change in gene relative to normalize.

It is interesting to note that the increase in PSA-NCAM expression is only significant in the basolateral amygdala, a region considered critical in the pathophysiology of schizophrenia (Benes, 2010). This increase in PSA-NCAM expression may be due to increase in polysialylation of pre-existing NCAM molecules, because we found an increment of this protein only in the medial amygdala of the isolation-reared rats. However, we have not detected parallel increments in any of the two NCAM polysialyltransferases, which may mean that the increased polysialylation of NCAM in the basolateral amygdala has occurred at an earlier age.

Previous studies have shown that an enhanced dopaminergic activity exists in the amygdala of isolation-reared rodents. This procedure increases basal dopamine turnover (Heidbreder et al., 2000), dopaminergic presynaptic function (Lapiz et al., 2003) and D2 receptor binding in the amygdala. It is possible that the increases that we observed in GAD67 and PSA-NCAM expression respond to this enhanced dopaminergic activity through D2 receptors, because, at least in the prefrontal cortex, chronic treatment with a D2 receptor agonist produces the same effects (Castillo-Gomez et al., 2008).

In summary, our results indicate that discrete but significant changes occur in the amygdala of isolation-reared rats, involving molecules related to structural plasticity and inhibitory neurotransmission. However, the direction of these differences is not similar to that observed in schizophrenic patients. Consequently, although this paradigm has been confirmed as a suitable model to study schizophrenia, because it reproduces some of its core defects (Fone and Porkess, 2008), it also may present some differences, which should be taken into account and explored further when establishing comparisons.

4. Experimental procedures

4.1. Animals

Eight pregnant Lister Hooded rats were purchased from Jackson laboratories (Bar Harbor, Maine, USA) and bred in our animal facility. Pregnant rats were housed individually in a controlled temperature room (25±1 °C) and on a 12-h light/dark cycle with food and water available ad libitum. 27 male rats were born from the pregnant rats and were used for the experiments. All efforts were made to minimize the number and suffering of animals used. All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València.

4.2. Social isolation-rearing

Newborn rats remained with their mothers until weaning (21 days); at this moment, male rats were randomly divided into two groups: social and isolated group. Socially housed rats (n=15), used as controls, were housed 3 per cage (215×465×145 mm) and isolated rats (n=12) were housed in individual cages (220×220×145 mm) and reared in isolation individually. All rats were housed in the same room, and sharing the same light, temperature and humidity. Rats reared in isolation could hear and smell the other rats, but were unable to see or have physical contact with them. All animals were handled once a week by the same person, who replaced the bedding of the cage and added food and water. Rats were reared in these conditions during 8 weeks.

4.3. Quantitative retrotranscription-polymerase chain reaction

The rats used for qRT-PCR were sacrificed by decapitation using a guillotine. After that, brains were removed from the skull and the whole Amygdala of each brain of 9 control and 7 isolated rats were extracted. Total mRNA was extracted using TriPure reagent (Roche Applied Science, Indianapolis, IN) following manufacturer's instructions. The concentration and purity of total RNA were determined by Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). cDNA synthesis was performed using the Expand reverse transcriptase (Roche Applied Science).

Specific primers to rat *GAD67*, *GAD65*, *NCAM*, *ST8SIAII*, *ST8SIAIV* and *SYN* genes (Table 1) were designed from public sequences, which were obtained from Ensembl Genome Browser data base (http://www.ensembl.org/) using Primer-Blast free software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed between exons to avoid DNA contamination, when possible. The primer sequence for the reference gene (*ywhaz*) was obtained from Bonefeld et al. (2008). Primers were tested for nonspecific products and correct amplicon size by electrophoresis in 1.5% EtBr agarose gel. qPCR was carried out in triplicates with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems), primers at a concentration of 240 nM, and 4 μl of cDNA (25 ng/μl). Following a 95 °C denaturation for 10 min, the reactions were cycled 40 times with a 95 °C denaturation for 15 s and a 60 °C annealing step for 1 min. After this, a melt curve was performed to assess the specificity of primers.

Relative quantification was performed using the comparative threshold (Ct) method according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Changes in gene expression were reported as fold changes relative to controls. An unpaired *t*-test was performed to analyze the statistical significance of results.

4.4. Immunohistochemistry

Six control rats and five isolated rats were perfused transcardially with a 4% paraformaldehyde solution in phosphate buffer (PB, 0.1 M, pH 7.4). Brains were removed from the cranium and the hemispheres were separated. The right hemisphere was cryoprotected in a 30% sucrose solution in PB and cut in a sliding microtome at 50 μm. These sections were destined for immunohistochemical analyses. The contralateral hemisphere was stored.

The immunohistochemistry protocol was performed as follows: Briefly, floating sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6 at 100 °C). After cooling down the sections to room temperature, they were incubated with 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 10% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton-X-100 (Sigma-Aldrich) and they were incubated overnight at room temperature with one of these antibodies: anti-PSA-NCAM (AbCys, 1:700), anti-NCAM (DSHB, 1:500), anti-GAD65 (Millipore, 1:500), anti-GAD67 (Chemicon, 1:500), anti-synaptophysin (Sigma, 1:200) with PBS containing 0.2% Triton-X-100 and 3% NDS. The second day, sections were incubated for 1 h with either donkey anti-mouse IgM or IgG biotinylated antibodies (1:200; Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton-X-100 and 5% NDS. Then, sections were incubated in an avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min in PBS. Color development was achieved by incubating with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.033% hydrogen peroxide in PB for 4 min. Finally, sections were mounted on slides, dried for one day at room temperature, dehydrated with ascending alcohols and rinsed in xylene. After this, sections were coverslipped using Eukitt mounting medium.

All the studied sections passed through all procedures simultaneously in order to minimize any difference from immunohistochemical staining itself. To avoid any bias in the analysis, all slides were coded prior to analysis and the codes were not broken until the experiment was finished. All the sections were analyzed by the same researcher.

4.5. Quantification of neuropil immunoreactivity

From each immunostaining (PSA-NCAM, SYN, GAD65, GAD67 and NCAM), three sections per animal containing the 3 amygdaloid nuclei analyzed (CeM, BLa and Me) were selected randomly from the following coordinate interval: Bregma −1.80 to 2.80 mm (Paxinos and Watson, 1986), in order to measure immunoreactivity as previously described (Varea et al., 2007).

Sections of each immunostaining were examined in one single session with an Olympus CX41 microscope under bright-field illumination, homogeneously lighted and digitalized using a CCD camera. Photographs to the different regions were taken at 20× magnification. Gray levels were converted to optical densities (OD) using Image J software (NIH). In order to normalize the values, the gray levels obtained from photographs of the corpus callosum in each section were subtracted from those obtained in the different amygdaloid nuclei. Means were determined for each experimental group, using the number of animals as the "n", and data were analyzed by means of unpaired Student's *t*-test.

Acknowledgments

Spanish Ministry of Science and Innovation (MICINN-FEDER) BFU2009-12284/BFI, MICINN-PIM2010ERN-00577/NEUCONNECT in the frame of ERA-NET NEURON and the Stanley Medical Research Institute supported JN. Javier Gilabert-Juan has a FPU predoctoral fellowship from the Spanish Ministry of Education (AP2008-00937).

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Article 4: A "double hit" murine model for schizohphrenia shows alterations in the structure and neurochemistry of the medial prefrontal cortex and the hippocampus

TITLE: A "DOUBLE HIT" MURINE MODEL FOR SCHIZOPHRENIA SHOWS ALTERATIONS IN THE STRUCTURE AND NEUROCHEMISTRY OF THE MEDIAL PREFRONTAL CORTEX AND THE HIPPOCAMPUS

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NUMBER OF FIGURES AND TABLES: 7 figures, 4 supplemental figures and 1 table.

ABSTRACT

Schizophrenia is a very complex psychiatric disorder in which both alterations in neurodevelopment and aversive experiences during adolescence seem to be important risk factors. Animal models reproducing these types of alterations mimic some of the symptoms found in patients, constituting a valid approach to study the etiopathology of this disorder and a good platform to test the validity of new therapeutic approaches. Among these models, the perinatal injection of NMDA receptor antagonists and the postweaning social isolation rearing are among the most widely used. Each of them has reproduced different behavioral, structural and neurochemical alterations resembling those found in schizophrenic patients. Our aim is to combine them in a "double hit" model, which should produce a wider spectrum of alterations. Lister Hooded rats have been subjected to a single injection of MK-801 at P7 and have been socially isolated from postweaning to adulthood. We have found that these animals present increased weight gain and volume reductions in their medial prefrontal cortex (mPFC) and hippocampus. They also show an increased number of activated pyramidal cells and a decrease of parvalbumin expressing cells in the mPFC. The expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), a molecule related to neuronal structural plasticity and that of GAD67 are decreased in the mPFC. qRT-PCR analysis revealed that the mRNA of *calbindin* was decreased in the mPFC while, that of *calretinin* was increased. Alterations in the expression of the ERbB4 mRNA, a gene associated to schizophrenia, were also found in this region. All these structural and neurochemical alterations, specially in cortical inhibitory circuits, are similar to those found in schizophrenic patients and are more numerous than those found in each of the single models. Consequently, we consider that the present "double hit" model is a better tool to study the neurobiological basis of schizophrenia and to explore new pharmacological approaches to treat this disorder.

KEYWORDS: Schizophrenia, animal model, inhibitory neurotransmission, adult neurogenesis interneuron.

INTRODUCTION

Schizophrenia is a highly complex, multifactorial disease, which results in dramatic changes in behavior, perception and cognition. These changes are paralleled by alterations in the structure, neurochemistry and physiology of certain cerebral regions, specially the prefrontal cortex (PFC) and the hippocampus, two regions critically involved in the etiopathology of this psychiatric disorder.

Several structural studies have shown that patients with schizophrenia have lower volumes of PFC and hippocampus than normal control subjects (for review see (Levitt et al. 2010, Phillips et al. 2003). Another aspect of structural plasticity, which may be relevant to schizophrenia, given the involvement of the hippocampus in this disorder, is the apparent presence of alterations in adult neurogenesis in the dentate gyrus of schizophrenic patients (Reif et al. 2006).

A closer view to the cerebral cortex of schizophrenic patients has also revealed alterations in neuronal circuitry, specially affecting the structure of neuronal inhibitory networks and their neurotransmission. In fact, current pathophysiological theories of schizophrenia are pointing to the GABAergic system as responsible for some of the alterations in schizophrenic brains (Benes and Berretta 2001, Lewis and Gonzalez-Burgos 2008). The inhibitory neurotransmitter GABA and some genes implicated in its metabolism have been associated with schizophrenia (Straub et al. 2007, Zai et al. 2009), specially the 67-kDa isoform of glutamic acid decarboxylase (GAD67) (See Akbarian and Huang 2006, Beneyto and Lewis 2011, Curley et al. 2011 for review). These alterations in inhibitory neurotransmission appear to affect particularly certain interneuronal populations, specially those expressing parvalbumin. Decreased density of neurons expressing the phenotypic markers of cortical GABAergic interneurons, parvalbumin and calbindin have been found in the PFC (Akbarian et al. 1995, Beasley et al. 2002, Chance et al. 2005, Sakai et al. 2008) and the hippocampus (Zhang and Reynolds 2002) in post-mortem studies of subjects with schizophrenia. Interestingly, a recent study suggests that these alterations in inhibitory circuitries may be mediated by neuregulin 1 (Nrg1) and its receptor ErbB4, two important risk genes for schizophrenia, since their signaling controls the development and connectivity of these circuitries in the cerebral cortex (Fazzari et al. 2010).

The structural alterations in cortical inhibitory neurons found in schizophrenia may be mediated by the polysialylated form of the neural cell

adhesion molecule (PSA-NCAM), which, through its antiadhesive properties, facilitates neuronal and synaptic remodeling (see Bonfanti 2006, Rutishauser 2008 for review), or the partial isolation of neuronal elements (Gomez-Climent et al. 2011). The addition of PSA to NCAM is mediated by the two polysialyltransferases St8SiaII and St8SiaIV (see Hildebrandt et al. 2008 for review). PSA-NCAM is expressed in a subpopulation of interneurons, both in the PFC and the hippocampus of different mammalian species, including humans (Gilabert-Juan et al. 2012a, Gilabert-Juan et al. 2012b, Gomez-Climent et al. 2011, Mikkonen et al. 1998, Mikkonen et al. 1999, Nacher et al. 2002, Varea et al. 2005, Varea et al. 2007b), which have more reduced structural features than those lacking this molecule (Gomez-Climent et al. 2011). Interestingly, both *NCAM* and *ST8SIAII* genes have been associated with schizophrenia and alterations in the expression of NCAM and PSA-NCAM have been found in postmortem studies of this disorder, including some on the hippocampus and the PFC (Barbeau et al. 1995, Brennaman and Maness 2010, Gilabert-Juan et al. 2012b, Sullivan et al. 2007, Tao et al. 2007).

To circumvent the intrinsic problems of studying human brains and to explore new experimental therapeutic approaches, several animal models of schizophrenia have been developed during the recent years. Obviously, none of these models mimics completely the disorder, but all of them can reproduce some of its core symptoms. Given the importance of altered neurodevelopment on the etiopathogenesis of schizophrenia, some of these models consist in experimental interventions during embryogenesis or early postnatal development. One of the most used of such models is the administration of NMDA receptor antagonists during the perinatal period, which produces certain cognitive and social impairments similar to those found in schizophrenia, (Abdul-Monim et al. 2006, Beninger et al. 2002, Hickey et al. 2012, Rung et al. 2005). Perinatal NMDA glutamate receptor antagonist administration also reduces GABAergic neurotransmission and the number of parvalbumin expressing neurons in the PFC and the hippocampus in adulthood (Rotaru et al. 2012). The existence of adverse experiences during early-life markedly influences the development of the nervous system and may facilitate, in genetically pre-disposed individuals, the development of psychiatric disorders such as schizophrenia. In this line, it is known that exposing rodents to postweaning social isolation affects brain development and leads to behavioral, morphological and neurochemical alterations during adulthood, which resemble core symptoms of schizophrenic patients (Fone and Porkess 2008, McLean et al. 2010, Simpson et al. 2010). These alterations include reduced cortical

volume (Day-Wilson et al. 2006), as well as deficits in the number of parvalbumin and calbindin interneurons in the hippocampus (Harte et al. 2007).

During the recent years there has been an effort to combine some of the previous animal models of schizophrenia to better reproduce the disorder. A recent report has tested the hypothesis that a “double-hit” model combining MK-801 administration during adulthood and postweaning social isolation rearing of Sprague-Dawley rats, produces greater behavioral and neurochemical effects than either insult alone, with limited results (Hickey et al. 2012). In the present study, we have developed a similar “double hit” model in Lister Hooded rats in order to find whether the combination of an earlier injection of MK-801 (P7), which may alter different neurodevelopmental processes, and a postweaning social isolation rearing reproduces some of the structural and molecular changes found in the mPFC and the hippocampus of schizophrenic patients, particularly in their inhibitory networks. We have analyzed the volume of these regions, the presence of alterations in the expression of the immediate early gene c-fos in their pyramidal neurons and have studied putative differences in the number of proliferating cells and of immature neurons in the hippocampal dentate gyrus. We have also analyzed changes in the number of parvalbumin expressing interneurons and the expression of different molecules involved in synaptic/structural plasticity and inhibitory neurotransmission, such as GAD67, synaptophysin, NCAM and PSA-NCAM by means of immunohistochemistry and optical densitometry. Finally, we have quantified and compared the expression of mRNAs for *GAD67*, *synaptophysin*, *NCAM*, *parvalbumin*, *calretinin*, *calbindin*, *ErbB4*, *Nrg1* and the polysialyltransferases (*St8SiaII* and *St8SiaIV*) using quantitative real-time PCR (qRT-PCR).

EXPERIMENTAL PROCEDURES

Animals

Fifteen pregnant Lister Hooded rats were purchased from Jackson laboratories (Bar Harbor, Maine, USA) and bred in our animal facility. Pregnant rats were housed individually in a controlled temperature room (25º C) and on a 12-h light/dark cycle with food and water available *ad libitum*. After a week, 60 male rats were born from the pregnant rats and were used for the experiments. These animals were assigned randomly to the vehicle or the MK-801 groups. The weight of the rats was determined at postnatal day 7 (P7), P21 and after the 8 weeks of isolation (P77), right before their sacrifice. All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València.

Isolation and MK-801 Acute Treatment

P7 male rat pups were randomly assigned to 2 groups. 28 rats were intraperitoneally injected with a solution of the non-competitive antagonist of the N-methyl-d-aspartate (NMDA) receptor dizocilpine (MK-801, 1mg/Kg, Ascent Scientific, Princeton, USA) and 32 rats were injected with saline solution (0.9% NaCl). All rat pups remained with their mothers until weaning (21 days); at this postnatal day, rats were housed in groups or reared in isolation, thus forming 4 new groups: Socially housed and vehicle injected (n=15), socially housed and MK-801 injected (n=12), isolated and vehicle injected (n=17) and finally isolated and MK-801 injected (n=16). Rats reared in group were housed 3 per cage (215 × 465 × 145 mm), while isolated rats were housed in individual cages (220 × 220 × 145 mm). All rats were housed in the same room, sharing the same controlled light, temperature and humidity. Rats reared in isolation could hear and smell other rats, but were unable to see or have physical contact with them. All animals were handled once a week by the same person, who replaced the bedding of the cage and added food and water. Rats were reared in these conditions during 8 weeks.

Gene Expression

Thirty-two rats were used for qRT-PCR analysis and were sacrificed by decapitation using a guillotine. After this, their brains were removed from the skull and the whole medial prefrontal cortex (mPFC) and hippocampus of each brain were microdissected. Total mRNA was extracted using TriPure reagent (Roche Applied Science, Indianapolis, IN) following manufacturer's instructions. The concentration and purity of total RNA was determined with an Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). cDNA synthesis was performed using the Expand Reverse Transcriptase (Roche Applied Science).

qRT-PCR analyses were performed in triplicates. Specific primers for all genes (Table 1) at a concentration of 240 nM, and 4 µl of cDNA (50ng) were used. Primers were designed between exons to avoid DNA contamination, when possible. *Ywhaz* was used as a reference gene based on the study of Bonefeld et al. (2008). Primers were tested for nonspecific products and correct amplicon size by electrophoresis in 1.5% EtBr agarose gel. qPCR was carried out with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems), following a 95ºC denaturation for 10 minutes, the reactions were cycled 40 times with a 95ºC denaturation for 15 seconds, and a 60ºC annealing step for 1 minute. After this, a melt curve was performed to assess the specificity of primers.

Relative quantification was performed using the comparative threshold (Ct) method according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Changes in gene expression were reported as fold changes relative to controls.

Immunohistochemistry

Twenty-eight rats were perfused transcardially with a 4% paraformaldehyde solution in phosphate buffer (PB, 0.1M, pH 7.4). Brains were removed from the cranium and the hemispheres were separated. The right hemisphere was cryoprotected in a 30% sucrose solution in PB (3-4 days) and cut frozen in a sliding microtome at 50 µm in coronal sections. These sections were destined for immunohistochemical analyses. The contralateral hemisphere was stored.

The immunohistochemistry protocol was performed as follows: Briefly, floating sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6 at 100 °C). After cooling down the sections to room temperature, they were incubated with 3% H_2O_2 in phosphate buffered saline

(PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 10% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton-X-100 (Sigma-Aldrich) and they were incubated overnight at room temperature with the primary antibody in each case; anti-PSA-NCAM (AbCys, 1:700), anti-NCAM (DSHB, 1:500), anti-GAD67 (Chemicon, 1:500), anti-parvalbumin (SWANT, 1:2000), anti-Ki67 (Abcam, 1:500) and anti-DCX (Abcam, 1:1000) with PBS containing 0.2% Triton-X-100 and 3% NDS. The second day, sections were incubated for 1 hour with either donkey anti-mouse IgM or IgG, and donkey anti-rabbit IgG biotinylated antibodies (1:200; Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton-X-100 and 5% NDS. Then, sections were incubated in an avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min in PBS. Color development was achieved by incubating with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.033% hydrogen peroxide in PB for 4 min. Finally, sections were mounted on slides, dried for one day at room temperature, dehydrated with ascending alcohols and rinsed in xylene. After that, sections were coverslipped using Eukkit mounting medium. All the slides containing sections destined to quantitative analysis were coded and their code was not broken until the analyses were finished.

Quantification of Neuropil Immunoreactivity

From the immunostainings of PSA-NCAM, GAD67 and NCAM, the 5 layers of the mPFC and the lucidum, radiatum and oriens layers of the hippocampus were selected in order to measure immunoreactivity as previously described (Varea et al. 2007a). Sections of each immunostaining were examined in one single session with an Olympus CX41 microscope under bright-field illumination, homogeneously lighted and digitalized using a CCD camera. Five photographs of the different areas and layers were taken for each animal at 20X magnification. Grey levels were converted to optical densities (OD) using Image J software (NIH). In order to normalize the values, the gray levels obtained from photographs of the corpus callosum in each section were subtracted from those obtained in the different layers. Means were determined for each experimental group, using the number of animals as the “n”.

Estimation of the total number of PV+ cells in mPFC and hippocampus and of Ki67+ and DCX+ cells in the dentate gyrus

The total number of neurons expressing parvalbumin (PV) protein in their somata was estimated in the different regions of the mPFC and in the hippocampus as described before (Varea et al. 2007a); the same procedure was used for Ki67 and doublecortin (DCX) expressing cells in the dentate gyrus of the hippocampus. Briefly, sections were selected by a 1:10 fractionator sampling covering the whole rostral to caudal extension of the different regions of interest studied and on each section all labeled cells within the region were counted. Cell somata were identified and counted with a 40X objective using an Olympus CX41 microscope.

Quantification of the activation of CaMKII expressing pyramidal neurons in the mPFC

In order to estimate the proportion of activated pyramidal cells in the mPFC and hippocampus, we performed a double fluorescence immunohistochemistry in one in ten subseries of brain sections from each animal. The protocol used was similar to that described above, omitting the endogenous peroxidase block. Sections were incubated overnight at room temperature in polyclonal rabbit IgG against c-Fos (1:20, Santa Cruz Biotechnology Inc.) and mouse IgGs1 against CaMKII (1:500, Abcam) primary antibodies. Then they were incubated for 1 hour in Dylight 649 conjugated secondary antibody against rabbit IgG (Jackson ImmunoResearch,1:200) and Dylight 488 conjugated secondary antibody against mouse IgGs1 (Jackson ImmunoResearch, 1:200). Fifty CAMKII expressing pyramidal neurons per animal/region were randomly selected from each animal. Then, the percentage of c-Fos co-expressing cells was determined.

Volumetry

In order to estimate the volumes of the regions studied, one in ten subseries of fixed brains was stained with cresyl violet, mounted and coverslipped with Eukitt. Volumetries of the different mPFC regions (prelimbic, infralimbic, cingulate 1 and 2) and the total hippocampus were performed by

means of Volumest plugin in the Image J Software (NIH). First, pictures of all slices were acquired with an Olympus CX41 microscope under bright-field illumination, homogeneously lighted and digitalized using a CCD camera. After that, the measures of the areas were estimated in pictures containing: prelimbic cortex in Bregma 4.70mm to 2.20mm; infralimbic cortex in Bregma 3.20mm to 2.20mm; cingulated 1 cortex in Bregma 3.70mm to -1.40mm; cingulate 2 cortex in Bregma 1.70mm to -1.40mm and hippocampus in Bregma -1.80mm to -6.80mm. The volumes were estimated for the right hemisphere using the Cavalieri's principle (Gundersen and Jensen, 1987), outlining the region of interest digitally and combining the cut section (50μm) and the section increment (10 μm), to compute volumes automatically.

Data analysis

For body weight analysis, body weight differences at P21 were subjected to *t*-test analysis, and at P77, the differences were assessed by means of 2-way analyses of variance (ANOVA). All the data from the volumetries, counts, gene expression or densitometries were analyzed separately in each layer studied in the mPFC or the hippocampus, using 2-way ANOVA considering treatment (vehicle vs. MK-801) and housing (social vs. isolated) such as between-subjects factors. When appropriate, tests of simple main effects and pair-wise comparisons were performed. An alpha level of 0.05 was used for all statistical tests and all tests were conducted using Statview software.

RESULTS

Body weight is affected by MK-801 treatment and by postweaning isolation rearing

Body weight changes were measured at P7 (the day of MK-801 treatment), P21 (the weaning day) and at the end of the experiment (P77). There were no differences in weight between the two groups (MK-801 or Vehicle solution) of rat pups injected in P7. The *t*-test at P21 day showed less weight in individuals treated with MK-801 than those injected with the vehicle solution (0.9% NaCl) $t = 11.01$, $p = 0.0016$. In contrast, the 2-way ANOVA performed at P77 showed an increased weight gain in rats reared in isolation compared to those reared in group, indicating an effect of the housing $F(1,55) = 5.36$, $p = 0.024$ (Supplemental figure 1).

Volume reduction of mPFC and hippocampus

The volume measures showed a significant reduction of the prelimbic region caused by the housing $F(1, 21) = 4.96$, $p = 0.037$ and the treatment $F(1, 21) = 7.22$, $p = 0.014$. Similarly, the infralimbic region was significantly reduced by the housing $F(1, 21) = 4.52$, $p = 0.045$ and the treatment $F(1, 21) = 5.5$, $p = 0.029$. No significant changes in volume were detected in the cingulate cortices. However, when the total mPFC was taken into account, significant reductions were found again, caused by the housing $F(1, 21) = 4.48$, $p = 0.046$ and the treatment $F(1, 21) = 6.91$, $p = 0.016$ (Figure 1).

The hippocampal volume was also reduced, although this reduction was only caused by the treatment $F(1, 21) = 11.36$, $p = 0.0029$ (Figure 1).

c-Fos expression reveals an increase in the proportion of activated excitatory neurons in the infralimbic cortex

In order to know the proportion of principal neurons activated in the different groups of rats, we have estimated the percentage of cells coexpressing CaMKII (a marker of excitatory neurons) and c-Fos in the different regions of the mPFC. We observed a significant increase in the percentage of activated cells in the infralimbic region due to the housing $F(1, 21) = 11.18$, $p = 0.0031$,

but no changes were found in the rest of the mPFC regions or when the whole mPFC was taken into account (Figure 2).

The total number of parvalbumin expressing somata is decreased in the infralimbic cortex

The total number of parvalbumin (PV) expressing somata was estimated in the four regions of the mPFC and the CA1, CA3 and dentate gyrus regions of the hippocampus. In the mPFC regions, there was a decrease in the number of PV expressing cells in the infralimbic cortex caused by the treatment F (1, 20) = 5.7, p = 0.027, and an interaction housing x treatment F (1, 20) = 17.17, p = 0.0005. Follow up tests showed decreased number of PV expressing cells in Iso-Veh, Soc-MK-801, Iso-MK-801 groups respect to Soc-Veh group (p = 0.0015; 0.0004; 0.03 respectively) and an increase of cells in Iso-MK-801 group respect to Soc-MK-801 (p = 0.044). Trends towards a decrease in the number of PV immunoreactive cells were observed in the other three studied mPFC regions and when considering the mPFC as a whole (Figure 3).

Regarding the hippocampal measures, no significant changes were observed when analyzing the CA1 and CA3 regions or the dentate gyrus of the hippocampus or when analyzing this structure as a whole (Supplemental figure 2).

The expression of PSA-NCAM and GAD67 is decreased in deep layers of the mPFC and that of PSA-NCAM is decreased in the stratum lucidum of the hippocampus.

To study the alterations in the expression of GAD67, PSA-NCAM and NCAM in the mPFC and hippocampus, we have performed a comparison of the immunoreactivity in the neuropil of each layer of these brain regions. Our results showed a significant decrease of GAD67 expression in layers V and VI of the mPFC induced by the treatment (F (1, 21) = 4.56, p = 0.045; F (1, 21) = 4.80, p = 0.039 respectively). A similar decrease in PSA-NCAM expression was found in these two deep layers (V and VI), but induced by the housing (F (1, 20) = 6.06, p = 0.023; F (1, 21) = 8.41, p = 0.0088 respectively). No significant changes in GAD67 or PSA-NCAM expression were found in the rest of layers of

the mPFC. No changes in the expression of NCAM were found in any layer of the mPFC (Figure 4).

In the hippocampus, PSA-NCAM expression was altered in the stratum lucidum induced by housing x treatment interaction (F (1, 23) = 4.54, p = 0.044). Follow up tests revealed a decreased PSA-NCAM expression in the Soc-MK-801group compared to the Soc-Veh group (p = 0.026). The expression of GAD67 and NCAM did not change in any region of the hippocampus (Figure 5).

Altered expression of *calbindin*, *calretinin* and *ErbB4* genes in the mPFC

In order to study the expression of different genes related to inhibitory synapses, plasticity and cell signaling, we performed quantitative RT-PCR of some transcripts (table 1) in the whole mPFC and hippocampus, using as a control gene the *Ywhaz* mRNA. The qRT-PCR analysis on the mPFC revealed a significant increase in the mRNA of the calcium binding protein *calbindin* (*CB*) in isolated animals when compared to socially reared animals. Two-way ANOVA analysis revealed significant effects of housing (F (1, 27) = 8.66, p = 0.006). By contrast, the expression of the gene of the calcium binding protein *calretinin* (*CR*) was significantly reduced in animals treated with MK-801 when compared with those injected with vehicle (F (1, 27) = 6.13, p = 0.019), showing an effect of the treatment. For the *ErbB4* receptor gene, there was a significant housing x treatment interaction (F (1, 27) = 4.97, p = 0.034). Follow up tests of simple main effects revealed that Iso-Veh animals had lower mRNA expression than Soc-Veh and Iso-MK-801 animals (p = 0.02; p = 0.041 respectively). There was no significant effect of housing or treatment in the rest of the genes studied, neither in the mPFC nor in the hippocampus (Figure 6 & supplemental figure 3).

Increased number of immature neurons in the hippocampus

The total number of doublecortin (DCX) and Ki67 expressing cells was estimated in the granule cell layer of the hippocampus of the 4 groups studied. DCX is considered a solid marker of immature neurons in this region (Brown et al. 2003) and Ki67 expression is present in proliferative cells (Kee et al. 2002). The total number of DCX expressing cells was significantly increased in isolated individuals when compared to those reared in groups (F (1, 22) = 5.6, p = 0.027). No significant changes were found in the total number of Ki67

expressing cells. Nevertheless, the correlation between the numbers of DCX and Ki67 expressing cells was high (R = 0.52, p = 0.0084) (Figure 7).

Absence of alterations in apoptotic markers in the adult mPFC and hippocampus

In order to determine the expression of apoptotic markers in the two studied areas we performed a qRT-PCR of the proapoptotic gene *Bax* and the antiapoptotic gene *Bcl2*. The *Bax*/*Bcl2* genes ratio represents a critical balance of regulatory pro-apoptotic and anti-apoptotic proteins in normal living cells, being an increase in *Bax*/*Bcl2* ratio a trigger for the apoptotic pathway. There were no significant alterations in the fold change of *Bax* or *Bcl2* expression in the mPFC or in the hippocampus. When the *Bax*/*Bcl2* ratio was analyzed, there were no changes in the different groups in any of the two brain region studied (Supplemental figure 4).

DISCUSSION

In the present study we report alterations in the volume and different parameters related to structural plasticity and inhibitory neurotransmission in a "double hit" model of schizophrenia in Lister Hooded rats, which combines a perinatal injection of the NMDA receptor antagonist MK-801 and a postweaning social isolation rearing. The combined model shows a wider spectrum of schizophrenia-like symptoms than each model by itself. The postweaning isolation rearing contributes to the increased body weight during adulthood, the increase in the activation of mPFC pyramidal neurons, the decrease in the expression PSA-NCAM and the increase in the expression of *Calbindin* in this neocortical region, as well as the increase in the number of immature neurons in the dentate gyrus. On the other hand, the perinatal NMDA antagonist treatment contributes to the decrease in hippocampal volume and to the decreases in the expression of GAD67 and *Calretinin* in the mPFC. The interaction of the two interventions contributes to the decrease in the volume and the number of parvalbumin expressing cells in the mPFC, as well as to the decrease in PSA-NCAM expression in the hippocampal stratum lucidum. In the following paragraphs we discuss these data, comparing them with previous results obtained independently in each treatment and with data obtained from postmortem studies on schizophrenic patients.

The present results show an increased weight gain in rats reared in isolation compared to those reared in groups, indicating an effect of the housing. These results are in agreement with previous studies, which reported similar changes in female Sprague Dawley rats (Hermes et al. 2011, Ness et al. 1995). Although we have not found changes in body weight in adulthood induced by the perinatal MK-801 injection, treated rats showed less weight when weighed at P21. This is in accordance with other reports using different perinatal MK-801 treatments, which have consistently found transient lower body weights that normalized in adolescence or in adulthood (Stefani and Moghaddam 2005, Su et al. 2011). The previous studies using dual animal models of schizophrenia combining postweaning social isolation rearing and acute MK-801 treatment did not explore changes in body weight (Ashby et al. 2010, Hickey et al. 2012).

In regard to volumetric changes, our results are in agreement with previous reports describing a decrease in mPFC volume in Lister Hooded rats reared in isolation (Day-Wilson et al. 2006, Schubert et al. 2009). Our study

expands these previous findings, showing that the volume changes in the mPFC appear to be due to reductions in the prelimbic and infralimbic cortices, but not in the cingulate cortices. Moreover, we have found that these volumetric changes are caused both by the social isolation rearing and by the perinatal MK-801 treatment. Another interesting result of the present study is that the hippocampal volume was also reduced in the "double hit" model, although this effect was only caused by the MK-801 injection. These volumetric reductions in the "double hit" model are extremely important, because they are very similar to those found consistently in schizophrenia: Reductions in the volume of the PFC and hippocampus have been found in these patients, even in first episodes, suggesting a marked progression at the initial stage of the disease (Levitt et al. 2010, Yoshida et al. 2011).

The volume reductions found in the hippocampus and the mPFC in our study may well be correlated with structural changes in the neuropil. In fact, reduced dendritic length has been reported in pyramidal neurons of the PFC and hippocampus, and decreased spine density was found in hippocampal pyramidal neurons in rats reared in social isolation (Silva-Gomez et al. 2003). Moreover, similar changes in dendritic atrophy and spine density reduction have been found in the PFC of schizophrenia patients (Black et al. 2004, Broadbelt et al. 2002, Glantz and Lewis 2000) and diminished spine density has been observed in the subicular pyramidal neurons (Rosoklija et al. 2000). The presence of reduced neuronal and glial size and reduced glial cell density in the PFC and the hippocampus of schizophrenic patients (Benes et al. 1991, Rajkowska et al. 1998, Stark et al. 2004, Schmitt et al. 2009) may also be responsible for the volume reductions found in these cortical regions.

Another aspect of structural plasticity, which may be relevant to the hippocampus, is the presence of alterations in adult neurogenesis. Although these alterations are far from explaining the etiology of schizophrenia, they may contribute to the hippocampal aspects of this disorder (Kempermann 2011). A study in adult human postmortem tissue has found reduced amounts of proliferating cells in the hippocampus of schizophrenic patients (Reif et al. 2006). This is in contrast with our results in the "double hit" model, showing an increase of immature granule neurons (due to social isolation rearing) and no changes in the number of proliferating cells. These results may appear to be in conflict with those found in schizophrenic patients. However, it has to be noted that a significantly higher incidence of granule cells with basal dendrites has been found in these brains (Lauer et al. 2003) and that the presence of basal

dendrites has been described as a characteristic of immature granule cells, at least in rodents (Nacher et al. 2001, Shapiro et al. 2005).

Several lines of evidence point to alterations in inhibitory circuits as one of the main factors to explain the neurobiological basis of schizophrenia (Benes and Berretta 2001, Lewis et al. 2005). In this regard, the 67-kDa isoform of glutamic acid decarboxylase (GAD67), one of the enzymes responsible of GABA synthesis, is one of the most affected molecules (See Curley et al. 2011, Akbarian and Huang 2006, Beneyto and Lewis 2011 for review). Reduced expression of the GAD67 mRNA in the PFC (Akbarian et al. 1995, Guidotti et al. 2000, Hashimoto et al. 2008, Torrey et al. 2005) and the hippocampus (Thompson Ray et al. 2011) is one of the most consistent findings in postmortem studies of individuals with schizophrenia. Similar decreases in GAD67 protein expression in the PFC and the hippocampus of schizophrenics have been found (Torrey et al. 2005), including those reported recently by our group (Gilabert-Juan et al. 2012b). The present results are partially in accordance with the findings in schizophrenic brains, showing a significant decrease of GAD67 protein expression in layers V and VI of the mPFC, although no changes were detected in the hippocampus. No differences in the expression of GAD67 mRNA were detected in our study, suggesting the presence of posttranscriptional alterations or the presence of downregulations of mRNA expression prior to the age at which our animals were sacrificed. The alterations in mPFC GAD67 protein expression were present in the “double hit” model, although they were induced only by MK-801 perinatal injection. Interestingly, previous reports using perinatal treatments with NMDA receptor antagonists have failed to find significant differences in GAD67 expression (Facchinetti et al. 1993). It is possible that this discrepancy with our results may be due to differences in the strain (Wistar) or the dosis/duration of the treatment (chronic treatment for 22 days) used by Facchinetti et al. (1993).

Our results are in agreement with those reported recently by Hickey et al. (2012) using a similar “double hit” model combining social isolation rearing and MK-801 treatment, which describe increases in the activity of the GABA transporter (GAT1) and the expression of the $GABA_A$ receptor, both in the PFC and the hippocampus. Their results also suggest a reduction in inhibitory neurotransmission, together with a compensatory response of GABA receptors to the decreased GABA availability. However, one should be cautious when comparing the two models, because Hickey and collaborators injected MK-801 at P56 (after the isolation paradigm) and twice daily for 7 days.

In addition to these changes in GAD67 expression, recent findings indicate that schizophrenia is associated with different alterations in certain interneuronal subpopulations, specially parvalbumin expressing cells, which may have an important impact on the physiology of pyramidal cells (see Lewis et al. 2012 for review). The number of parvalbumin expressing interneurons does not appear to be reduced in schizophrenic patients, at least in the PFC, but they exhibit reduced expression of parvalbumin mRNA and lower density of parvalbumin expressing puncta in certain layers, among other abnormalities at the presynaptic and postsynaptic level (see Beneyto and Lewis 2011 for review). Our “double hit” model also shows alterations in parvalbumin expressing cells in the mPFC. The number of these interneurons is reduced significantly in the infralimbic cortex, although trends toward decreases were also found in the rest of regions studied. This effect was due to MK-801 treatment and it is in accordance with previous reports describing similar reductions after acute perinatal treatment with MK-801 (Coleman et al. 2009, Wang et al. 2008). It is probable that this reduction in the number of parvalbumin expressing cells is due to the extensive cell death caused by the perinatal NMDA antagonist administration during perinatal development (see Lim et al. 2012 for review); we have not found evidence of cell death at the time of sacrifice. Although we have not found alterations in the expression of parvalbumin mRNA in the present study, we have observed differential changes in calbindin and calretinin expression, which certainly make necessary future experiments to evaluate more closely the subpopulations of interneurons expressing these calcium binding proteins. Although apparently the calretinin subpopulation is not affected in schizophrenic patients, the calbindin subpopulation may be altered (see Lewis and Hashimoto 2007 for review).

Our study on the expression of c-Fos in the mPFC also gives support to the idea that prefrontocortical inhibition is decreased in the “double hit” model and, consequently, this may lead to an excessive activation of excitatory neurotransmission, since we have found an increase in the expression of this marker of cell activity in the nuclei of pyramidal neurons. Interestingly, the increase in c-Fos expression is found in the same region, the prelimbic cortex, where a significant reduction in parvalbumin expressing interneurons has been observed.

Although our results show an interaction, which prevents the observation of changes in *ErbB4* mRNA in the “double hit” model, we still find very interesting that social isolation alone is capable of decreasing its expression.

This is the first report describing this decrease in this schizophrenia model. A previous report found decreased ErbB4 and p-ErbB4 expression in the adult mPFC after perinatal treatment with an NMDA receptor antagonist (phencyclidine) (du Bois et al. 2012). However, it has to be noted that in this model the animals received three injections of the NMDA receptor antagonist, on postnatal days 7, 9 and 11, while only one injection was used in our model. *ErbB4* and its ligand *Nrg1* are important risk genes for schizophrenia (Buonanno 2010, Norton et al. 2006) and their signaling controls the development of inhibitory cortical networks, regulating the connectivity of certain interneuronal populations, particularly parvalbumin expressing basket and chandelier cells (Fazzari et al. 2010). Consequently, alterations in *ErbB4* during postnatal development and adolescence, such as those induced by post-weaning social isolation rearing, must interfere with the final establishment of cortical connectivity, specially that involving inhibitory neurons.

In connection with the changes induced by the “double hit” model discussed above regarding inhibitory circuits, we find very interesting our result indicating a similar decrease in PSA-NCAM expression and GAD67 in the deep layers (V and VI) of the mPFC. This provides a putative link between changes in inhibitory neurotransmission and structural plasticity. In this regard, previous work in our laboratory has found that a subpopulation of interneurons in the mPFC of adult humans and rodents expresses PSA-NCAM (Gomez-Climent et al. 2011, Varea et al. 2005, Varea et al. 2007b) and that these inhibitory neurons display reduced dendritic arborization and spine density when compared with interneurons lacking this molecule (Gomez-Climent et al. 2011). Parallel alterations in the expression of GAD67, synaptophysin and PSA-NCAM have been observed in the mPFC in rats with pharmacological manipulations of dopamine D2 receptors and these changes are blocked when PSA is depleted from the mPFC (Castillo-Gomez et al. 2008, Castillo-Gomez et al. 2011). Changes in PSA-NCAM expression also occur in parallel to the stress-induced dendritic remodeling of interneurons, at least in the amygdala (Gilabert-Juan et al. 2011). Moreover, the reductions in PSA-NCAM and GAD67 expression observed in the “double hit” model are similar to those we have found in the mPFC of schizophrenic patients (Gilabert-Juan et al. 2012b). We have not found any change in the expression of NCAM or the polysialyltransferases, suggesting that this cell adhesion molecule is apparently unaffected in this model and that changes in the expression of the enzymes responsible for the addition of PSA to NCAM may have occurred previously to sacrifice or that the

reduction of PSA-NCAM is due to other factors, such as an enhancement of its removal from the plasma membrane.

We have not performed behavioral analysis in the present study, but a similar “double hit” model shows alterations in locomotor activity, which may be the result of decreased inhibitory neurotransmission (Hickey et al. 2012). Future experiments should explore whether other behavioral alterations resembling some of those observed in schizophrenia, which have been described independently in the perinatal MK-801 injections and in the social isolation rearing models, persist in the “double hit” model. Such alterations include impaired social interactions, deficits in sensory motor gating, hyperactivity in a novel environment, as well as impaired cognitive flexibility, reversal learning, and novel object discrimination (for review see Fone and Porkess 2008, Lim et al. 2012).

Although many of the parameters analyzed in our study appear to act through independent mechanisms, we find that, using their combination, this “double hit” model can be a very valuable experimental tool to mimic a wider spectrum of specific symptoms and alterations of schizophrenia, specially those affecting inhibitory neurotransmission, and to serve as a testing platform for novel treatments directed to this devastating disorder.

ACKNOWLEDGEMENTS: Spanish Ministry of Science and Innovation (MICINN-FEDER) BFU2009-12284/BFI, MICINN-PIM2010ERN-00577/NEUCONNECT in the frame of ERA-NET NEURON", Generalitat Valenciana ACOMP/2012/229 and the Fundación Alicia Koplowitz to JN. Javier Gilabert-Juan has a FPU predoctoral fellowship from the Spanish Ministry of Education (AP2008-00937).

FIGURE LEGENDS

Figure 1. Stereological estimates of mPFC and hippocampal volumes. Histograms show the estimated volumes of the prelimbic, infralimbic, cingulate 1 and cingulate 2 cortices, as well as those of the whole mPFC and hippocampus in Soc-Veh, Iso-Veh, Soc-MK-801 and Iso-MK-801groups. Values represent mean±S.E.M. # $p < 0.05$ by housing. * $p < 0.05$ by treatment. ** $p < 0.01$ by treatment.

Figure 2. Histograms showing the percentages of CaMKII expressing pyramidal neuron somata colocalizing c-fos in their nuclei in the prelimbic, infralimbic, cingulate 1, cingulate 2 cortices and in the whole mPFC. # $p < 0.05$ by housing. Images show cells expressing CaMKII (green) in their soma and c-fos (blue) in their nucleus in the infralimbic region of the mPFC. Scale bar: 10 μm.

Figure 3. Somata expressing parvalbumin protein in the mPFC. Histograms show the differences in the total number of cells expressing parvalbumin in their somata. Values represent mean±S.E.M. * $p < 0.05$ by treatment. ‡ $p < 0.05$ in the Iso-MK801group compared to the Iso-Veh group. Images show parvalbumin expressing somata in the four groups (Soc-Veh, Iso-Veh, Soc-MK-801 and Iso-MK-801) in the infralimbic region. Scale bar: 200 μm.

Figure 4. Neuropil immunoreactivity of GAD67, PSA-NCAM and NCAM in the mPFC. Histogram bars show the grey level measured in the five layers (I, II, III, V and VI) of Soc-Veh, Iso-Veh, Soc-MK-801 and Iso-MK-801 groups. Data are the mean±S.E.M. # $p < 0.05$ by housing, * $p < 0.05$ by treatment, ** $p < 0.01$ by treatment.

Figure 5. Neuropil immunoreactivity of GAD67, PSA-NCAM and NCAM in the hippocampus. Histogram bars show the grey level measured in the three regions (radiatum, oriens and lucidum) of Soc-Veh, Iso-Veh, Soc-MK-801 and Iso-MK-801 groups. Data are the mean±S.E.M. ‡ $p < 0.05$ in the Soc-MK801group compared to the Soc-Veh group.

Figure 6. Relative gene expression. qRT-PCR mRNA fold change data for the four studied groups (Soc-Veh, Iso-Veh, Soc-MK801 and Iso-MK-801) in the mPFC for the genes *St8siaII, St8siaIV, Gad67, NCAM, SYN, parvalbumin, calbindin,calretinin, CaMKII, ErbB4* and *Nrg1*. The expression of all studied genes was normalized using *Ywhaz* as a control gene. # # $p < 0.01$ by housing, * $p < 0.05$ by treatment. ‡ $p < 0.05$ by single group effect.

Figure 7. DCX and Ki67 expression in the hippocampus. Histograms show the differences in the total number of cells expressing DCX and Ki67 in their somata and nuclei respectively. Values represent mean±S.E.M. # $p < 0.05$ by housing. The graph on the right side of the figure shows the correlation of the number of DCX expressing somata with Ki67 expressing nuclei in each individual. White circles represent Soc-Veh individuals, black circles represent Iso-Veh

individuals, white squares represent Soc-MK-801 individuals and black squares represent Iso-MK801 individuals (R = 0.52, p = 0.0084).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental figure 1. Histograms showing the weight of animals at 21 PND in the group injected with saline solution (Control) and the group injected with MK-801 (MK801). Values represent mean±S.E.M. ** $p < 0.01$. The final weight graph shows the histograms indicating the weight at the end of the experiment for each group (Soc-Veh, Iso-Veh, Soc-MK801 and Iso-MK-801). Values represent mean±S.E.M. # $p < 0.05$ by housing.

Supplemental figure 2. Somata expressing parvalbumin protein in the hippocampus. Histograms show the differences in the total number of cells expressing parvalbumin in their somata. Values represent mean±S.E.M.

Supplemental figure 3. Relative gene expression. qRT-PCR mRNA fold change data for the four studied groups (Soc-Veh, Iso-Veh, Soc-MK801 and Iso-MK-801) in the hippocampus for the genes *St8siaII, St8siaIV, Gad67, NCAM, SYN, parvalbumin, calbindin, calretinin, CaMKII, ErbB4* and *Nrg1*. The expression of all studied genes was normalized using *Ywhaz* as a control gene.

Supplemental figure 4. Expression of apoptosis-related genes. Histograms show the fold change of the proapoptotic gene *Bax*, the antiapoptotic gene *Bcl2* and the *Bax/Bcl2* relationship in the mPFC and the hippocampus for each group (Soc-Veh, Iso-Veh, Soc-MK-801 and Iso-MK-801).

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TABLES

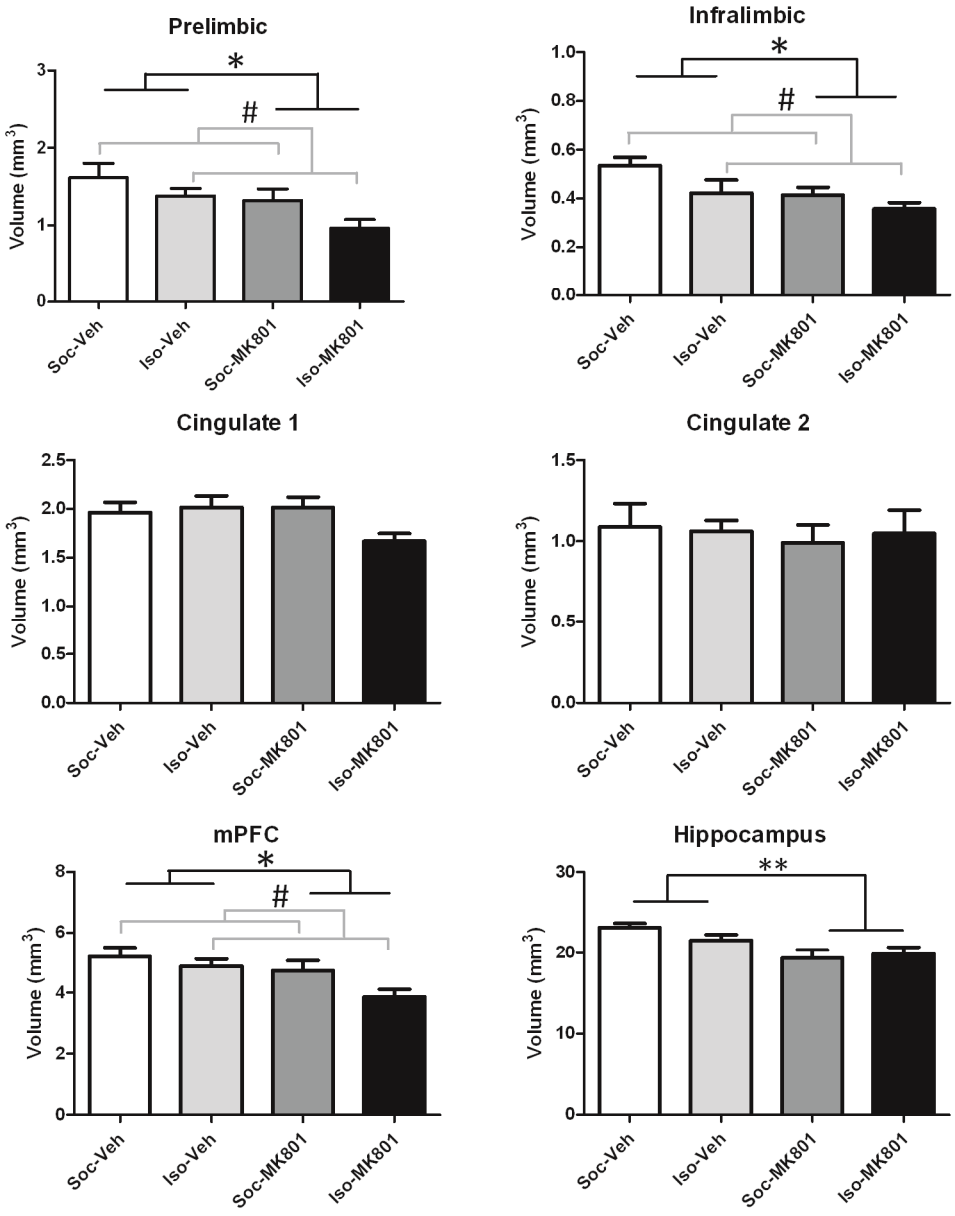
Table 1. Sequences of gene specific primers and associated amplicon lengths for qRT-PCR.

Target gene	Primers	Sequence (5' → 3')	Amplicon size(1)
ST8SiaII	Forward	GGCAACTCAGGAGTCTTGCT	123
	Reverse	GTCAGTCTTGAGGCCCACAT	
ST8SiaIV	Forward	CCTTCATGGTCAAAGGAGGA	125
	Reverse	CCAGTAACCTCTGACCGCAT	
GAD67	Forward	CTGGAGCTGGCTGAATACCT	120
	Reverse	TCGGAGGCTTTGTGGTATGT	
NCAM	Forward	AACGGACTCCAAACCATGAC	123
	Reverse	CTGGCTTTGCTTCTGACTCC	
SYN	Forward	CTATGGGCAGCAAGGCTATG	120
	Reverse	CAGGCCTTCTCTTGAGCTCTT	
Pvalb	Forward	AAGAGTGCGGATGATGTGAAG	150
	Reverse	AGCCATCAGCGTCTTTGTTT	
Calbindin	Forward	AGGGATGTGCTTCTGCTTGT	171
	Reverse	CATCTGGCTACCTTCCCTTG	
Calretinin	Forward	GTGGTGGGTGGGTCACACGG	179
	Reverse	GGAATTGCGGGGGTCAGTGGG	
CaMKII	Forward	ACCATCAACCCGTCCAAAC	152
	Reverse	ATGGCTCCCTTCAGTTTCCT	
ErbB4	Forward	CAGTCGCCCAGGGTGCAACG	133
	Reverse	GCGAACACTGTGGGGTCGGC	
Nrg1	Forward	GCTCCGGTGCGAAACCAGCT	133
	Reverse	TCGAAGCTCTGACTTCCCTGGCT	
BAX	Forward	AAACTGGTGCTCAAGGCCCT	92
	Reverse	AGCAGCCGCTCACGGAG	
BCL2	Forward	CCGGGAGAACAGGGTATGATAA	81
	Reverse	CCCACTCGTAGCCCCTCTG	
Ywhaz	Forward	TTGAGCAGAAGACGGAAGGT	136
	Reverse	GAAGCATTGGGGATCAAGAA	

(1) Amplicon length in base pairs.

FIGURES

Figure 1



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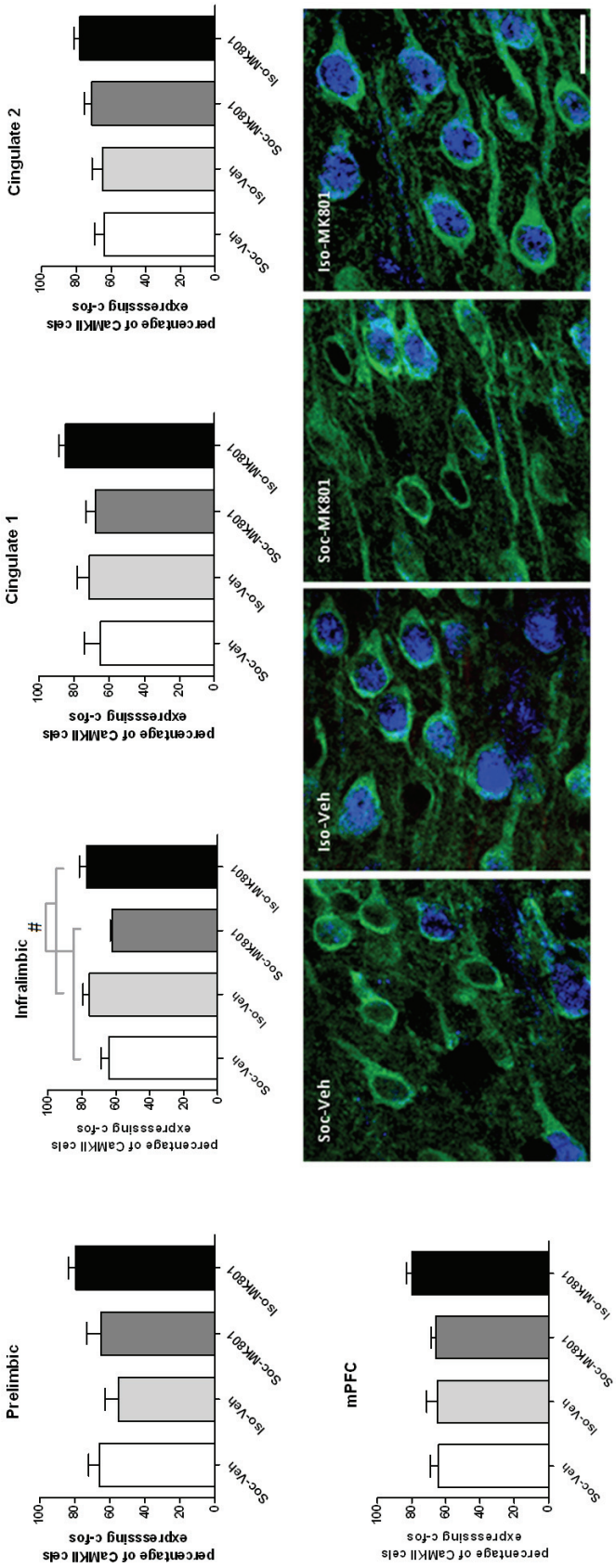

Cingulate 2
Cingulate 1
Infralimbic
Prelimbic
mPFC
percentage of CaMKII cells expressing c-fos
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801
#

Figure 3

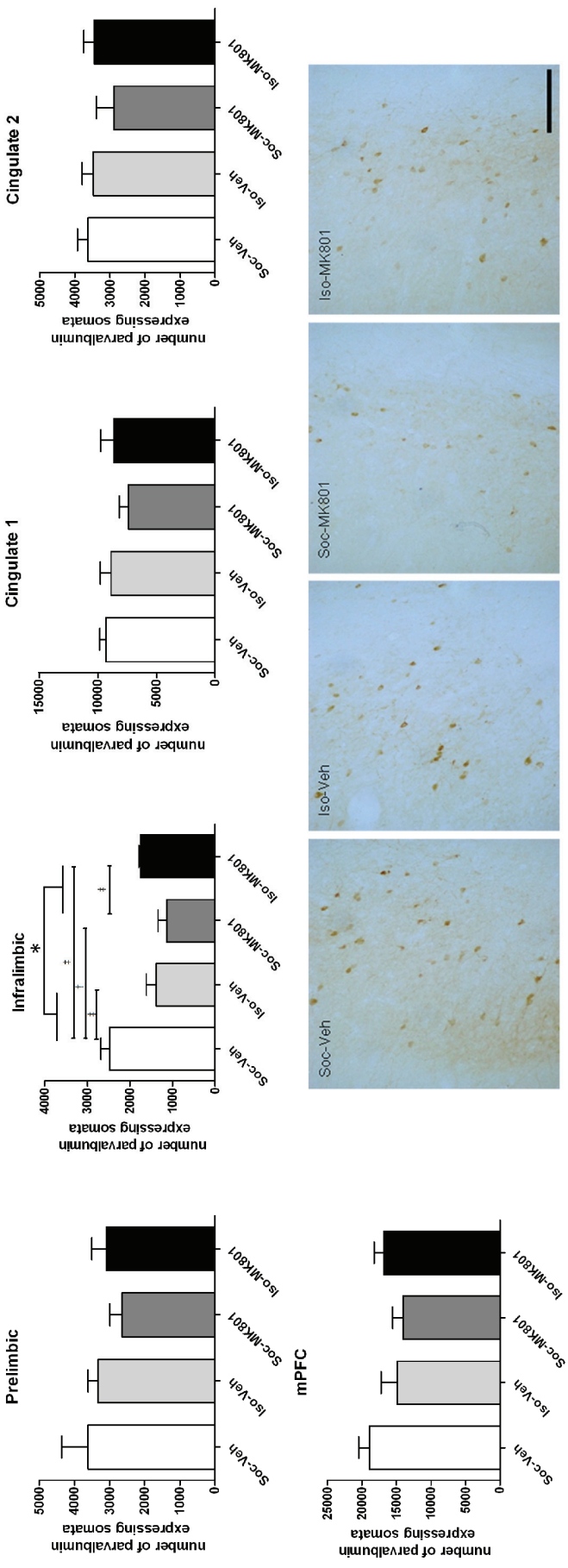

Cingulate 2
Cingulate 1
Infralimbic
Prelimbic
mPFC
number of parvalbumin expressing somata
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801

Figure 4

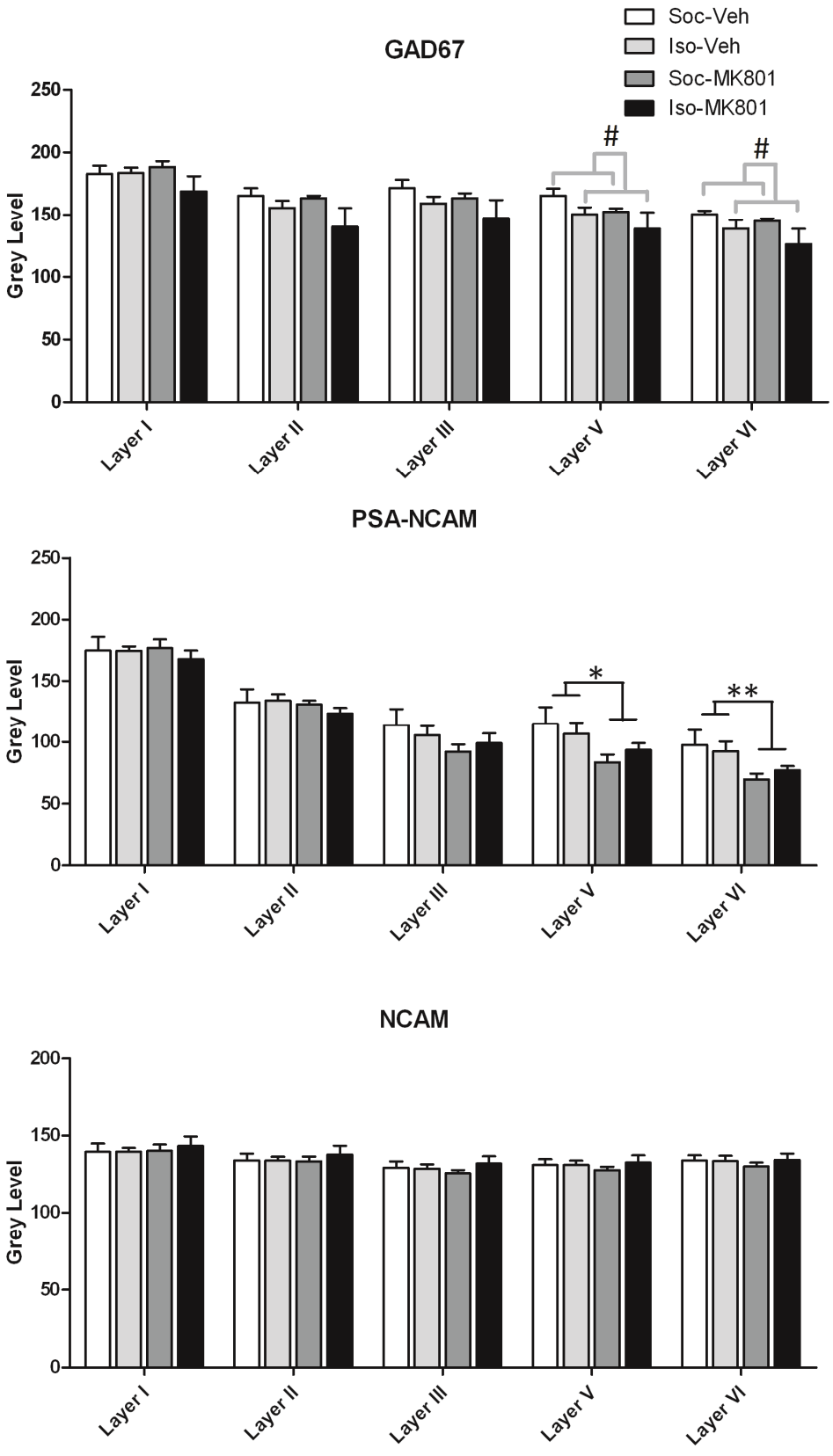
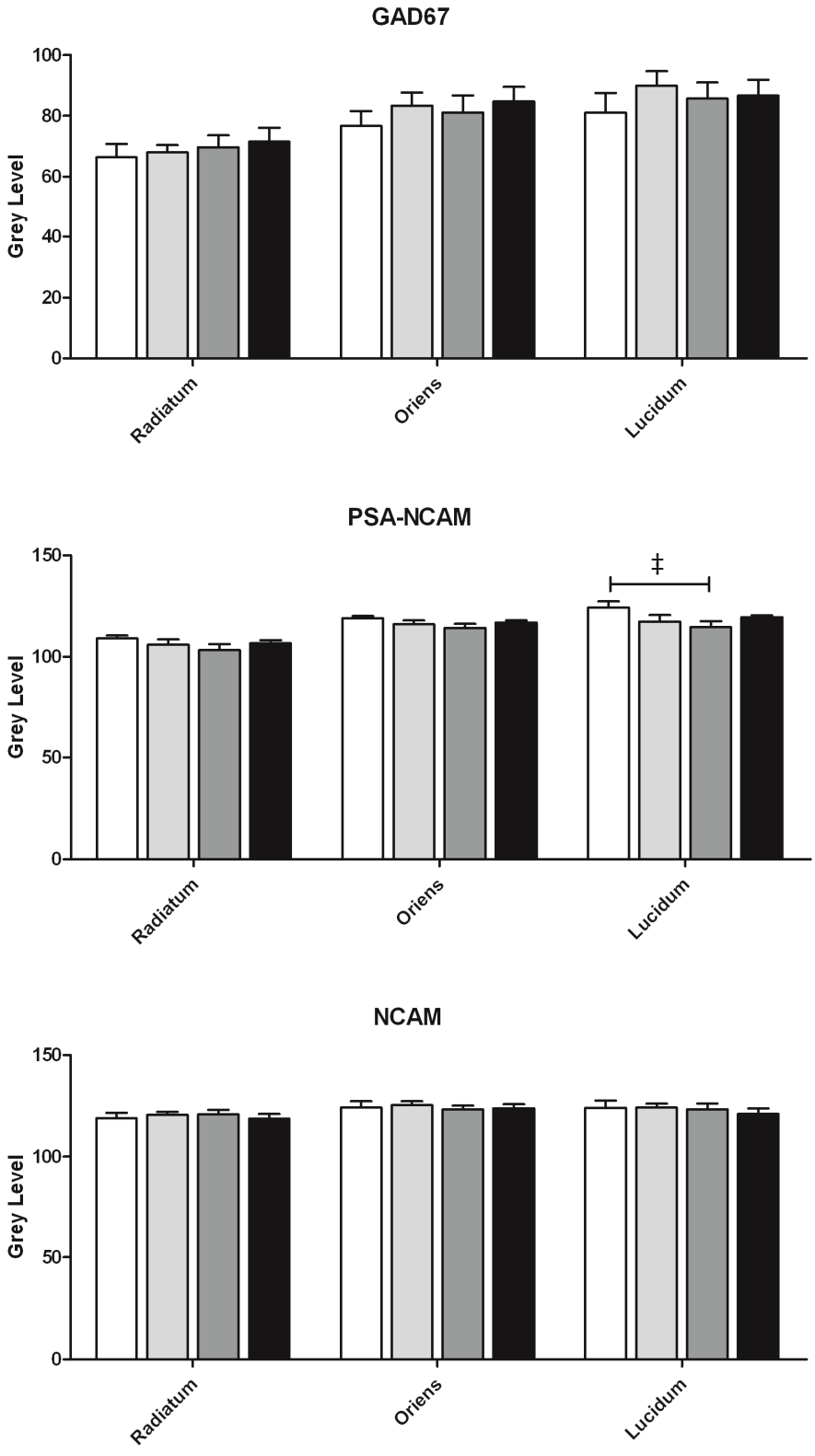

GAD67
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801
Grey Level
250
200
150
100
50
0
#
#
Layer I
Layer II
Layer III
Layer V
Layer VI
PSA-NCAM
Grey Level
250
200
150
100
50
0
*
**
Layer I
Layer II
Layer III
Layer V
Layer VI
NCAM
Grey Level
200
150
100
50
0
Layer I
Layer II
Layer III
Layer V
Layer VI

Figure 5


GAD67
Grey Level
100
80
60
40
20
0
Radiatum
Oriens
Lucidum
PSA-NCAM
Grey Level
150
100
50
0
‡
Radiatum
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Grey Level
150
100
50
0
Radiatum
Oriens
Lucidum

Figure 6

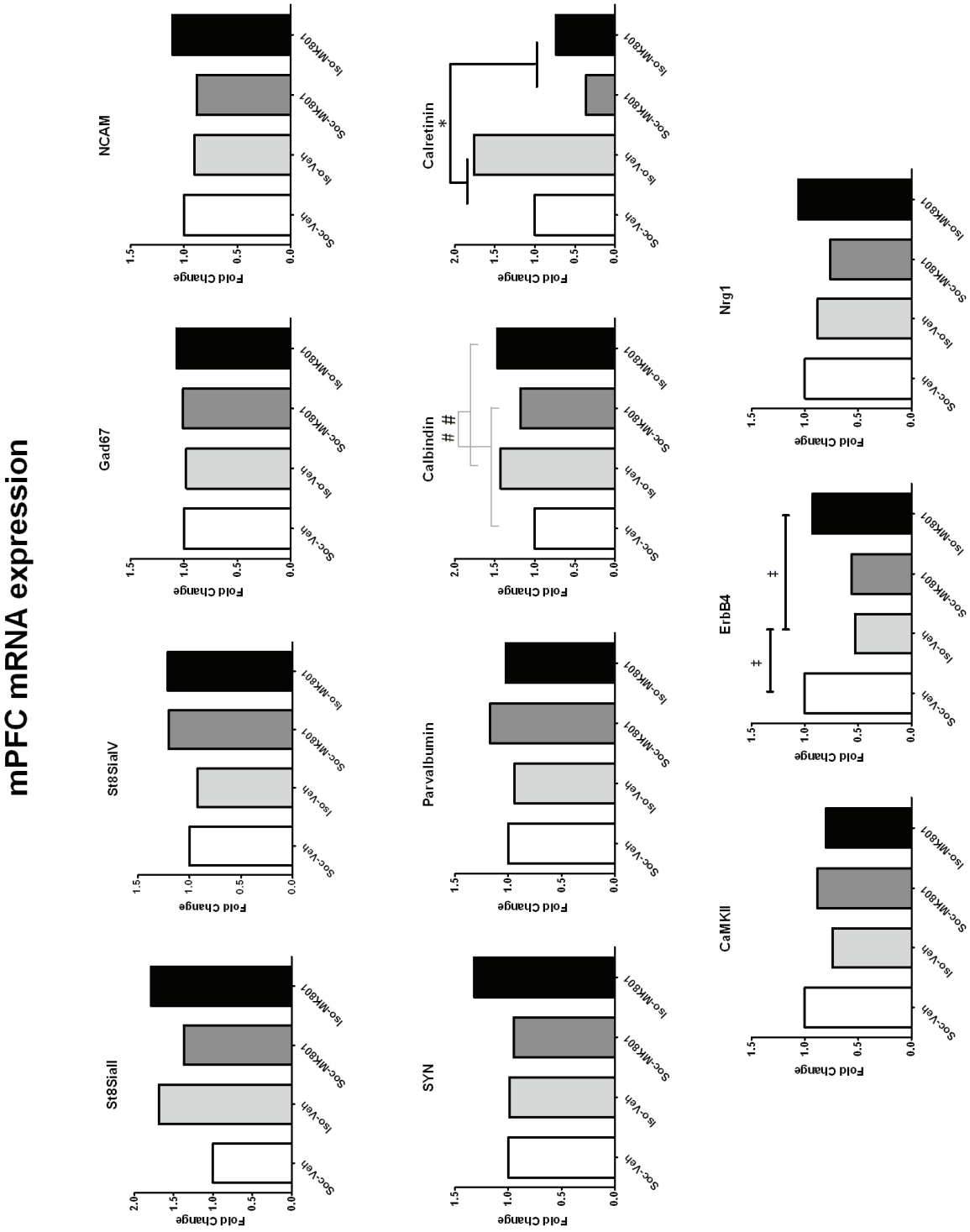
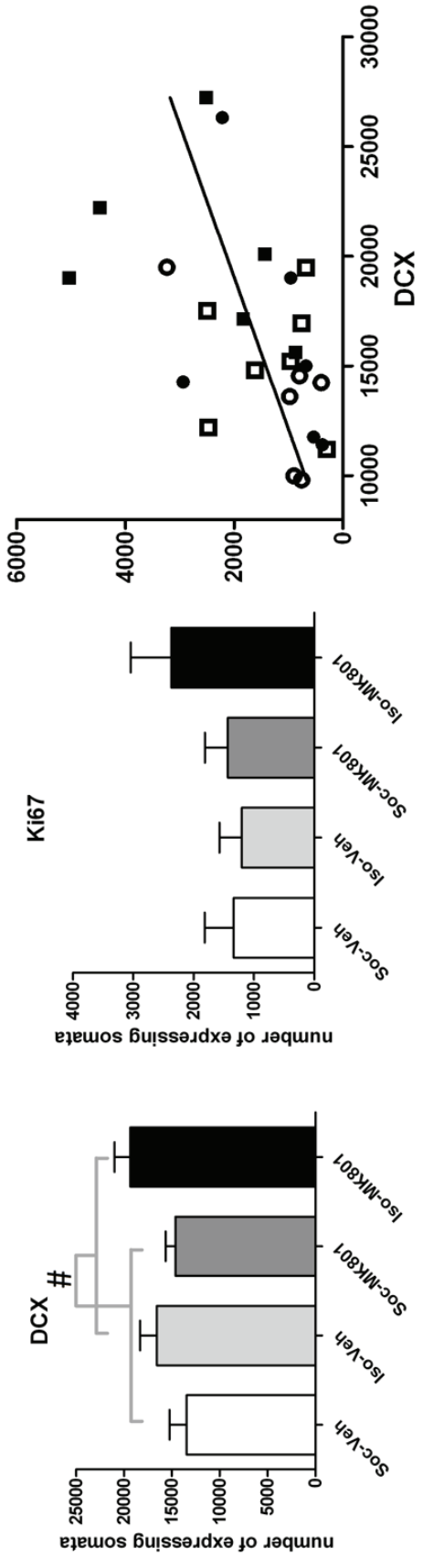

mPFC mRNA expression
NCAM
Calretinin
Nrg1
Gad67
Calbindin
ErbB4
St8SiaV
Parvalbumin
CaMKII
St8SiaII
SYN
Fold Change
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801
*
# #
+
+

Figure 7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Ki67
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2000
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0
Soc-Veh
Iso-Veh
Soc-MK801
Iso-MK801
DCX #
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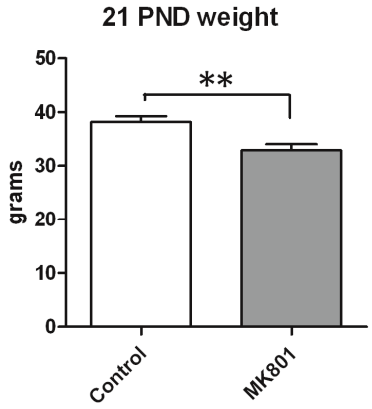
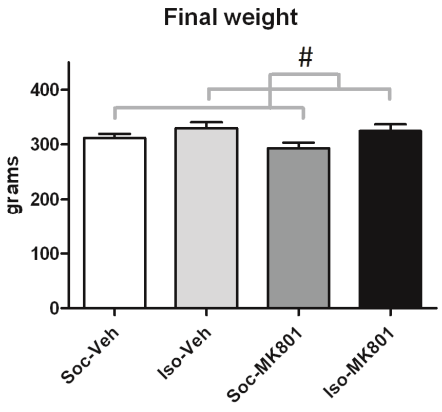
Iso-Veh
Soc-MK801
Iso-MK801

Supplemental figure 1





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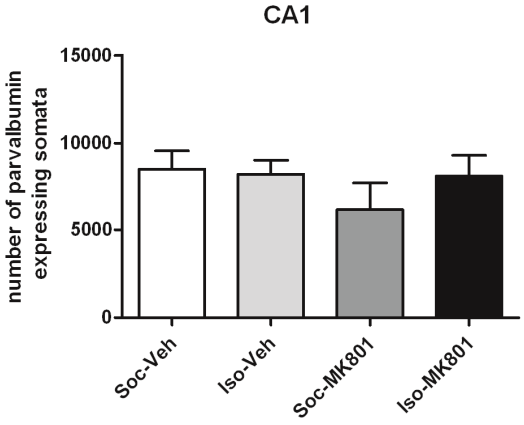

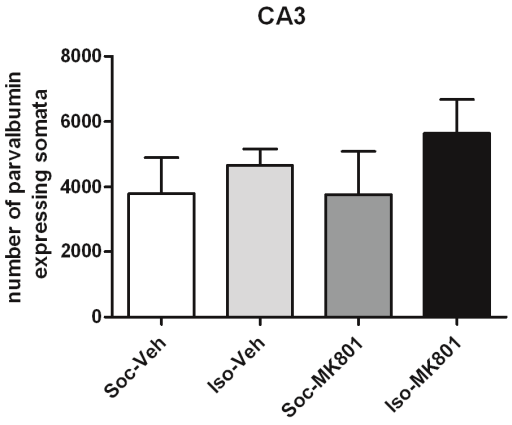

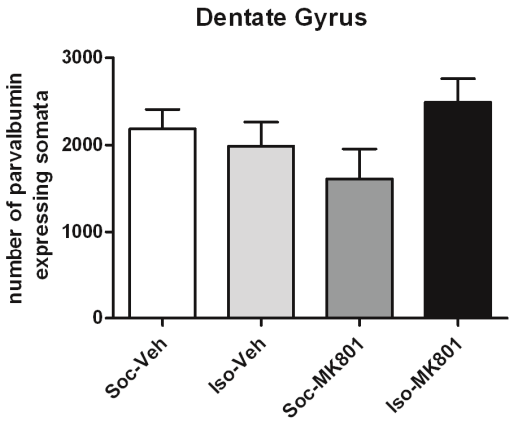

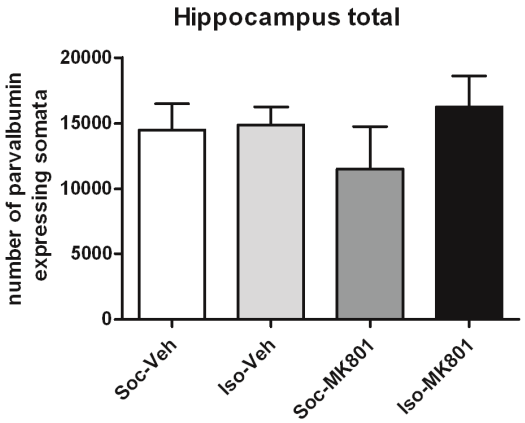

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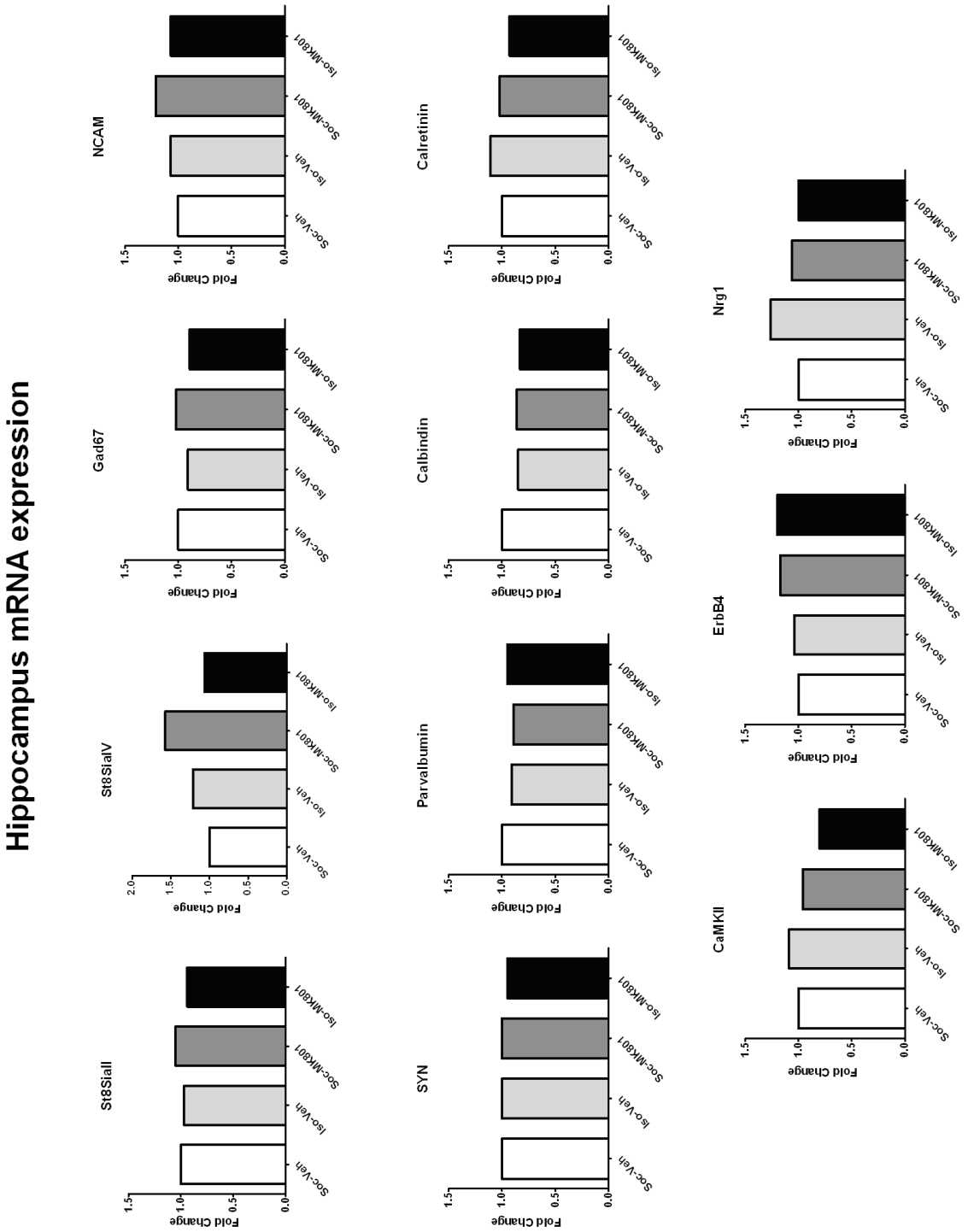

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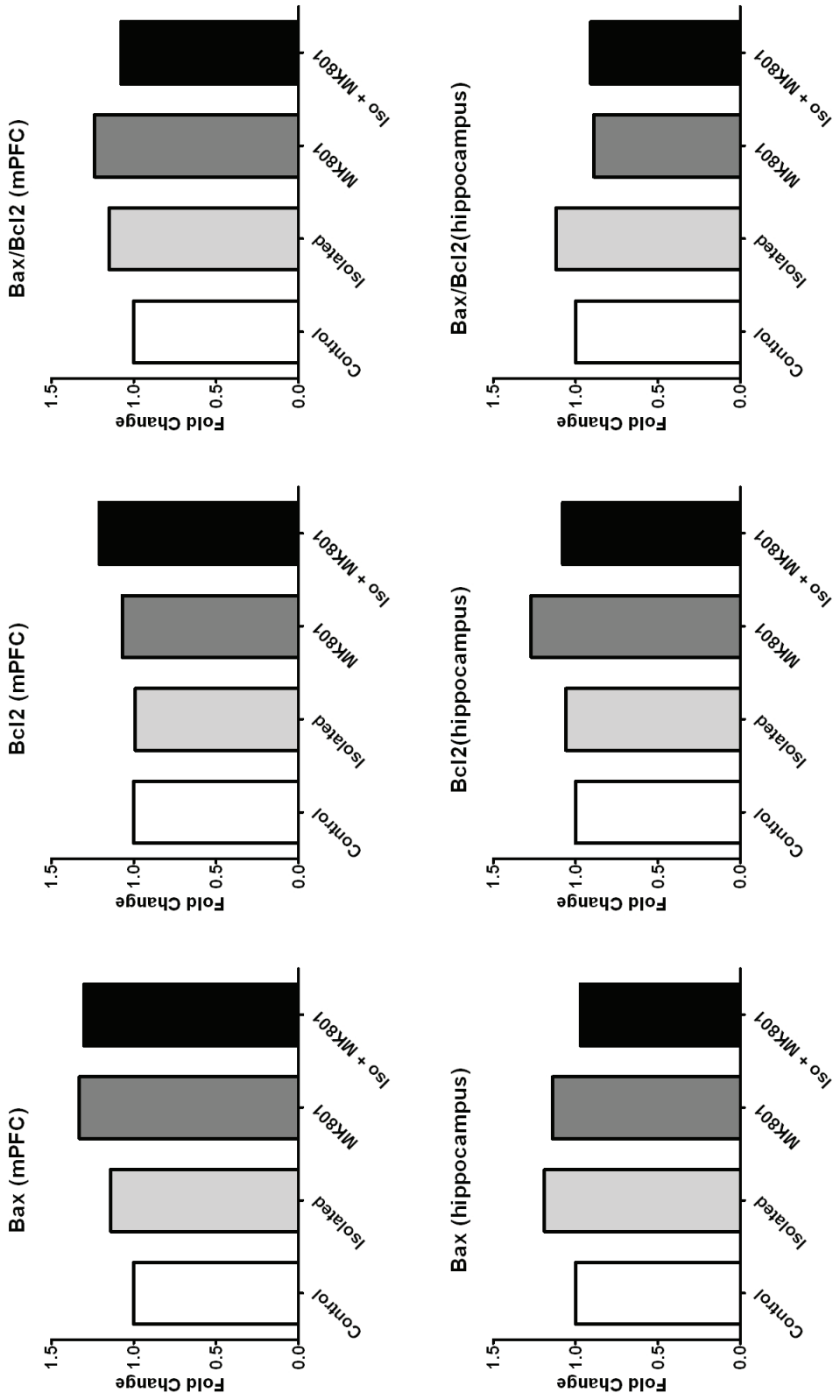

Article 5: Alterations in the expression of PSA-NCAM and synaptic proteins in the dorsolateral prefrontal cortex of psychiatric disorder patients

Neuroscience Letters 530 (2012) 97–102

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Alterations in the expression of PSA-NCAM and synaptic proteins in the dorsolateral prefrontal cortex of psychiatric disorder patients

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HIGHLIGHTS

- Synaptic and plasticity markers are altered in the DLPFC in psychiatric disorders.
- There is a reduction in the complexity of the circuitry for all these disorders.
- In schizophrenic patients there is a reduction in the plasticity molecule PSA-NCAM.

ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form
12 September 2012
Accepted 18 September 2012

Keywords:
Major depression
Schizophrenia
Synaptophysin
Glutamate decarboxylase
Structural plasticity
PSA-NCAM

ABSTRACT

Alterations in the structure and physiology of the prefrontal cortex (PFC) have been found in different psychiatric disorders and some of them involve inhibitory networks, especially in schizophrenia and major depression. Changes in the structure of these networks may be mediated by the polysialylated neural cell adhesion molecule (PSA-NCAM), a molecule related to neuronal structural plasticity, expressed in the PFC exclusively by interneurons. Different studies have found that PSA-NCAM expression in the hippocampus and the amygdala is altered in schizophrenia, major depression and animal models of these disorders, in parallel to changes in the expression of molecules related to inhibitory neurotransmission and synaptic plasticity. We have analyzed post-mortem sections of the dorsolateral PFC from the Stanley Neuropathology Consortium, which includes controls, schizophrenia, bipolar and major depression patients, to check whether similar alterations occur. PSA-NCAM was found in neuronal somata and neuropil puncta, many of which corresponded to interneurons. PSA-NCAM expression was only reduced significantly in schizophrenic patients, in parallel to a decrease in glutamic acid-decarboxylase-67 (GAD67) and to an increased expression of vesicular glutamate transporter 1 (VGLUT1) in the white matter. Depressed patients showed significant decreases in synaptophysin (SYN) and VGLUT1 expression. Whereas in bipolar patients, decreases in VGLUT1 expression have also been found, together with a reduction of GAD67. These results indicate that the expression of synaptic proteins is altered in the PFC of patients suffering from these disorders and that, particularly in schizophrenia, abnormal PSA-NCAM and GAD67 expression may underlie the alterations observed in inhibitory neurotransmission.

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1. Introduction

Schizophrenia, bipolar disorder, and major depression are devastating mental diseases, each with distinctive yet overlapping characteristics. Alterations in the structure and function of the prefrontal cortex (PFC) seem to be common features to all of them. Interestingly, these disorders, specially schizophrenia and major depression, as well as animal models mimicking some of their symptoms, are also particularly characterized by abnormalities involving prefrontocortical inhibitory networks. Schizophrenic patients have impaired cognitive and executive functions associated with the PFC [19,28,41], which correlate with a reduction in the number of interneurons [3,21]. Moreover, other studies have also revealed a reduction in the expression of the transcript for the 67-kDa isoform of glutamic acid decarboxylase (GAD67) [1,12,39,40]. Several lines of evidence coming from both animal and human studies also indicate the involvement of the GABAergic system in the pathophysiology of major depression [20,23,27,29].

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http://dx.doi.org/10.1016/j.neulet.2012.09.032

These alterations in the inhibitory neurotransmission of the PFC may be mediated by changes in the structure and connectivity of interneurons. The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is an extremely interesting candidate to mediate these changes, because it is expressed in the PFC of humans, specifically in inhibitory neurons [35]. These interneurons have more reduced structural features and synaptic input than those lacking this molecule [16]. Moreover, changes in the levels of PSA-NCAM expression occur in parallel to the structural remodeling of interneurons in the mouse amygdala after chronic stress, an animal model of depression [14]. In addition, manipulation of serotoninergic [34] or dopaminergic [6] neurotransmission, two monoamines critically involved in the etiology and treatment of psychiatric disorders, induces alterations in PSA-NCAM expression in the PFC. These changes are accompanied by alterations in the expression of proteins related to general and inhibitory neurotransmission and may indicate the presence of synaptic remodeling in inhibitory networks. Finally, a recent report from our laboratory using the same brain collection of the present study, has found alterations in the expression of PSA-NCAM, synaptophysin (SYN) and/or GAD67 in the amygdala of major depression, bipolar and schizophrenic patients [37].

In order to know whether similar changes can be detected in the PFC using immunohistochemistry, we have analyzed the expression of PSA-NCAM and that of SYN, vesicular glutamate transporter type 1 (VGLUT1) and GAD67, markers of generic, excitatory and inhibitory synapses respectively. We have performed our analyses in the dorsolateral PFC from post-mortem samples of the Stanley Foundation Neuropathology Consortium, which includes tissue from control, schizophrenia, major depression and bipolar disorder patients. In order to confirm whether, as in rodents [6], puncta expressing PSA-NCAM in the neuropil have an inhibitory nature, we have studied the neurochemical phenotype of these elements using fluorescence immunohistochemistry and confocal analysis.

2. Materials and methods

2.1. Samples and histological processing

Frozen 14 μm thick coronal sections containing the dorsolateral prefrontal cortex (DLPFC) of patients diagnosed with schizophrenia, bipolar disorder, or major depression and normal controls were obtained from the Stanley Medical Research Institute (Bethesda, MD, USA). All patient records were reviewed by one psychiatrist and summarized in narrative form, and the information was entered into a computerized database by identifying number only. When all the information was collected, a DSM-IV psychiatric diagnosis was made independently by two senior psychiatrists. If there was disagreement between them, the records were given to a third senior psychiatrist and a consensus diagnosis was arrived at [33]. The cohort consists of 15 individuals in each group. The demographic data and recruitment of these patients has been described earlier [33] and are summarized in supplemental table 1. The average pH measurements of each group was 6.2 and the post-mortem interval (PMI) for the bipolar, major depressive, schizophrenia and control groups were 32.5, 27.5, 33.7, and 23.7 h, respectively. All brains underwent clinical neuropathological examination by two neuropathologists, none demonstrated evidence of neurodegenerative changes or other pathological lesions.

Sections were thawed and immediately fixed by immersion in a solution of paraformaldehyde 2.5% in a lysine–phosphate buffer, pH 7.4 for 20 min at room temperature. The lysine–phosphate buffer was prepared 1:1 from a solution of phosphate buffer 0.1 M pH 7.4 (PB) and a solution 0.2 M of lysine adjusted to pH 7.4 using a solution of Na_2HPO_4 0.1 M. The buffer was mixed with a concentrated solution of paraformaldehyde 3:1 and 0.214 g of sodium peryodate was added for each 100 ml just before use. After fixation, sections were washed in phosphate buffer (PB, 0.1 M, pH 7.4) and processed immediately for immunohistochemistry.

Frozen human samples from the DLPFC, Brodmann area 9, were also obtained from the Neurological Tissues Bank of the University of Barcelona. This tissue was used to perform the phenotypical analysis of PSA-NCAM immunoreactive puncta. Samples were obtained from five subjects without any neurological abnormality, the average age was 59.6 years (42–74) and the time post-mortem before freezing the samples was 8.5 h (3.5–17.5). The tissue was unfrozen and fixed by immersion in a solution of paraformaldehyde 2.5% in a lysine–phosphate buffer, as described above. After fixation, samples were washed using PB and 50 μm sections were obtained using a vibratome. Sections were then postfixed in the same solution for 20 min. After fixation, sections were washed in PB and maintained in PB with sodium azide 0.05% at 4 °C until used.

All the sections studied passed through the procedures simultaneously, to minimize any difference from histochemical and immunohistochemical protocols themselves.

2.2. Immunohistochemistry for conventional light microscopy

Tissue was processed for immunohistochemistry as follows. Briefly, sections were incubated for 1 min in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100 °C. After cooling down the sections to room temperature, they were incubated with 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 5% normal donkey serum (NDS) (Jackson Laboratories) in PBS with 0.2% Triton-X100 (Sigma) and then incubated at 4 °C during 72 h in mouse monoclonal Men-B anti-PSA-NCAM IgM (1:1400, Abcys), mouse monoclonal anti-SYN IgG (SYN, 1:200, Sigma), rabbit polyclonal anti-GAD67 (1:500, Chemicon Int.) or guinea pig anti-VGLUT1 (1:1000, Chemicon Int.) antibodies. After washing, sections were incubated for 60 min in biotinylated donkey anti-mouse IgM, donkey anti-mouse IgG, donkey anti-rabbit IgG or donkey anti-guinea pig IgG antibodies respectively (Jackson Laboratories, 1:250), followed by an avidin–biotin–peroxidase complex (ABC, Vector Laboratories) for 30 min in PBS. Color development was achieved by incubating in 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and H_2O_2 for 4 min. PBS containing 0.2% Triton-X100 and 3% NDS was used for primary and secondary antibodies dilution. After staining, sections were washed, dehydrated and mounted with Eukitt.

2.3. Quantification of neuropil immunoreactivity

We determined PSA-NCAM, SYN, GAD67 and VGLUT1 immunoreactivity intensity in the neuropil of the different regions studied using a previously described methodology [34]. Sections were examined under bright-field illumination, homogeneously lighted and digitalized using a CCD camera. Photographs were taken at 20× magnification and grey levels (GL) were evaluated using Image J software (NIH). Five images were taken from each sample.

2.4. Characterization of the phenotype of PSA-NCAM expressing elements in the neuropil of the human amygdala

In order to characterize the inhibitory nature of the PSA-NCAM positive elements in the neuropil of the human DLPFC, according to previous results observed for neuronal somata in this region [36] and the results obtained in other human regions, such as the amygdala [37], double fluorescence immunohistochemistry was performed using antibodies against PSA-NCAM and the marker

for inhibitory synaptic terminals GAD67. In general, sections were processed as described above, but the endogenous peroxidase block was omitted. The sections were incubated overnight with mouse monoclonal IgM anti-PSA-NCAM antibody (Men-B, 1:1400) and rabbit polyclonal anti-GAD67 (1:500, Chemicon Int.). After washing, sections were incubated with donkey anti-mouse IgM and donkey anti-rabbit IgG secondary antibodies conjugated with Alexa 488 or Alexa 555 (Molecular Probes, Eugene, OR, USA; 1:200) in PBS containing 0.2% Triton X-100 and 3% NDS. All sections were observed under a confocal microscope (Leica, SPE). Z-series of optical sections (0.5 μm apart) were obtained using sequential scanning mode. One hundred PSA-NCAM immunoreactive puncta were analyzed in the DLPFC of different control human samples ($n = 4$) to determine the co-expression of PSA-NCAM and GAD67.

2.5. Data analyses

Data analyses were performed with SPSS v14.0 software (SPSS, Chicago, IL). All values are given as mean densities ± standard error of the mean (S.E.M.). Effect of post-mortem interval (PMI), brain pH, brain weight, age, suicide, substance-alcohol abuse, side, onset, lifetime neuroleptic use (in fluphenazine mg equivalents) or sex was assessed by Spearman's correlations in every layer and every diagnostic group prior to the main analysis. None of the conditions displayed any correlation with the values obtained with the different markers analyzed.

Significant interactions of diagnosis with layer were investigated using multivariate ANOVA of layer. This allowed the effects of diagnosis to be examined separately in each layer, with planned comparisons within a single analysis.

3. Results

The study of the expression of PSA-NCAM in the dorsolateral prefrontal cortex (DLPFC) confirmed our previous observations [36], showing a laminated pattern of staining in the neuropil. Layers III–V displayed intense PSA-NCAM expression, while the other layers showed only faint expression, which was almost absent in the white matter. We have found a similar distribution of expression when studying the synaptic protein SYN in all the layers of the human DLPFC, finding only a slightly lower expression in layer I. Similar results have been observed for GAD67 and VGLUT1, markers of inhibitory and excitatory synapses respectively.

PSA-NCAM immunoreactive structures were found in all the layers of the DLPFC. Most of these structures were characterized as individual puncta, although some processes, which most likely were truncated dendrites, could also be found (Fig. 1A). Some immunoreactive somata, similar to those we previously described as belonging to interneurons [36], were occasionally observed in the DLPFC. The characterization of the phenotype of PSA-NCAM immunoreactive puncta in the neuropil reflected a high level of colocalization with GAD67 in the DLPFC (30.9 ± 6.8%, Fig. 1A).

The study of the expression of PSA-NCAM and different synaptic markers in the DLPFC (Fig. 1B) showed that PSA-NCAM was only reduced significantly in layers IV and V ($p < 0.05$) of schizophrenic patients. For SYN expression, we observed a reduction in layers III ($p < 0.05$) and IV ($p < 0.01$) of patients suffering major depression. Regarding VGLUT1, we observed decreases in its expression in layer V of major depression ($p < 0.05$) and bipolar disorder patients ($p < 0.05$). Interestingly, we also found a significantly increased expression of VGLUT1 in the white matter of schizophrenic patients. Finally, we observed a reduction in the expression of GAD67 in layers II, III, and IV of bipolar disorder patients ($p < 0.05$) and a reduction in layers II, and IV of schizophrenic patients ($p < 0.05$).

4. Discussion

The phenotypic characterization of PSA-NCAM immunoreactive puncta in the human DLPFC has revealed their inhibitory nature. This result is in accordance with a previous study of our laboratory [36], where we have demonstrated the inhibitory nature of PSA-NCAM expressing somata in this cortical region. PSA-NCAM expressing somata and neuropil structures displaying an inhibitory phenotype can also be found in the hippocampus [22], the septum [13] and the paleocortex and neocortex of adult rats and mice [16], as well as in the human amygdala [37].

The study of the expression of the different synaptic markers showed that major depression patients presented a reduction in SYN (layers III, IV), VGLUT1 (layer V) and GAD67 (layer V) expression. The reductions in SYN and VGLUT1 expression seem to be in accordance with the dendritic atrophy of medial PFC (mPFC) pyramidal neurons observed in animal models of depression, such as chronic stress [10,25,26]. Although such dendritic atrophy still remains to be demonstrated in major depression patients, volume reductions have been reported in this region [24]. These results give support to the neuroplastic hypothesis of depression, which poses that changes in neuronal structure and connectivity may underlie the etiology of this disorder and that these changes may be reverted by antidepressants [7]. In fact, treatment with fluoxetine increases the level of expression of SYN [34,35] and pharmacological manipulation of dopamine D2 receptors with specific agonists induces increases in SYN and GAD67 expression in the rat PFC [6]. Our results showing an increase in GAD67 expression are also in accordance with previous lines of evidence coming from animal and human studies indicating the involvement of the GABAergic system in the pathophysiology of major depression [20,30]. Using western blot analysis, GAD67 protein expression was also found significantly reduced in the DLPFC of depressed subjects [18]. Moreover, neuroimaging studies have reported reductions in GABA levels [17,29] in the PFC and post-mortem immunohistochemical analyses have found reductions in the density and size of certain interneuronal subpopulations in this region [23,27].

Although in a recent study our laboratory has found that changes in SYN and GAD67 expression in the amygdala of major depression patients were accompanied by parallel changes in PSA-NCAM expression [37], apparently this does not occur in the PFC. Interestingly, similar results have been obtained by our group in mice subjected to chronic stress, in which PSA-NCAM expression was altered by this aversive experience in the amygdala [14] but not in the mPFC (Gilabert-Juan et al., unpublished results).

In schizophrenic patients we have observed a reduction in the expression of PSA-NCAM (layers IV and V), and GAD67 (layers II and IV). These parallel changes of PSA-NCAM and GAD67 may reflect the already reported alterations of cortical inhibitory circuits in schizophrenia (for a review see [21]); in fact, a recent report has found that GAD67 mRNA and protein levels are significantly lower in the DLPFC of schizophrenic patients [11]. Additionally, they also point to the possible involvement of PSA-NCAM in these alterations in inhibitory neurotransmission, since we have previously demonstrated that this molecule is exclusively expressed by interneurons in the PFC of humans [36] and rodents [16,38]. Interestingly, these interneurons have more reduced structural features and synaptic input than those lacking PSA-NCAM expression [16]. Moreover, both *NCAM* and the polysialyltransferase *ST8SIAII* genes have been associated to schizophrenia [5,31,32] and the number PSA-NCAM expressing neurons is reduced in the hippocampus of schizophrenic patients [2]. The present results are similar to those we have recently found in an animal model for schizophrenia (postweaning isolation rearing), in which we have observed similar decreases in PSA-NCAM and GAD67 expression in the mPFC (Gilabert-Juan, Nacher, unpublished results). These results

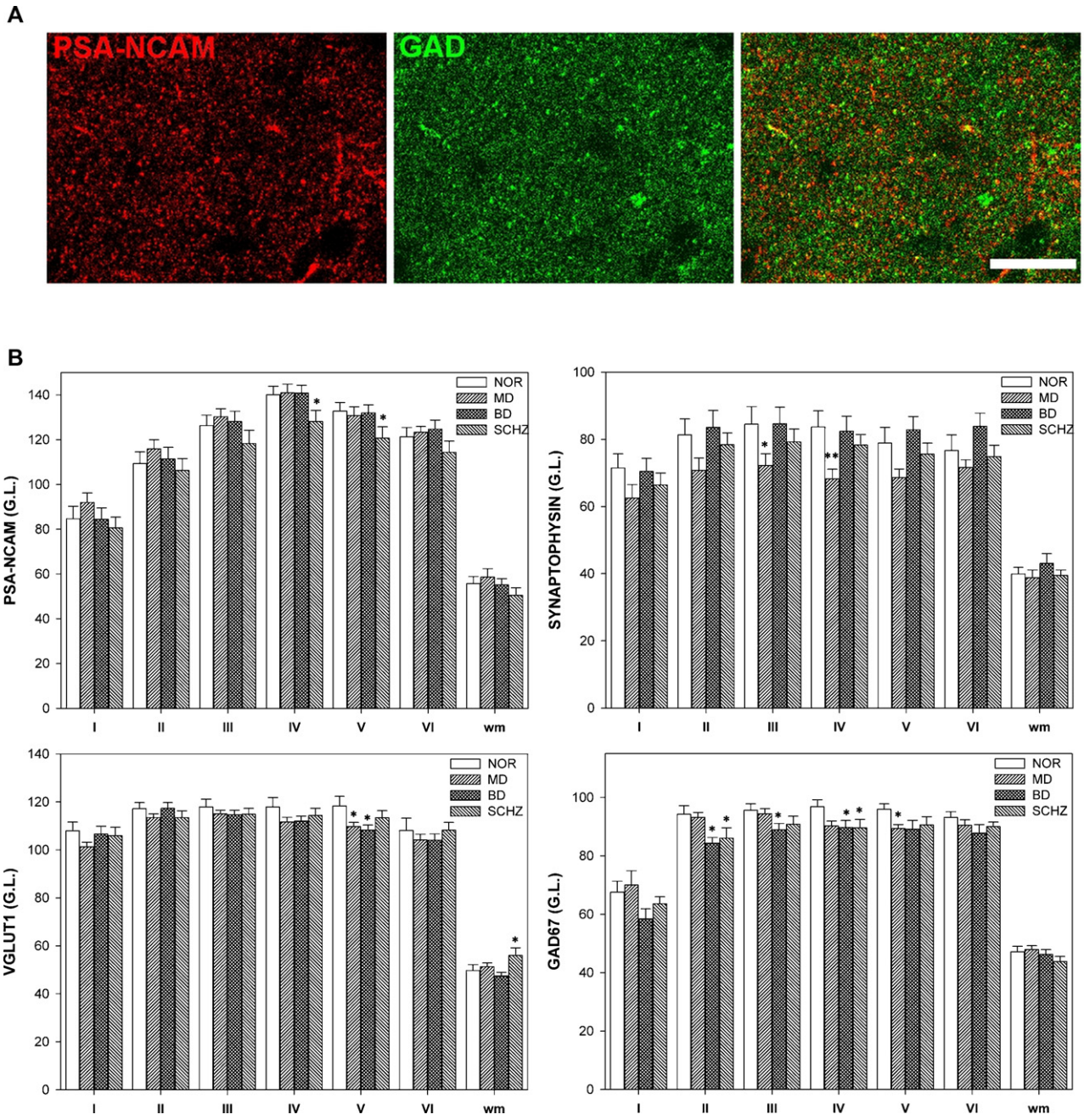

Fig. 1. (A) Phenotypic characterization of PSA-NCAM expressing puncta in the human dorsolateral prefrontal cortex. Confocal imaging of double-labeled puncta for PSA-NCAM (in red) and GAD67 (in green) in layer III of the human dorsolateral prefrontal cortex. Scale bar: 25 μm. (B) Changes in the expression of PSA-NCAM and synaptic markers in the human dorsolateral prefrontal cortex of patients suffering from different psychiatric disorders: major depression (MD), bipolar disorder (BD) and schizophrenia (SCHZ). (A) PSA-NCAM. (B) SYN. (C) VGLUT1. (D) GAD67 expression (*$p < 0.05$; **$p < 0.01$).

are opposite to those found in the amygdala of this animal model, in which we have found increases in the expression of PSA-NCAM and GAD67 [15]. Although we have not observed differences in the expression of PSA-NCAM in the amygdala of schizophrenic patients, decreases in GAD67 expression were found in its basolateral and basomedial nuclei [37], in agreement with previous studies showing reduced GAD activity [4] and GABA concentration [30] in this region.

The increased expression of VGLUT1 observed in white matter could be related with the higher density of neurons observed in this region in the brain of schizophrenic patients [8,9], supporting the hypothesis of alterations in the normal development and positioning of cortical neurons in this disorder.

Finally, the samples from bipolar disorder patients displayed reduction in the expression of VGLUT1 (layer V) and GAD67 (layers II, III and IV). The explanation for these results is more complicated because bipolar patients have periods of mania followed by periods of deep depression, which may result in cyclic changes in the expression of molecules related to neurotransmission and neural plasticity, as well as in the structure of neurons in the PFC. However, the reduction observed in the expression of both excitatory and inhibitory contacts indicates that this region is clearly affected in this disorder.

The limitations of this study are those that are common to all human post-mortem brain research, which require accounting for various demographic and clinical variables that may influence

molecular preservation in the tissue. The variables analyzed in our study included effect of post-mortem interval, brain pH, brain weight, age, suicide, substance–alcohol abuse, side, onset, lifetime neuroleptic use (in fluphenazine mg equivalents) and sex; none of the conditions displayed any correlation with the values obtained with the different markers analyzed. However it has to be noted that some other variables, specially chronic treatments with other typical or atypical antipsychotics and antidepressants have not been controlled. In this regard, it is important to notice that these drugs may also influence the expression of the different markers analyzed in our study. In fact, chronically administered antidepressants increase the expression of PSA-NCAM and SYN in the mPFC of rodents [34,35]. Similarly, the antipsychotic haloperidol decreases the expression of PSA-NCAM, SYN and GAD67 in the rodent mPFC [6].

5. Conclusions

Synaptic and plasticity markers are altered in the human dorsolateral prefrontal cortex of psychiatric disorder patients, showing that changes in prefrontocortical networks, specially those involving interneurons, may underlie the etiology of these disorders. The results point to an impoverishment in the complexity of the circuitry in the prefrontal cortex for all these disorders and, in the case of schizophrenia, also to an impairment in the plasticity necessary for normal function.

Authors' disclosure

The authors of the manuscript disclose any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations concerning the work submitted that could inappropriately influence (bias) their work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Acknowledgements

Spanish Ministry of Science and Innovation (MICINN-FEDER) BFU2009-12284/BFI, MICINN-PIM2010ERN-00577/NEUCONNECT in the frame of ERA-NET NEURON", Generalitat Valenciana ACOMP/2012/229 and the Stanley Medical Research Institute to JN. Javier Gilabert-Juan has a predoctoral fellowship from the Spanish Ministry of Education and Science (AP2008-00937).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2012.09.032.

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Article 6: Sex-specific association of the ST8SIAII gene with schizophrenia in a Spanish population

TITLE: **Sex-specific Association of the *ST8SIAII* Gene with Schizophrenia in a Spanish population.**

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NUMBER OF TABLES: 2

KEYWORDS: Schizophrenia, association study, *ST8SIAII*, PSA-NCAM, Caucasian population.

RUNNING TITLE: Association of *ST8SIAII* with Schizophrenia

Sex-specific Association of the *ST8SIAII* Gene with Schizophrenia in a Spanish population.

ABSTRACT

The alpha-2,8-sialyltransferase II gene (*ST8SIAII*) map to chromosomal region reported as a susceptibility locus to schizophrenia. The promoter region of *ST8SIAII* has been also associated with schizophrenia in different Asian and Australian population samples. *ST8SIAII* encodes an enzyme that catalyzes the transfer of polysialic acid to the neural cell adhesion molecule (NCAM), regulating finely the function of this molecule which contributes to neuronal plasticity. To further support for the involvement of *ST8SIAII* in the etiology of schizophrenia, we carried out a case-control association study with rs3759916, rs3759915, rs3759914 and rs2305561 SNPs, in a Caucasian sample of Spanish origin, consisting of 508 unrelated schizophrenic patients and 428 unrelated healthy subjects. In addition, we explored the effect of sex in the association between *ST8SIAII* and schizophrenia, because it has been reported that estrogens regulate the post-translational modifications of NCAM by controlling the transcription of both polysialyltransferases genes. We did not replicate in our sample the positive results found by previous association studies using the same SNPs. However when the analysis was carried out taking into account the sex of the subjects, the *G* allele (P = 0.044) and *AG* genotype (P = 0.04) of rs3759916 were significantly associated with the disease in the female sample. In the male sample, the *ACAG* four marker haplotype was associated with schizophrenia (P = 0.028). Our study shows *ST8SIAII* as a gene containing susceptibility alleles for schizophrenia with notable differences between men and women.

INTRODUCTION

The alpha-2,8-sialyltransferases II and IV (st8siaII and st8siaIV) are two enzymes that catalyze the transfer of polysialic acid (PSA) to the neural cell adhesion molecule (NCAM). These enzymatic activities are crucial in neural development, modulating the adhesive properties of NCAM, which are involved in cell-cell and cell-extracellular matrix recognition [Rutishauser 2008]. NCAM plays important roles in neuronal migration, neurite growth, axon guidance, synaptic plasticity [Maness and Schachner 2007], regulation of circadian cues, and learning and memory processes [Conboy et al. 2010]. The functions of NCAM are fine regulated by the post-translational addition of PSA conferring new anti-adhesive properties to the molecule which contributes to neuronal plasticity [Rutishauser 2008].

Different studies in human postmortem samples and in animal models have suggested that alterations in PSA-NCAM expression in the Central Nervous System (CNS) increases the vulnerability to several psychiatric disorders [Barbeau et al. 1995; Gilabert-Juan et al. 2011a; Gilabert-Juan et al. 2012; Varea et al. 2012]. In addition, the genes coding for both polysialyltransferases, *ST8SIAII* and *ST8SIAIV* respectively, map to chromosomal regions reported as susceptibility loci for schizophrenia and bipolar disorders [Maziade et al. 2004; McAuley et al. 2008]. *ST8SIAII* has been also associated to these psychiatric disorders in different studies. In a sample from the Japanese population, some single nucleotide polymorphisms (SNPs) located in the promoter region of *ST8SIAII* were associated with schizophrenia [Arai et al. 2006]. Moreover, haplotypes constructed with these SPNs and others belonging to the same linkage disequilibrium region were associated to the disease [Arai et al. 2006]. A similar result was found in a Han Chinese sample [Tao et al. 2007] providing further support on the one hand, for the potential involvement of this gene in schizophrenia, and on the other hand, that an altered level of *ST8SIAII* expression may be critical, since significant association was found with SNPs located in the promoter of this gene. In fact, *in vitro* functional assays showed that a risk promoter haplotype of *ST8SIAII* has significantly higher transcriptional activity than a protective one [Arai et al. 2006]. Recently, a risk SNP haplotype spanning from 16 kb upstream of *ST8SIAII* to intron 2 was reported in two Australian cohorts suffering from schizophrenia and bipolar disorder respectively [McAuley et al. 2012].

PSA-NCAM is highly expressed in the CNS during embryonic development, being dramatically down regulated after the perinatal period [Hildebrandt et al. 1998]. However in adult brains, PSA-NCAM expression is

present in several regions where neural plasticity persist, as the hypothalamus, the olfactory bulb, the medial prefrontal cortex (mPFC), the hippocampus or the amygdale [reviewed in Bonfanti 2006]. PSA-NCAM expression has been associated with different sexual hormones, being the most interesting the gonadotropin-releasing hormone (GnRH) [Parkash and Kaur 2005], one of the most important molecules in the reproductive life in vertebrates. It has also been reported that estrogens regulate the post-translational modifications of NCAM by controlling the transcription of both polysialyltransferases genes [Tan et al. 2009]. Therefore PSA-NCAM may have different impact in brain development and maintenance in each sex. Schizophrenia occurs 1.4 times more frequently in men than women and typically appears earlier in men [McGrath et al. 2008]. The course of the disease is also different between the two sexes, displaying premenopausal women a more benign course of disease than men, with less severe levels of psychopathology and disability, and with better response to antipsychotic medication [reviewed in Kulkarni et al. 2012]. This data suggests an important hormonal component in the development of the disease, showing a scenario where neural genes, such as *ST8SIAII*, regulated by sexual hormones may contribute to the vulnerability to schizophrenia.

To further support for the involvement of *ST8SIAII* in the etiology of schizophrenia, we carried out a case-control association study using a sample of Spanish origin. Taking into account that this gene is regulated by estrogens, we also explored the effect of sex in the association study.

MATERIAL AND METHODS

Subjects

The sample consisted of 508 unrelated psychotic patients, 185 females (36.4%) and 323 males (63.6%) and 428 healthy control subjects, 132 females (30.8%) and 296 males (69.2%). Patients came from the psychiatric in-patient and out-patient units of the Mental Health Service 4 of the Clinical Hospital, University of Valencia, Spain. All patients met DSM-IV criteria for schizophrenia. The diagnoses for every patient were confirmed by a consensus meeting with the treating psychiatrist and one of the psychiatrists of our research group. Patients also had a minimum one-year evolution of the illness and were on antipsychotic treatment at evaluation time. The 428 healthy unrelated subjects had no history or familial background of psychiatric disorders. To avoid sample stratification, these subjects had similar demographic characteristics (Caucasian ethnic group, similar age) to the schizophrenic group. They were also of Spanish origin. No stratification has been found in the Spanish population with the exception of the Canary Islands [Laayouni et al. 2010], therefore no allelic differences due to ethnic procedure were expected. Drug abuse was also considered among the exclusion criteria.

All the participants in the study gave their written informed consent to participate in this study, approved by the Ethical Committee of Valencia University.

SNP Genotyping

To replicate positive results found between schizophrenia and the *Sialyltransferase II* gene (*ST8SIAII)* in the Japanese [Arai et al. 2006] and the Chinese Han [Tao et al. 2007] populations, three SNPs located in the promoter region of *ST8SIAII* were selected: rs3759914, rs3759915 and rs3759916. In addition one SNP, rs2305561, situated in the coding region of *ST8SIAII* affecting the NCAM polysialylation efficiency [Isomura et al. 2011] was also analyzed.

Genomic DNA was isolated from the peripheral blood of patients and controls according to standard procedures. SNP genotyping was performed through the iPLEX assay on the Mass ARRAY platform (Sequenom, Santiago de Compostela, Spain), which allows high throughput genotyping through multiplex reactions. Exclusion criteria during quality control of the genotyping procedure were the following: (i) Genotyping call rate lower than 99%; (ii) Deviations from Hardy-Weinberg Equilibrium (HWE) in the control sample ($P<0.05$).

Statistical Analysis

QUANTO software v. 1.2.4 [Gauderman 2002] was used to calculate the statistical power to find association between the genetic polymorphisms and the risk for schizophrenia in our study. This power was 54% to detect a risk allele over rare allele frequencies (0.05-0.5) and assuming an odds ratio (OR) of 1.5 with 95% confidence intervals (CI). We also set the prevalence of schizophrenia at 1% and the inheritance model as overdominant.

Genotypes were assessed for HWE in both patient and control samples by applying a χ2 test implemented with SNPator software [Morcillo-Suarez et al. 2008]. Differences in the allelic and genotypic frequencies between patients and controls were evaluated with a χ2 test via SNPator. Bonferroni test for multiple comparisons was applied to correct all the reported *P*-values.

Regarding the haplotype analysis, haplotypes were constructed with the four SNPs and compared between patients and controls using SNPator package. Frequencies of the four-marker haplotypes were estimated through a retrospective likelihood algorithm and compared between patients and controls. A Bonferroni multiple test correction was applied taken into account the number of haplotypes in each case.

RESULTS

Table 1 shows the allelic and genotypic frequencies of the *ST8SIAII* polymorphisms analyzed, as well as the *P*-values obtained from association analyses between these polymorphisms and schizophrenia. Whole sample and the sample grouped by gender were considered to perform the association study. No significant differences in the allelic or genotypic frequencies between cases and controls were detected in the total sample. Meanwhile some positive results were found when we carried out the analyses taking into account the sex of the subjects. At the allelic level, there was a significant association between the *G* allele of rs3759916 and the disease in the female sample (χ^2 = 6.514, *P* = 0.011; corrected *P* = 0.044). Among 132 control women none of them has this allele, while nine of the 185 women with schizophrenia are *G* carriers. In the male subgroup, a significant association was found with the *G* allele of rs2305561 polymorphism, but this association did not overcome the multiple test correction (χ^2 = 4 .201, *P* = 0.04; corrected *P* = 0.16). Concerning the genotypic analyses, the *AG* genotype of rs3759916 was associated significantly

to the disease in women respect to the *AA* genotype of this SNP (χ^2 = 6.609, *P* = 0.01; corrected *P* = 0.04). As can be seen in Table 1, there were no individuals with the *GG* genotype in our sample. In men, a positive association was observed between schizophrenia and the *GG* genotype respect to *CC+CG* genotypes of rs2305561, but this association was not significant after Bonferroni correction (χ^2 =3 .966, *P* = 0.046; corrected *P* = 0.18).

Haplotype analysis showed no significant differences between cases and controls in the total sample. However, we found again positive association when the sample was grouped by gender (Table 2). In women, the *GCAG* haplotype covering rs3759916, rs3759915, rs3759914 and rs2305561 was associated to the disease, but it lost the statistical significance after Bonferroni correction (χ^2 = 5.018, *P* = 0.025; corrected *P* = 0.15). In the men subsample, the *ACAG* haplotype was associated with the disease retaining the significant association after multiple test corrections (χ^2 = 8.007, *P* = 0.0047, corrected *P* = 0.028).

DISCUSSION

Schizophrenia is considered as a sexually dimorphic disorder because significant differences in the incidence and course of disease between men and women are reported [Kulkarni et al. 2012]. Besides gender, many other factors are associated with augmented risk to suffer schizophrenia [van Os and Kapur 2009], although the overall mechanism underlying this disease is poor understood. According to the neuro-developmental hypothesis of schizophrenia [Weinberger 1996], abnormalities in neural plasticity during certain stages of brain development may increase significantly the risk of developing the disease. Because PSA-NCAM is a major molecular actor of plasticity of the nervous system, it has become an attractive candidate to explore. Even more since *ST8SIAII* and *ST8SIAIV*, which encode the enzymes that catalyze the transfer of PSA to NCAM, are regulated by estrogens [Tan et al. 2009]. *ST8SIAII* map to chromosome 15q25-26, a region reported as including a susceptibility gene for schizophrenia [Maziade et al. 2004]. Furthermore, a significant association was found between *ST8SIAII* and the disease in two Asian samples [Arai et al. 2006; Tao et al. 2007] and recently in one Australian cohort [McAuley et al. 2012]. These associations were found with SNPs located in the promoter region of *ST8SIAII*, suggesting that the dysregulation of the expression of this gene may increase the risk to suffer schizophrenia.

Because no association between *ST8SIAII* and schizophrenia was reported in the Caucasian population, we explored in a Spanish sample the involvement of this gene as a risk factor for schizophrenia. A previous attempt to replicate in European ancestry samples the significant results found in the Australian schizophrenia cohort was not successful [McAuley et al. 2012] because data did not reach statistical significance. Nevertheless, we did not replicate the positive results found either by Arai et al. [2006] with respect to rs3759916 and rs3759914 or by Tao et al [2007] regarding rs3759915 in our sample. Differences in the allelic frequencies between the different population samples might explain these results. In fact, the alleles associated with schizophrenia in each of the two Asian samples, show lower frequencies in the Spanish cohort. Alternatively, allelic heterogeneity that characterizes complex disease could also explain these discrepancies. However when the association analysis was carried out taking into account the sex of the subjects, interesting results were found. In the women sample, the *G* allele (P = 0.044) and the *AG* genotype (P = 0.04) of rs3759916 was significantly associated with schizophrenia, suggesting that this allele is a risk factor for the disease in females of the Spanish population. Therefore the *A* allele might be a protective factor for schizophrenia in females of this population. In the men subgroup, the frequency of the *G* allele is very similar in healthy controls and in patients (1.18% and 0.92% respectively). This was also reported in the Chinese cohort with frequencies of 35.6% in controls and 33.9% in patients, so that no association was obtained for this SPN in this population [Tao et al. 2007]. It would be interesting to know whether in the Chinese population also occur an interaction between sex and the polymorphism rs3759916 concerning the vulnerability to schizophrenia. This polymorphism is located 200 base-pairs downstream from a binding sequence of a glucocorticoid receptor (GR) gene, a transcriptional factor implicated in sexual features.

To further confirm the involvement of *ST8SIAII* in schizophrenia, rs2305561 was also genotyped in the Spanish population. This SNP is located in the coding region of this gene, concretely in exon 5 [Arai et al. 2006], and shows functional significance because each allele has different efficiency of NCAM polysialylation [Isomura et al. 2011]. Therefore chain length and quantity of NCAM polysialylation could vary depending on the rs2305561 genotype affecting its biological function. Nevertheless, no association between rs2305561 and schizophrenia has been reported [Arai et al. 2006]. The same result was found in our sample, although the *G* allele and the *GG* genotype were more frequent in the cases than in controls in the male subset. However,

this association was lost after the Bonferroni test correction (*P* = 0.16 and 0.18 for allelic and genotypic frequencies respectively). Since this test is particularly stringent, significance may be lost for this polymorphism that may be a real risk factor. In fact, cells expressing the *G* allele of rs2305561 may have a significantly decreased amount of PSA on NCAM when compared with cells expressing the *C* allele [Isomura et al. 2011]. All these results suggest, on the one hand, that the *G* allele of the rs2305561 polymorphism is a likely risk factor for schizophrenia and on the other hand, that it has become a risk factor in males of the Spanish population. Again it would be interesting to analyze this interaction in other populations in order to confirm our results.

The haplotypic study indicated a risk haplotype *ACAG* in the men sample of the Spanish population (*P* = 0.028), while no haplotypes significantly associated with schizophrenia were found in the women sample of this population. Interestingly, the risk haplotype reported in Japanese sampled population [Arai et al. 2006] share the same alleles at rs3759916, rs3759915 and rs3759914 positions, although the frequency of the haplotype *ACA* is lower in our sample than in the Japanese one.

In this paper we point out the importance of taking into account the gender of the subjects in association studies in mental illness in general and in schizophrenia in particular. Several mental disorders affect differently each sex, specifically there are more men than women affected in schizophrenia and the contrary is observed in depression [Viveros et al. 2012]. Regarding PSA-NCAM, a correlation between this molecule and the cells secreting GnRH has been demonstrated [Chalivoix et al. 2010]. Furthermore, estrogen is one of the transcriptional regulators of both polysialiltransferases genes, *ST8SIAII* and *ST8SIAIV* [Tan et al. 2009]. Besides *ST8SIAII*, there are several studies pointing out sex differences in other genes involved in schizophrenia [Gilabert-Juan et al. 2011b; Goes et al. 2010; Hoenicka et al. 2010].

Finally, we acknowledge that our study has the typical limitations of a small sample study. The sample size became reduced when the sample was divided by gender decreasing the power of the analysis. Nevertheless we found interesting sex-specific associations to schizophrenia in *ST8SIAII* and we replicated partially former studies studying this gene in other populations. Our study shows *ST8SIAII* alleles as a susceptibility factors for schizophrenia with notable differences between genders. The role of this gene and the high number of interactions that it has with different neuronal pathways suggest

polysialiltransferases as attractive molecules to be studied in the field of the psychiatric disorders. Deep studies in the global expression mechanisms and in the regulation of polysialiltransferases have to be done in order to achieve more knowledge about their role in the neurodevelopment and in the brain maintenance.

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ACKNOWLEDGEMENTS

Spanish Ministry of Science and Innovation (MICINN-FEDER) BFU2009 - 12284/BFI, MICINN-PIM2010ERN- 00577/NEUCONNECT in the frame of ERA-NET NEURON", Generalitat Valenciana ACOMP/2012/229 to JN.

Conflict of Interest

The authors have no conflict of interest including any financial, personal or other relationships with other people or organizations that could influence, or be perceived to influence this work.

TABLES

Table1. Allelic and Genotypic Frequencies of the *ST8SIAII* Polymorphisms analyzed.

	Polymorphisms	Allele Counts (frequency)		*P* value	*P* value after Bonferroni correction	Genotype Counts (frequency)			*P* value[a]	*P* value after Bonferroni correction
TOTAL SAMPLE	rs3759916 Schizophrenia Control	A 1001(0.98) 849(0.99)	G 15(0.02) 7(0.01)	0.19	0.76	AA 493(0.97) 421(0.98)	AG 15(0.03) 7(0.02)	GG 0(0.0) 0(0.0)	0.19	0.76
	rs3759915 Schizophrenia Control	C 62(0.06) 37(0.04)	G 954(0.94) 815(0.96)	0.091	0.36	CC 4(0.01) 0(0.0)	CG 54(0.11) 37(0.09)	GG 450(0.86) 389(0.91)	0.066	0.26
	rs3759914 Schizophrenia Control	A 1008(0.99) 854(0.99)	G 2(0.002) 2(0.002)	0.87	1	AA 503(0.99) 426(0.99)	AG 2(0.004) 2(0.005)	GG 0(0.0) 0(0.0)	0.87	1
	rs2305561 Schizophrenia Control	C 140(0.14) 140(0.16)	G 872(0.86) 710(0.84)	0.11	0.44	CC 7(0.01) 6(0.01)	CG 126(0.25) 128(0.3)	GG 373(0.74) 291(0.69)	0.97	1
FEMALES	rs3759916 Schizophrenia Control	A 361(0.98) 264(1.0)	G 9(0.02) 0(0.0)	**0.011**	**0.044**	AA 176(0.95) 132(1.0)	AG 9(0.05) 0(0.0)	GG 0(0.0) 0(0.0)	**0.01**	**0.04**
	rs3759915 Schizophrenia Control	C 17(0.05) 8(0.03)	G 353(0.95) 254(0.97)	0.33	1	CC 2(0.01) 0(0.0)	CG 13(0.07) 8(0.06)	GG 170(0.92) 123(0.94)	0.23	0.92
	rs3759914 Schizophrenia Control	A 366(0.99) 264(1.0)	G 2(0.005) 0(0.0)	0.23	0.92	AA 182(0.99) 132(1.0)	AG 2(0.01) 0(0.0)	GG 0(0.0) 0(0.0)	0.23	0.92
	rs2305561 Schizophrenia Control	C 55(0.15) 38(0.15)	G 311(0.85) 224(0.85)	0.85	1	CC 5(0.03) 1(0.007)	CG 45(0.25) 36(0.27)	GG 133(0.73) 94(0.72)	0.21	0.84
MALES	rs3759916 Schizophrenia Control	A 640(0.99) 585(0.99)	G 6(0.01) 7(0.01)	0.66	1	AA 317(0.98) 289(0.98)	AG 6(0.02) 7(0.02)	GG 0(0.0) 0(0.0)	0.66	1
	rs3759915 Schizophrenia Control	C 45(0.07) 29(0.05)	G 601(0.93) 561(0.95)	0.13	0.52	CC 2(0.006) 0(0.0)	CG 41(0.13) 29(0.1)	GG 280(0.86) 266(0.99)	0.26	1
	rs3759914 Schizophrenia Control	A 642(1.0) 590(0.99)	G 0(0.0) 2(0.003)	0.14	0.56	AA 321(1.0) 294(0.99)	AG 0(0.0) 2(0.007)	GG 0(0.0) 0(0.0)	0.14	0.56
	rs2305561 Schizophrenia Control	C 85(0.13) 102(0.17)	G 561(0.87) 486(0.83)	**0.04**	0.16	CC 2(0.006) 5(0.02)	CG 81(0.25) 92(0.31)	GG 240(0.74) 197(0.67)	**0.046**	0.18

[a]Genotype *P* value with the overdominant model of inheritance

Table 2. *ST8SIAII* haplotypes significantly associated to schizophrenia in the Spanish population sample.

	Haplotype				Frequency			
	rs3759916	rs3759915	rs3759914	rs2305561	Control	Schizophrenia	*P* value	*P* value after Bonferroni correction
FEMALES	G	C	A	G	0%	1.89%	**0.025**	0.15
MALES	A	C	A	G	0.92%	3.56%	**0.0047**	**0.028**

Results and Discussion

CHRONIC STRESS MODEL

In the first and second articles, a model of chronic stress was performed based on one of the most accepted strategy to generate chronic anxiety and depression models. The stressor consisted in the immobilization of mice for 1 hour per day during 21 days. Transgenic mice expressing GFP in a subset of inhibitory neurons (GIN mice) were used. In these animals we have studied by means of qRT-PCR the expression of several genes related to inhibitory transmission and of PSA synthesizing enzymes. The expression of GAD67, synaptophysin and PSA-NCAM was also studied in Medial (Me), CentroMedial (CeM) and BasoLateral (BLa) amygdaloid nuclei using immunohistochemistry. Furthermore, we have analyzed the dendritic arborization and the density of dendritic spines of interneurons in the BLa nucleus of the amygdala and in the mPFC. The number of GAD67 and PSA-NCAM expressing somata was also estimated in the mPFC.

The analysis showed that chronic stress did not induce changes in body weight gain in our model. Considering the whole amygdala, mRNA expression analysis revealed a reduced expression of *St8SiaII* and *GAD67* genes. These alterations were in contrast with those found in the mPFC, were we found an increase in the expression of *NCAM*, *synaptophysin* and *GABAA*a_1 genes. PSA-NCAM expression was significantly reduced in the CeM nucleus, GAD67 and synaptophysin in the Me nucleus. Finally, chronic stress also reduced dendritic arborization in amygdala GAD-GFP expressing interneurons, while increasing it in the mPFC. No changes in dendritic spine density were found in GAD-GFP expressing interneurons in any of these two regions.

The present results in mice confirm previous studies on the effects of chronic stress on amygdaloid PSA-NCAM expression in rats and expand them studying the expression of the enzymes responsible of its polysialylation. These effects on PSA-NCAM expression are paralleled by changes in molecules related to inhibitory transmission, but not by structural changes in amygdaloid interneurons. For the mPFC, the results show that interneurons in the mPFC of adult mice undergo dendritic remodeling after chronic stress. This remodeling is accompanied by significant changes in the number of neurons expressing GAD67 and in the expression of different molecules related to inhibitory

neurotransmission and neuronal plasticity. Apparently, the decrease in GAD67 expressing neurons is not due to apoptosis but to changes in the expression of this GABA synthesizing enzyme.

Therefore, our studies support the idea that inhibitory networks in the mPFC are also targets of chronic stress and that their alteration may also contribute to the behavioral and cognitive impairments induced by this aversive experience.

PSA-NCAM is implicated in the response to chronic stress in the amygdala

The distribution of PSA-NCAM expression in the amygdala and mPFC of GIN mice is similar to that described previously in a different mouse strain (Nacher et al., 2010) and in rats (Nacher et al., 2002b). As it has been demonstrated for many PSA-NCAM expressing structures in the cerebral cortex (excluding those of immature neurons) (Gomez-Climent et al., 2011; Nacher et al., 2002a; Nacher et al., 2002b; Varea et al., 2005), many PSA-NCAM expressing neurons in the amygdala express markers of interneurons and lack expression of molecules exclusively found in principal neurons. Consequently, changes in PSA-NCAM expression should primarily affect the structure of interneurons, rather than that of principal neurons. It is possible then, that the stress-induced reductions in PSA-NCAM expression showed by this study in the amygdala affect the connectivity of certain amygdaloid interneurons, leaving more plasma membrane extension free for the establishment of new synaptic contacts. Another non-excluding possibility is that, given its anti-adhesive properties, the reduction in PSA-NCAM expression may limit the ability of certain interneurons to remodel their structure in response to different stimuli. However, it is possible that these structural changes occur in an earlier time window during the stress procedure, in which changes in PSA-NCAM expression occur in a different direction, or only in certain amygdaloid nuclei and, consequently, we may have missed them studying the amygdala as a whole. The influence of PSA-NCAM on amygdaloid interneurons may also occur by the interference of the PSA in certain signaling cascades mediated by NCAM, especially those affecting inhibitory circuits (see Maness and Schachner, 2007; Rutishauser, 2008 for review). Future experiments manipulating PSA-NCAM expression are needed to understand whether this molecule plays a role in the remodeling of amygdaloid interneurons.

Our results also suggest that the decrease in polysialylation detected in the amygdala after chronic stress may be caused by the observed downregulation of polysialyltransferase *St8SiaII* expression, since *St8SiaIV* mRNA levels are not affected. These results are interesting, because the analysis of single polysialyltransferase knockout mice revealed that most PSA-NCAM expressing structures in the amygdala of adult control mice are polysialylated by St8SiaIV (Nacher et al., 2010). However, it is possible that, although St8SiaIV may function as the "main" polysialyltransferase in the amygdala during normal circumstances, St8SiaII may take care of the addition of PSA to NCAM when the system is challenged, for instance by stress. The observed reductions in PSA-NCAM expression in the amygdala after chronic stress must have consequences on behaviors dependent on this cerebral region. In fact, previous studies in naive rodents with reduced PSA-NCAM expression indicate a potentiation of amygdala-dependent behaviors: PSA depletion in the amygdala of rats results in enhanced fear extinction (Markram et al., 2007) and *St8SiaII* knockout mice displayed increased aggression (Calandreau et al., 2010).

Regarding the mPFC, the present results indicate that PSA-NCAM is not directly implicated in the changes we have described in this region. First, the GAD67-EGFP expressing interneurons in which the structural features have been analyzed did not show PSA-NCAM expression in their somata, neurites or in the puncta located in their projection fields in layers I and II. This absence of PSA-NCAM expression has been observed both in control and in chronically stressed mice. Second, no changes in the number of PSA-NCAM expressing cells or in the expression of the mRNA of polysialyltransferases have been observed after chronic stress. It is, however, possible that changes in PSA-NCAM expression in the mPFC occur before 21 days of chronic stress in interneuronal populations different from the one studied in the present study.

Synaptophysin is altered under the stress conditions

There are no previous reports showing changes in synaptophysin expression or in synaptic density in the amygdala after chronic stress. Our finding of a reduction in synaptophysin expression in the medial amygdala may indicate a reduction in active synapses, because the expression of this synaptic vesicle membrane protein is linked to synaptic remodeling (Greengard et al., 1993) and it is considered a reliable index of synaptic density (Eastwood

and Harrison, 2001; Masliah et al., 1990). Since this decrease coincides with the previously reported reduction in dendritic spine density in principal neurons of the medial amygdala (Bennur et al., 2007), it is possible that some of the lost synapses corresponded to those established on the lost spines. However, we have not observed increases in synaptophysin expression in the basolateral amygdala, where the density of spines in principal neurons is increased after chronic stress (Mitra et al., 2005). Another possibility is that the synapses that disappear after stress were inhibitory. This would be in agreement with the parallel reductions observed in GAD67 expressing elements in the medial amygdala. In fact, different studies have demonstrated that stress can induce amygdala activation, affecting inhibitory neurotransmission, which in turn has an important role in stress-induced synaptic plasticity (Davis et al., 1994). Moreover, an inverse relationship has been found between inhibitory tone and behavioral anxiety in the basolateral nucleus of the amygdala (Roozendaal et al., 2009). In fact, electrophysiological experiments in amygdala slices have shown that stress levels of corticosterone can reduce inhibitory neurotransmission and increase the excitability of principal amygdaloid neurons (Duvarci and Pare, 2007). However, these results and our findings on the expression of molecules related to inhibitory neurotransmission are apparently in contrast with reports of a reduced response of the amygdala to corticotropin releasing factor (CRF) after chronic stress (Sandi et al., 2008). CRF increases the excitability of principal neurons in the BLA (Rainnie et al., 1992) and, consequently, a reduced response to this factor may result in decreased excitability. Further analyses evaluating the expression of CRF and their receptors in relation to inhibitory neurotransmission during stress are necessary to understand these complex interactions.

Regarding the alterations in synaptophysin expression in the mPFC, it is important to note that although a previous report has failed to find changes in the levels of this synaptic protein (Carvalho-Netto et al., 2011), a significant increased expression of the *SYN* gene in the total mPFC has been found in this thesis. This difference may be due to the fact that Carvalho-Netto et al. studied the whole PFC, while we only focused in its medial region. In fact, the effects of chronic stress on the structure of pyramidal neurons in the orbitofrontal cortex, another region of the prefrontal cortex, are opposite to those in the mPFC (Liston et al., 2006) and this may mask the overall results in synaptophysin

expression. However, detailed studies on the synaptic input of pyramidal and inhibitory neurons must be performed to shed light on this intricate matter.

The chronically stressed mice show changes in the expression of molecules related to inhibitory transmission

The changes in GABAergic neurotransmission described in this thesis were only restricted to GAD67 expression in the amygdala, whereas in the mPFC they affected the numbers of GAD67 and GAD67-EGFP expressing somata, *GABAAa1* mRNA expression but not to *GAD67* expression.

The effects observed in GAD67 expression in the amygdala may be mediated by the monoaminergic system, since it is known that stress enhances the release of monoamines in the amygdala (Goto et al., 2007; Maier and Watkins, 2005) and these monoamines, in turn, affect amygdaloid inhibitory circuits (Braga et al., 2004; Marowsky et al., 2005). The stress-induced changes in the expression of GAD67 in the amygdala occur in parallel to the downregulation of PSA-NCAM expression. Changes in PSA-NCAM expression may promote remodeling of inhibitory circuits, which may lead to the observed decrease in GAD67 expression.

A previous report has described increases of GAD65 or GAD67 mRNAs in the hippocampus after chronic immobilization stress (Bowers et al., 1998), but we have not found differences in the expression of these mRNAs in this thesis. It is possible that changes in mRNA expression occur before 21 days, since the study of Bowers et al., used a 15 days paradigm. On the other hand, previous studies, found decreased GABA levels measured with HPLC in the PFC after 3 weeks of chronic mild stress (Shalaby and Kamal, 2009). A previous report has also found a decrease in the number of parvalbumin cells in the hippocampus of tree shrews after chronic stress (Czeh et al., 2004). Another possible explanation for the decrease in GAD67 expressing interneurons found in our study may be cell death. In fact, a previous study found that the number of apoptotic cells was increased in the cerebral cortex and the hilus of adult tree shrews after chronic psychosocial stress (Lucassen et al., 2001) and this has been suggested as an explanation for the loss of hippocampal parvalbumin expressing cells (Czeh et al., 2004). However, we find this possibility unlikely, unless it occurred sooner during the stress procedure, since we have not found

evidences of apoptosis or of degenerated interneurons in our material. In other way, it may occur an overactivation of a subpopulation of interneurons and the consequent inhibition of excitatory neurons and other subpopulations of interneurons. Our results in the mPFC showing a decrease in the number of GAD67 and GAD67-EGFP expressing somata suggest an imbalance in the expression of this GABA synthetizing enzyme.

The analysis of the molecules related to inhibitory neurotransmission by qRT-PCR in the mPFC has only found a significant increase in the expression of the *GABAAa1* receptor gene. The function of this receptor appears to be necessary to mediate the effects of chronic stress in the structural remodeling of principal neurons, at least in the hippocampus, because treatment with specific agonists prevents dendritic atrophy in CA3 pyramidal neurons (Magarinos et al., 1999). It may be possible that the increase in expression of $GABA_A$ receptor constitutes an adaptive response directed to augment the function of these receptors and to counteract deleterious effects of stress on mPFC circuitry. This response may also counteract the decreased binding to $GABA_A$ receptors described after chronic stress in rodents (Gruen et al., 1995) and in post-traumatic stress disorder (PTSD) patients (Bremner et al., 2002; Geuze et al., 2008).

Our results in the mPFC show an increase in the expression of *NCAM* mRNA after chronic stress. A similar study did not find changes in this parameter in the PFC using *in situ* hybridization, although it described a reduction of *NCAM* mRNA in the hippocampus (Venero et al., 2002), a region where decreases in NCAM protein expression have also been reported after chronic stress (Sandi et al., 2001). However, it has to be taken into account that these measures were obtained in the whole prefrontal cortex and not only in the mPFC as in our study. The increase in *NCAM* expression after chronic stress may lead to increased cell adhesion and it can influence the different intracellular signaling cascades mediated by this protein (Maness and Schachner, 2007). This increase in *NCAM* expression may have a neuroprotective role against the effects of stress, since reduced levels of this protein have been found to increase the vulnerability to behavioral alterations induced by this aversive experience: *NCAM* heterozygous mice (Jurgenson et

al., 2012) and conditional NCAM-CAMKII mice (Bisaz and Sandi, 2012) display increased immobility in the tail suspension test.

The effects of chronic stress in mPFC interneuron structure are opposite to those found in the basolateral amygdala

This aversive experience induces opposite effects on the structure of principal neurons in these two regions (Radley et al., 2004; Vyas et al., 2002). In this thesis opposite effects have also been observed in interneurons: a significant reduction in dendritic arborization in interneurons of the basolateral and lateral amygdala and a significant increase in the dendritic arborization of mPFC interneurons. This is, to our knowledge, the first report describing dendritic remodeling in interneurons after chronic stress. All the previous studies have been focused on the structure of principal neurons. Particularly, in the basolateral amygdala, chronic stress induces dendritic hypertrophy of stellate and pyramidal neurons (Vyas et al., 2002), and a chronic stress paradigm similar to that used in our study induces dendritic atrophy and reductions in spine density in pyramidal neurons of mPFC of rats (Radley et al., 2004). However, we have not found differences in spine density in the subpopulation of interneurons studied in the mPFC or amygdala suggesting that the structure of these postsynaptic elements is not affected by this stress paradigm in this interneuron subpopulation.

The dendritic growth of principal neurons in the basolateral amygdala has been interpreted as a structural strengthening of excitatory neurotransmission, which may represent a cellular substrate for enhanced anxiety (Roozendaal et al., 2009). In the same way, the retraction of the dendrites of interneurons, which may also reduce inhibition on principal cells, can also contribute to this strengthening of excitatory neurotransmission. Unfortunately, we have not been able to study interneuron structure in the centromedial nucleus, where significant changes in PSA-NCAM expression have been detected, because very few interneurons express GFP in this nucleus in GIN mice. The atrophy of pyramidal neurons in the mPFC has been interpreted as a structural weakening of excitatory neurotransmission, which may represent an adaptive cellular substrate for responding to the increase in excitatory neurotransmission elicited during the first phases of stress (Lowy et al., 1995). In fact, the dendritic atrophy of principal neurons in the hippocampus

after chronic stress is paralleled by a decrease in the density of excitatory synapses on these cells (Sousa et al., 2000; Sandi et al., 2003).

It is tempting to interpret the hypertrophy of the dendrites of mPFC interneurons, as another attempt of mPFC circuitry to minimize this overexcitation. An increased dendritic surface may favor the formation of synaptic contacts on mPFC interneurons. However, we do not know yet whether new synapses are established on this expanded dendritic surface and, if so, whether they are excitatory or inhibitory. Solving these questions is essential to understand what is the role of the stress-induced interneuronal remodeling. It is also very important to determine the sequence of the events that lead to the scenario that we observe after 21 days of stress: Is the interneuronal hypertrophy subsequent to the decrease in inhibitory neurotransmission suggested by the reduction in the number of GAD67 and GAD67-GFP expressing somata or viceversa? Are these two independent phenomena? Do changes in interneuron structure in the mPFC occur before, simultaneously or after the changes described in pyramidal neuron structure? Although, obviously, further experiments analyzing different time points along the chronic stress are necessary to elucidate these questions, it is interesting to note that a recent study by Keck et al. (2011) suggests that structural changes in inhibitory neurons may precede structural changes in excitatory circuitry in the visual cortex following sensory deprivation.

Our analysis of the phenotype of enhanced green fluorescence protein (EGFP) expressing neurons in the mPFC of GIN mice reveals that they belong exclusively to those expressing somatostatin. According to a recent report (Xu et al., 2010), the interneurons analyzed structurally in our study cannot be chandelier or basket cells, which always express parvalbumin. Since most of the neurons in the mPFC of GIN mice are located in layers II, III and upper V, and their axons arborize profusely in superficial layers, we are confident that most of them have to be Martinotti cells, as it has previously suggested in the somatosensory cortex of this strain of transgenic mice (Ma et al., 2006). Martinotti cells can also express calbindin and calretinin in addition to somatostatin and they never express NPY or parvalbumin (Xu et al., 2010). Martinotti cells are interneurons whose axons mainly target the apical dendritic tree of pyramidal neurons (Markram et al., 2004) and, interestingly, this apical

region is the one that shows the dendritic retraction after chronic stress (Radley et al., 2004). Consequently, the dendritic hypertrophy that we observe in these interneurons may be related to the shrinkage of the apical region of pyramidal neurons. However, studies directed to evaluate structural remodeling in the axonal projection of EGFP expressing interneurons in superficial layers are needed to understand their relationship to the dendritic atrophy of principal neurons.

Chronic stress in experimental animals is one of the most accepted models of chronic anxiety and depression (McEwen, 2000) and there is a clear link between prefrontal cortex and amygdala dysfunction with mood disorders, such as major depression (Brody et al., 2001) or posttraumatic stress disorder (Bremner, 2005). Consequently, the present results may increase our understanding of the molecular and structural plasticity associated to the development of anxiety and mood disorders. This plasticity may be a substrate for the increases in anxiety-like behaviors, cognitive changes and mood alterations observed in this animal model and in these psychiatric disorders. In fact, several lines of evidence coming from both animal and human studies indicate the involvement of the GABAergic system in the pathophysiology of major depression (Krystal et al., 2002; Sanacora et al., 1999). Neuroimaging studies have reported reductions in GABA levels in the prefrontal cortex (Hasler et al., 2007; Sanacora et al., 1999). Reduced GABA concentrations were also demonstrated in the plasma and cerebrospinal fluid in depression (Brambilla et al. 2003) and GAD67 protein expression was significantly reduced in depressed subjects (Karolewicz et al., 2010). Moreover, similar to what we have found in our chronically stressed mice, post-mortem morphometric analyses in major depression patients have found reductions in the density and size of GABAergic interneurons immunoreactive for calbindin (Rajkowska et al., 2006) and calretinin (Oh et al., 2012) in the PFC. Table 4 summarizes the main results obtained in this thesis about the chronic stress model.

Table 4. Summary of results in the chronic stress model

Stress	PSA-NCAM(+)	Arborization	Spines	Gene expression	Protein density	Somata
amygdala	38%	decrease	=	↓*St8SiaII, GAD67*	↓PSA-NCAM, GAD67, SYN	
mPFC	No	increase	=	↑*NCAM, SYN, $GABA_Aa1$*		↓GAD67 =PSA-NCAM

ISOLATION AND DUAL SCHIZOPHRENIA MODELS

In the third article the expression of molecules related to inhibitory neurotransmission and structural plasticity was studied in rats subjected to post-weaning isolation rearing, an animal model that reproduces several core symptoms of schizophrenia. The expression of PSA-NCAM, NCAM, synaptophysin, GAD67, GAD65, St8SiaII and St8SiaIV was studied using qRT-PCR and immunohistochemistry.

An increased expression of GAD67 was found in the three amygdaloid nuclei, Me, CeM and BLa, and the same increase was observed in the expression of PSA-NCAM in the BLa nucleus, without detectable changes in synaptophisin, NCAM or GAD65 expression. The mRNA measures did not find significant changes in any of the studied genes (*GAD67, GAD65, SYN, NCAM, St8SiaII, St8SiaIV*).

There is an overexpression of inhibitory molecules in the amygdala of social isolation rats

Very few studies have analyzed the expression of molecules related to synaptic transmission in the amygdala of isolation-reared rodents. This thesis shows that the expression of GAD67, but not GAD65, protein was increased in different amygdaloid nuclei in isolation-reared rats. By contrast, no differences were found in mRNA expression, which may be due to the masking effect of total *GAD67* mRNA when using whole amygdala extracts. Our findings are in agreement with those of a recent study, which did not find differences in GAD65 protein expression in the amygdala of isolation-reared Sprague–Dawley rats (Lim et al., 2011). The present results indicating an increase in GAD67 expression are in contrast with those reported in the amygdala of human schizophrenic patients. These postmortem studies showed reduced GAD activity (Bird et al., 1977), GABA concentration (Spokes et al., 1980) and GAD67 expression (Varea et al., 2012), which are consistent with an increased activation of the amygdala in schizophrenia. Consequently, we should be cautious when using isolation-reared rats as an animal model of schizophrenia, because while some of its features may correspond to those observed in human patients, some of them may be substantially different.

Also in agreement with Lim et al. (2011), no differences in the expression of synaptophysin were found in the amygdala of isolation-reared animals. These results are also consistent with previous studies describing no differences in the number of synapses in the medial amygdala (Ichikawa et al., 1993).

The differences in PSA-NCAM expression observed in our study may be related to structural changes in neurons, given the anti-adhesive properties of this molecule (Rutishauser, 2008; Sandi, 2004). However, this structural plasticity should be limited initially to interneurons, because, as it has been demonstrated for many PSA-NCAM expressing structures in the cerebral cortex (excluding those of immature neurons) (Gomez-Climent et al., 2011; Nacher et al., 2002a; Varea et al., 2005), many PSA-NCAM expressing neurons in the amygdala express markers of interneurons and lack expression of molecules exclusively found in principal neurons as it has been described in the stress model study. Consequently, changes in PSA-NCAM expression should primarily affect the structure of interneurons, rather than that of principal neurons. In this line, it has been recently reported in our laboratory that PSA-NCAM expressing cortical interneurons have reduced synaptic input and decreased dendritic arborization and spine density when compared with neighboring interneurons lacking PSA-NCAM (Gomez-Climent et al., 2011). It is possible then, that the increases in PSA-NCAM expression observed in the present thesis affect the connectivity of certain amygdaloid interneurons, leaving less plasma membrane extension free for the establishment of synaptic contacts. Whether these changes in PSA-NCAM expression are related to the increase in GAD67 expression still remains to be explored. Another non-excluding possibility is that, given its anti-adhesive properties, the increase in PSA-NCAM expression may facilitate the structural remodeling of certain interneurons in response to different stimuli.

It is interesting to note that the increase in PSA-NCAM expression is only significant in the basolateral amygdala, a region considered critical in the pathophysiology of schizophrenia (Benes, 2010). This increase in PSA-NCAM expression may be due to an increase in the polysialylation of pre-existing NCAM molecules, because an increment of this protein was only found in the medial amygdala of the isolation-reared rats. However, no parallel increments in any of the two NCAM polysialyltransferases were detected, which may mean

that the increased polysialylation of NCAM in the basolateral amygdala has occurred at an earlier age.

The present results indicate that discrete but significant changes occur in the amygdala of isolation-reared rats, involving molecules related to structural plasticity and inhibitory neurotransmission. However, the direction of these differences is not similar to that observed in schizophrenic patients. Consequently, although this paradigm has been confirmed as a suitable model to study schizophrenia, because it reproduces some of its core defects (Fone and Porkess, 2008), it also may present some differences, which should be taken into account and explored further when establishing comparisons.

The dual model of schizophrenia has weight alterations, volume reductions of mPFC and hippocampus and an increase of immature neurons in the hippocampus

In this thesis we have also developed a "double hit" animal model of schizophrenia using the same rat strain, combining both, isolation and N-methyl-D-aspartate (NMDA) receptor blockade during perinatal development. We have described an increased weight gain in rats reared in isolation compared to those reared in groups, indicating an effect of the housing. These results are in agreement with previous studies, which reported similar changes in female Sprague Dawley rats (Hermes et al., 2011; Ness et al., 1995). Although we have not found changes in body weight in adulthood induced by the perinatal MK-801 injection, treated rats showed less weight when weighed at P21. This is in accordance with other reports using different perinatal MK-801 treatments, which have consistently found transient lower body weights that normalized in adolescence or in adulthood (Stefani and Moghaddam, 2005; Su et al., 2011).

In regard to volumetric changes, our results are in agreement with previous reports describing a decrease in mPFC volume in Lister Hooded rats reared in isolation (Day-Wilson et al., 2006; Schubert et al., 2009). Our study expands these previous findings, showing that the volume changes in the mPFC appear to be due to reductions in the prelimbic and infralimbic cortices, but not in the cingulate cortices. Another interesting result of the present thesis is that the hippocampal volume was also reduced in the "double hit" model, although we found that this effect was only caused by the MK-801 injection.

These volumetric reductions in the "double hit" model are extremely important, because they are very similar to those found consistently in schizophrenia (Levitt et al., 2010; Yoshida et al., 2011).

One aspect of structural plasticity, which may be relevant to the hippocampus, is the presence of alterations in adult neurogenesis. Although these alterations are far from explaining the etiology of schizophrenia, they may contribute to the hippocampal aspects of this disorder (Kempermann, 2011). A study in adult human postmortem tissue has found reduced amounts of proliferating cells in the hippocampus of schizophrenic patients (Reif et al., 2006). This is in contrast with our results in the "double hit" model, showing an increase of immature granule neurons (due to social isolation rearing) and no changes in the number of proliferating cells. These results may appear to be in conflict with those found in schizophrenic patients. However, it has to be noted that a significantly higher incidence of granule cells with basal dendrites has been found in these human brains (Lauer et al., 2003) and that the presence of basal dendrites has been described as a characteristic of immature granule cells, at least in rodents (Nacher et al., 2001; Shapiro et al., 2005).

Inhibitory neurotransmission is also altered in the "double hit" model

Several lines of evidence point to alterations in inhibitory circuits as one of the main factors to explain the neurobiological basis of schizophrenia (Benes and Berretta, 2001; Lewis et al., 2005). Reduced expression of the GAD67 mRNA in the PFC (Akbarian et al., 1995; Guidotti et al., 2000, Hashimoto et al., 2007; Torrey et al., 2005) and the hippocampus (Thompson Ray et al., 2011) is one of the most consistent findings in postmortem studies of individuals with schizophrenia. Similar decreases in GAD67 protein expression in the PFC and the hippocampus of schizophrenics have been found (Torrey et al., 2005), including those reported in the fourth article of this thesis. The results of this model partially agree the findings in schizophrenic brains, showing a significant decrease of GAD67 protein expression in layers V and VI of the mPFC, although no changes were detected in the hippocampus. However we did not detect differences in the level of *GAD67* mRNA, suggesting a tissue-specific posttranscriptional regulation mechanism or mRNA downregulations prior to the age at which our animals were sacrificed. The alterations in mPFC GAD67 protein expression were present in the "double hit" model, although

they were induced only by MK-801 perinatal injection. Interestingly, previous reports using perinatal treatments with NMDA receptor antagonists have failed to find significant differences in GAD67 expression (Facchinetti et al., 1993). It is possible that this discrepancy with the present results may be due to differences in the strain (Wistar) or the dosis/duration of the treatment (chronic treatment for 22 days) used by Facchinetti et al. (1993).

Schizophrenia is associated with different alterations in certain interneuronal subpopulations, specially parvalbumin expressing cells, which may have an important impact on the physiology of pyramidal cells (see Lewis et al., 2012 for review). The number of parvalbumin expressing interneurons does not appear to be reduced in schizophrenic patients, at least in the PFC, but they exhibit reduced expression of parvalbumin mRNA and lower density of parvalbumin expressing puncta in certain layers, among other abnormalities at the presynaptic and postsynaptic level (see Beneyto and Lewis, 2011 for review). The "double hit" model developed in this thesis also shows alterations in parvalbumin expressing cells in the mPFC. The number of these interneurons is reduced significantly in the infralimbic cortex, although trends toward decreases were also found in the rest of regions studied. This effect was due to MK-801 treatment, as well as to the interaction of the two hits and it is in accordance with previous reports describing similar reductions after acute perinatal treatment with MK-801 (Coleman et al., 2009; Wang et al., 2007). It is probable that this reduction in the number of parvalbumin expressing cells is due to the extensive cell death caused by the perinatal NMDA antagonist administration during perinatal development (see Lim et al., 2012 for review). However, we have not found evidence of cell death at the time of sacrifice. Although we have not found alterations in the expression of *parvalbumin* mRNA in the present study, we have observed differential changes in *calbindin* and *calretinin* expression, which certainly make necessary future experiments to evaluate more closely the subpopulations of interneurons expressing these calcium binding proteins. Although apparently the calretinin subpopulation is not affected in schizophrenic patients, the calbindin subpopulation may be altered (see Lewis and Hashimoto, 2007 for review).

Our study on the expression of c-Fos in the mPFC also gives support to the idea that prefrontocortical inhibition is decreased in the "double hit" model

and, consequently, this may lead to an excessive activation of excitatory neurotransmission, since we have found an increase in the expression of this marker of cell activity in the nuclei of pyramidal neurons. Interestingly, the increase in c-Fos expression is found in the same region, the prelimbic cortex, where a significant reduction in parvalbumin expressing interneurons has been observed.

Although our results show an interaction, which prevents the observation of changes in *ErbB4* mRNA in the "double hit" model, we still find very interesting that social isolation alone is capable of decreasing *ErbB4* expression. This is the first report describing this decrease in the social isolation schizophrenia model. *ErbB4* and its ligand *Nrg1* have been described as risk genes for schizophrenia (Buonanno, 2010; Norton et al., 2006) and their signaling controls the development of inhibitory cortical networks, regulating the connectivity of certain interneuronal populations, particularly parvalbumin expressing basket and chandelier cells (Fazzari et al., 2010). Consequently, alterations in *ErbB4* during postnatal development and adolescence may interfere with the final establishment of cortical connectivity, specially connectivity involving inhibitory neurons. In addition, we found a similar decrease in PSA-NCAM and GAD67 expression in the deep layers (V and VI) of the mPFC in the "double hit" model. These results, in connection with those described above, provide a putative link between changes in inhibitory neurotransmission and structural plasticity. Changes in PSA-NCAM expression also occur in parallel to the stress-induced dendritic remodeling of interneurons, at least in the amygdala, as it has been described above. Moreover, the reductions in PSA-NCAM and GAD67 expression observed in the "double hit" model are similar to those found in the mPFC of schizophrenic patients. No changes in the expression of NCAM or that of the polysialyltransferases have been found, suggesting that this cell adhesion molecule is apparently unaffected in this model and that changes in the expression of the enzymes responsible for the addition of PSA to NCAM may have occurred previously to sacrifice or that the reduction of PSA-NCAM is due to other factors, such as an enhancement of its removal from the plasma membrane.

Although many of the parameters analyzed in the present study appear to act through independent mechanisms, we find that, using their combination, this "double hit" model can be a very valuable experimental tool to mimic a wider spectrum of specific symptoms and alterations in schizophrenia, specially those affecting inhibitory neurotransmission, and to serve as a testing platform for novel treatments directed to this devastating disorder. Table 5 summarizes the results obtained in the two studied models of schizophrenia.

Table 5. Summary of results in the two schizophrenia models.

Schizophrenia models	Volume	CaMKII (c-Fos +)	DCX(+)	PV (+) Somata	Gene Expression	Protein Density
amygdala	--	--	--	--	=	↓GAD67 ↓PSA-NCAM ↓NCAM
mPFC	↓	↑	--	↓IL	↑*CB* ↓*CR* ↑↓*ErbB4*	↓GAD67 ↓PSA-NCAM
hippocampus	↓	--	↑		=	↓PSA-NCAM

STUDIES IN HUMAN SAMPLES

The article "Alterations in the expression of PSA-NCAM and synaptic proteins in the dorsolateral prefrontal cortex of psychiatric disorder patients" is an analysis of post-mortem sections of the dorsolateral PFC samples from the Stanley Neuropathology consortium, which includes controls, schizophrenia, bipolar and major depression patients. The aim of this study is to find potential alterations in the expression of PSA-NCAM, GAD67, SYN and vesicular glutamate transporter 1 (VGLUT1) proteins.

In the human PFC PSA-NCAM is expressed in interneuronal somata and in neuropil elements belonging to interneurons. The analysis of PSA-NCAM and GAD67 expression in the material of the Stanley Neuropathology consortium revealed that the expression of both molecules were reduced in schizophrenic patients, while an increase in the expression of VGLUT1 was found in the white matter. Depressed patients showed a decrease in SYN and VGLUT1 expression. By contrast, bipolar disorder patients showed a reduction in VGLUT1 and GAD67 expression.

Major depression patients have a reduction in the expression of excitatory markers

The reductions in SYN and VGLUT1 expression found in the mPFC of major depression patients seem to be in accordance with the dendritic atrophy of mPFC pyramidal neurons observed in animal models of depression, such as chronic stress (Cook and Wellman, 2004; Radley et al., 2004; 2006). Nevertheless in our model of chronic stress we found an increase in *SYN* mRNA, which may be due to a feedback regulation effect of the gene expression. These results give support to the neuroplastic hypothesis of depression, which poses that changes in neuronal structure and connectivity may underlie the etiology of this disorder and that these changes may be reverted by antidepressants (Castren, 2005).

A previous study from our laboratory found that changes in SYN and GAD67 expression in the amygdala of major depression patients were accompanied by parallel changes in PSA-NCAM expression (Varea et al., 2012). However, this apparently does not occur in the PFC. In fact, we have obtained similar results in the mice subjected to chronic stress, in which PSA-NCAM expression was altered by this aversive experience in the amygdala, but not in the mPFC.

Schizophrenic patients have diminished expression of inhibitory markers

In schizophrenic patients we have observed a reduction in the expression of PSA-NCAM (layers IV and V), and GAD67 (layers II and IV). These parallel changes of PSA-NCAM and GAD67 may reflect alterations of cortical inhibitory circuits in schizophrenia (for a review see Lewis et al., 2012); in fact, a recent report has found that GAD67 mRNA and protein levels are significantly lower in the DLPFC of schizophrenic patients (Curley et al., 2011). Additionally, they also point to the possible involvement of PSA-NCAM in these alterations in inhibitory neurotransmission, since we have previously demonstrated that this molecule is exclusively expressed by interneurons in the PFC of humans (Varea et a., 2007c) and rodents (Gomez-Climent et al., 2011; Varea et al., 2005). Interestingly, as we have mentioned above, these interneurons have reduced structural features and synaptic input than those lacking PSA-NCAM expression (Gomez-Climent et al., 2011). Moreover, the number of PSA-NCAM expressing neurons is reduced in the hippocampus of schizophrenic patients

(Barbeau et al., 1995). These results are similar to those we have found in the "double hit" model for schizophrenia, in which we have observed decreases in PSA-NCAM and GAD67 expression in the mPFC. By contrast, they are opposite to those found in the amygdala of the social isolation model, in which we have found increases in the expression of PSA-NCAM and GAD67.

The increased expression of VGLUT1 observed in the white matter of the PFC of schizophrenic patients could be related to the higher density of neurons observed in this region (Connor et al., 2009; 2011), supporting the hypothesis of the existence of alterations in the normal development and positioning of cortical neurons in this disorder.

Bipolar disorder patients have reductions in the expression of excitatory and inhibitory markers

Finally, samples from bipolar disorder patients displayed a reduction in the expression of VGLUT1 (layer V) and GAD67 (layers II, III and IV). The explanation for these results is more complex, because bipolar patients have periods of mania followed by periods of deep depression, which may result in cyclic changes in the expression of molecules related to neurotransmission and neural plasticity, as well as in the structure of neurons in the prefrontal cortex. However, the reduction observed in the expression of both excitatory and inhibitory contacts indicates that this region is clearly affected in this disorder.

It is important to notice that the use of some antipsychotics or antidepressants by the patients included in the Stanley Neuropathology Consortium may also influence the expression of the different markers analyzed in this thesis. In fact, chronically administered antidepressants increase the expression of PSA-NCAM and SYN in the mPFC of rodents (Varea et al., 2007a; 2007b). Similarly, the antipsychotic haloperidol decreases the expression of PSA-NCAM, SYN and GAD67 in the rodent mPFC (Castillo-Gomez et al., 2008). Table 6 shows the differences in the protein expression in each layer of the mPFC of the postmortem brains.

Table 6. Summary of results in the postmortem brain study

Psychiatric Disorder	PSA-NCAM	SYN	VGLUT1	GAD67
Major Depression	=	=	↓V	↓V
Bipolar Disorder	=	↓III ↓IV	↓V	↓II ↓III ↓IV
Schizophrenia	↓IV ↓V	=	=	↓II ↓IV

II, III, IV and V refer to the layers of the mPFC

The last article included in this thesis describes an association study of the *ST8SIAII* gene with schizophrenia in a Spanish population. The objective of this study was to replicate the association found previously in Oriental populations, but taking into account the "sex" variable in the sample. Schizophrenia is considered a sexually dimorphic disorder because significant differences in the incidence and course of the disease between men and women have been reported (Kulkarni et al., 2012).

Previous studies found a significant association between *ST8SIAII* and schizophrenia in two Asian samples (Arai et al., 2006; Tao et al. 2007) and one Australian cohort (McAuley et al., 2012). These associations were found with SNPs located in the promoter region of *ST8SIAII*, suggesting that deregulation of *ST8SIAII* expression may increase the risk to suffer schizophrenia. In addition, *ST8SIAII* maps to chromosome 15q25-26, a region reported as including a susceptibility gene for schizophrenia (Maziade et al., 2005).

Allelic and genotyping association of rs3759916 SNP to schizophrenia in the Spanish female population

Because no association between *ST8SIAII* and schizophrenia was reported in the Caucasian population, we explored in a Spanish sample the involvement of this gene as a risk factor for schizophrenia. A previous attempt to replicate in European ancestry samples the significant results found in the Australian schizophrenia cohort was not successful (McAuley et al., 2012), because data did not reach statistical significance. Nevertheless, we did not replicate the positive results found either by Arai et al (2006) with respect to rs3759916 and rs3759914 or by Tao et al. (2007) regarding rs3759915 in our sample. Differences in the allelic frequencies between the different population samples might explain these results. In fact, the alleles associated with schizophrenia in each of the two Asian samples, show lower frequencies in the

Spanish cohort. Alternatively, allelic heterogeneity that characterizes complex disease could also explain these discrepancies. However, when the association analysis was carried out taking into account the sex of the subjects, interesting results were found. In the women sample, the *G* allele (P = 0.044) and the *AG* genotype (P = 0.04) of rs3759916 was significantly associated with schizophrenia, suggesting that this allele is a risk factor for the disease in females in the Spanish population. Therefore the *A* allele might be a protective factor for schizophrenia in females of this population. In the men subgroup, the frequency of the *G* allele is very similar in healthy controls and in patients (1.18% and 0.92% respectively). This was also reported in the Chinese cohort with frequencies of 35.6% in controls and 33.9% in patients, so that no association was obtained for this SPN in this population (Tao et al., 2007). It would be interesting to know whether in the Chinese population also occurs an interaction between sex and the polymorphism rs3759916 concerning the vulnerability to schizophrenia. This polymorphism is located 200 bp downstream from a binding sequence of a glucocorticoid receptor (GR), a transcriptional factor implicated in sexual features.

To further confirm the involvement of *ST8SIAII* in schizophrenia, rs2305561 was also genotyped in the Spanish sample. This SNP is located in the coding region of this gene, concretely in exon 5 (Arai et al., 2006), and shows functional significance because each allele has different efficiency of NCAM polysialylation (Isomura et al., 2011). Therefore chain length and quantity of NCAM polysialylation could vary depending on the rs2305561 genotype affecting its biological function. Nevertheless, no association between rs2305561 and schizophrenia has been reported (Arai et al., 2006). The same result was found in our sample, although the *G* allele and the *GG* genotype were more frequent in the cases than in controls in the male subset. However, this association was lost after the Bonferroni test correction (P = 0.16 and 0.18 for allelic and genotypic frequencies respectively). Since this test is particularly stringent, significance may be lost for this polymorphism that may be a real risk factor. In fact cells expressing the *G* allele of rs2305561 may have significantly decreased amount of PSA on NCAM when compared with cells expressing the *C* allele (Isomura et al., 2011). All these results suggest, on the one hand, that the *G* allele of the rs2305561 polymorphism is a likely risk factor for schizophrenia and on the other, that it has become a risk factor in males of the Spanish

population. Again it would be interesting to analyze this interaction in other populations in order to confirm our results.

A ST8SIAII haplotype is associated to schizophrenia in the Spanish male population

The haplotypic study indicated a risk haplotype *ACAG* in the men sample of the Spanish population (P = 0.028), while no significant haplotypes associated with schizophrenia were found in the women sample of this population. Interestingly the risk haplotype reported in Japanese sampled population (Arai et al., 2006) share the same alleles at rs3759916, rs3759915 and rs3759914 positions, although the frequency of the haplotype *ACA* is lower in our sample than in the Japanese one.

In this thesis we point out the importance of taking into account the gender of the subjects in association studies in mental illness in general and in schizophrenia in particular. Several mental disorders affect differently each sex, specifically there are more men than women affected in schizophrenia and the contrary is observed in depression (Viveros et al., 2012). Regarding PSA-NCAM, a correlation between this molecule and the cells secreting GnRH has been demonstrated (Chalivoix, 2010). Furthermore, estrogen is one of the transcriptional regulators of both polysialyltransferase genes *ST8SIAII* and *ST8SIAIV* (Tan, 2009). Besides *ST8SIAII*, several studies pointing out sex differences in other genes involved in schizophrenia (Goes et al., 2010; Hoenicka et al., 2010; Gilabert-Juan et al., 2011).

Finally, we acknowledge that the present study has the typical limitations of a small sample study. The sample size became reduced when the sample was divided by gender decreasing the power of the analysis. Nevertheless we found interesting sex-specific associations to schizophrenia in *ST8SIAII* and we replicated partially former studies analyzing this gene in other populations. Our study shows *ST8SIAII* as a susceptible gene for schizophrenia with notable differences between sexes. The role of this gene and the high number of interactions that it has with different neuronal pathways suggest polysialyltransferases as attractive molecules to be studied in the field of the psychiatric disorders. Deeper studies in the global expression mechanisms and in the regulation of polysialyltransferases have to be done in order to achieve

more knowledge about their role in the neurodevelopment and in the brain maintenance. Table 7 summarizes the association results in the Spanish sample.

Table 7. Association study results

Sample	rs3759916	rs3759915	rs3759914	rs2305561	Haplotype
Total	--	--	--	--	--
Females	**Allelic Genotypic**	--	--	--	*GCAG*
Males	--	--	--	Allelic Genotypic	***ACAG***

in bold significant results after multiple test correction

Conclusions

1. Enhanced green fluorescence protein (EGFP) expressing cells in the medial prefrontal cortex (mPFC) of adult mice correspond mainly to a subpopulation of Martinotti interneurons.

2. Chronic stress in adult mice induces dendritic hypertrophy in EGFP expressing neurons of adult GFP-expressing Inhibitory Neurons (GIN) mice.

3. Chronic stress in adult mice decreases the number of interneurons expressing GAD67 and GAD-EGFP in the mPFC.

4. Chronic stress causes dendritic hypotrophy of EGFP expressing BLa amygdaloid interneurons.

5. Chronic stress induces a decrease in molecular markers of plasticity and inhibitory neurotransmission (NCAM, synaptophysin and $GABA_A$ alpha receptor) in the amygdala of adult GIN mice.

6. Postweaning social isolation rearing in Lister Hooded rats induces increases in the expression of GAD67, PSA-NCAM and NCAM in different amygdaloid nuclei.

7. The combination of a perinatal injection of the NMDA receptor antagonist MK-801 and a postweaning social isolation rearing in Lister Hooded rats is a "double hit" animal model of schizophrenia, which reproduces a wider spectrum of structural and molecular alterations than any of the single models by itself.

8. The "double hit" model developed in this thesis, presents reductions in the volumes of the mPFC and the hippocampus.

9. The "double hit" model shows a reduction in the number of parvalbumin positive cells and alters *calbindin*, *calretinin* and *ErbB4* gene expression with reductions in PSA-NCAM and GAD67 molecules in the mPFC. PSA-NCAM is also decreased in the hippocampus.

10. The "double hit" model shows an increased number of immature granule neurons expressing doublecourtin in the hippocampus.

11. The "double hit" model shows an increased number of neurons coexpressing the immediate early gene "c-fos" and the marker of excitatory neurons "CaMKII" in the mPFC.

12. Synaptic and plasticity markers are altered in the human dorsolateral prefrontal cortex of psychiatric disorder patients. PSA-NCAM is reduced in schizophrenic patients, synaptophysin in patients of major depression, VGLUT1 in depressive and bipolar patients, and GAD67 in all of them.

13. rs3759916 polymorphism of *ST8SIAII* gene, situated in the promoter region, is associated to schizophrenia in the Spanish female population, and an haplotype of these gene is also associated in the Spanish male population. This result suggests *ST8SIAII* as a susceptibility factors for developing schizophrenia affecting differently depending on the sex of the individual.

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