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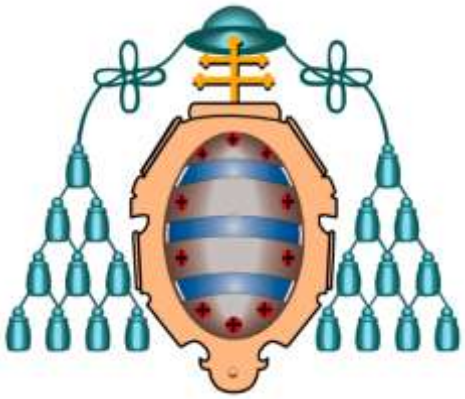

Departamento de Psicología

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Tesis doctoral

Envejecimiento y enriquecimiento ambiental: efecto sobre la conducta y mecanismos cerebrales

Patricia Sampedro Piquero

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Doctoranda

Patricia Sampedro Piquero

Directora

Azucena Begega Losa

Departamento de Psicología. Área de Psicobiología.

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PUBLICACIONES DE LA TESIS

Sampedro-Piquero, P., Begega, A., Zancada-Menéndez, C., Cuesta, M., Arias, J.L. (2013). Age-dependent effects of environmental enrichment on brain networks and spatial memory in Wistar rats. *Neuroscience*, *248*, 43-53. DOI: 10.1016/j.neuroscience.2013.06.003, IF=3.327.

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PUBLICACIONES RELACIONADAS CON LA TESIS

Sampedro-Piquero, P., Zancada-Menendez, C., Begega, A. (2015). More is better? Effect of different types of housing condition on spatial memory and Doublecortin and c-Fos expression in adult Wistar rats (en revisión).

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El cuerpo se me arruga, es inevitable, pero no el cerebro. Mantén tu cerebro ilusionado, activo, hazlo funcionar y nunca degenerará.

Rita Levi-Montalcini

(1909-2012)

ÍNDICE GENERAL

ABREVIATURAS

ACP: análisis de componentes principales.

ACTH: adenocorticotropina.

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.

AP: aprendizaje.

BDNF: factor neurotrófico derivado del cerebro (Brain derived neurotrophic factor).

CO: grupo control.

COx: citocromo c oxidasa (Cytochrome c oxidase).

CREB: elemento de respuesta a AMPc (camp response element-binding).

CRH: hormona liberadora de la corticotropina.

DCL: deterioro cognitivo leve.

DLP: depresión a largo plazo (DTP: Long-term depresion).

EAM: enriquecimiento ambiental.

EAM+AP: enriquecimiento ambiental + aprendizaje

GCs: glucocorticoides.

GD: giro dentado.

GFAP: proteína ácida fibrilar glial (Glial Fibrillary Acidic Protein).

HPA: eje hipotálamo-pituitario-adrenal (Hypothalamic-pituitary-adrenal axis).

MWM: laberinto de Morris (Morris water maze).

NGF: factor de crecimiento nervioso (nerve growth factor).

NMDA: N-methyl-D-aspartate.

NT: neurotrofina.

OMS: organización mundial de la salud.

PLP: potenciación a largo plazo.

PSD-95: proteína de densidad postsináptica (Postsynaptic density-95).

RAWM: laberinto radial acuático (radial arm-water maze).

RGs: receptores de glucocorticoides.

RESUMEN

En los últimos años ha crecido el interés sobre cómo el ambiente y nuestras experiencias pueden afectar a nuestro cerebro. Este interés se ha dado sobre todo en el campo del envejecimiento debido al hecho de que una vida mentalmente activa puede prevenir el deterioro cognitivo. Los mecanismos cerebrales que subyacen a los beneficios de una vida activa se han ido conociendo gracias a estudios con modelos animales expuestos a Enriquecimiento ambiental (EAM). El EAM es un paradigma experimental que consiste en modificar la condición de estabulación de los roedores, incluyendo factores como la exposición a la novedad o la estimulación física, social, sensorial y cognitiva. Con el fin de estudiar el efecto del EAM sobre la conducta y cognición en diferentes edades, ratas Wistar macho de 3 y 18 meses fueron estabuladas 3h/día durante 2 meses en EAM. Posteriormente, los niveles basales de ansiedad, como factor modulador del rendimiento cognitivo, y el aprendizaje de una prueba de memoria espacial fueron evaluados a través del laberinto elevado en cero y del laberinto radial acuático (4 brazos), respectivamente. En este último laberinto, los animales debían de aprender la localización de una plataforma oculta cuya posición fue constante a lo largo de todos los ensayos (6/día) y sesiones (4). Para ello, los roedores debían de utilizar una estrategia alocéntrica a través de pistas distales dispuestas en paneles.

Finalizado el aprendizaje, los cerebros fueron procesados para analizar la actividad Citocromo c oxidasa (COx) de diferentes regiones cerebrales con el fin de determinar los cambios en el metabolismo oxidativo relacionados con la edad, el aprendizaje y el EAM. También se realizó el Análisis de Componentes Principales (ACP) con el objetivo de estudiar las redes funcionales que subyacían a cada condición experimental. Del mismo modo, analizamos el efecto modulador del EAM sobre la plasticidad astrocítica (número y morfología de astrocitos positivos a la proteína ácida fibrilar glial (GFAP)), los niveles de la proteína presináptica Sinapsina I y de receptores de glucocorticoides (RGs), altamente expresados en el hipocampo y encargados de inhibir al eje neuroendocrino.

Nuestros resultados conductuales mostraron que el EAM fue capaz de reducir conductas de tipo ansioso tanto en ratas jóvenes como viejas cuando fueron expuestas a un ambiente nuevo y estresante. También, los grupos enriquecidos, independientemente de la edad, lograron un mejor rendimiento en el laberinto radial acuático. En cuanto a los resultados a nivel cerebral, el ACP reveló que la misma red metabólica funcional subyacía a ambos grupos de edad, pero el peso de las distintas regiones cerebrales en esta red fue ligeramente diferente según la edad y las condiciones de estabulación de los animales. Los análisis de la plasticidad astrocítica mostraron que las ratas viejas enriquecidas tenían un número superior de astrocitos positivos a la proteína GFAP en el Giro dentado dorsal (GD) y una mayor complejidad morfológica de estas células en CA1, CA3 y GD que aquellas estabuladas en condiciones estándar. Por último, el aprendizaje de una prueba de memoria espacial moduló de forma diferente el patrón de expresión de Sinapsina I y de los RGs en el hipocampo dorsal y ventral dependiendo de si los animales habían sido expuestos o no a EAM. Con todo ello, nuestros resultados parecen sugerir que el EAM es una intervención eficaz que mejora la memoria espacial

y reduce conductas ansiosas a cualquier edad, incluso durante el envejecimiento. Estos efectos positivos parecen estar relacionados, en parte, con una serie de cambios en mecanismos neurobiológicos implicados en estas funciones.

ABSTRACT

In recent years, there has been growing interest in how the environment and our experiences can modulate our brain. This interest has been mainly found in the field of aging due to the fact that an active lifestyle may prevent cognitive decline. The brain mechanisms underlying the benefits of an active lifestyle have been studied in different researches in which animal models are housed under environmental enrichment conditions (EE). EE is an experimental design in which the standard housing condition of laboratory rats is changed through the exposition to novelty and different types of stimulation (physical, social, sensory and cognitive). In order to clarify the effect of EE on behavior and cognition at different ages, male Wistar rats, aged 3 years and 18 months, were housed 3h/day for 2 months in an enriched condition. Subsequently, the anxiety basal levels, as a modulator factor in the cognition, and the performance on a spatial memory task were tested through the elevated zero-maze and the radial-arm water maze (RAWM, 4 arms), respectively. In the RAWM, the animals had to learn the position of a hidden platform, which was constant over the trials (6/day) and the 4 sessions. The animals had to apply an allocentric strategy by distal cues arranged in black panels around the maze.

After completing the spatial learning task, the brains were processed to analyse the Cytochrome c oxidase (COX) activity in different brain regions to determine the changes in the neuronal oxidative metabolism related to age, spatial learning and EE. Also, by the Principal Component Analysis (PCA), we studied the functional networks underlying each experimental condition. Similarly, we also analyzed the effect of EE on the astrocytic plasticity (number and morphology of Glial Fibrillary Acidic Protein (GFAP)-immunopositive cells), the expression of the presynaptic protein Sinapsin I and the glucocorticoid receptors (GRs), highly expressed in the hippocampus and involved in the inhibition of the neuroendocrine system.

Our results showed that EE reduced anxious behavior in the young and aged enriched rats, and regardless of the age, the enriched rats showed better performance in the RAWM. Respect to the brain results, the PCA revealed the same brain functional network in both age groups, but the relevance of the different brain regions to the network differed depending on the age and the housing condition of the rats. Also, our results showed that the aged enriched rats had a higher number of GFAP positive astrocytes in the dorsal dentate gyrus (GD) and greater morphological complexity of these cells in the CA1, CA3 and DG hippocampal subfields than those aged rats housed in standard conditions. Finally, the learning of a spatial memory task was able to modulate differently the pattern of expression of Synapsin I and the RGs in the dorsal and ventral hippocampus, depending on the previous housing condition of the aged rats. To sum up, our results seemed to suggest that EE is a successful behavioral protocol to improve the spatial memory and reduce the anxiety levels at different ages, even during aging. These positive effects could be related, in part, to the different changes in the neurobiological mechanisms involved in these functions.

MARCO TEÓRICO

ENVEJECIMIENTO

1.1. ENVEJECIMIENTO DE LA POBLACIÓN

El envejecimiento es un proceso natural que se caracteriza por ser universal, irreversible, progresivo, declinante, heterogéneo, y hasta el momento, también inevitable, en el que ocurren una serie de cambios consecuencia de la interacción de factores genéticos, sociales, culturales y del propio estilo de vida del individuo (Marín, 2003).

En la actualidad, el cambio demográfico de nuestro país y del resto del mundo ha hecho que la investigación en el área del envejecimiento sea fundamental y de gran necesidad. Uno de los factores a los que se debe este envejecimiento de la población es el aumento de la esperanza de vida, como recientemente ha puesto de manifiesto el Instituto Nacional de Estadística (INE) así como, la caída de la fecundidad. De este modo, a mitad de siglo XXI se espera que el 37,6% de los españoles tenga 65 años o más, lo que equivaldría a unos 16,4 millones de personas, y esta tendencia se mantendrá en los años siguientes (Christensen y cols., 2009) (Figura 1).

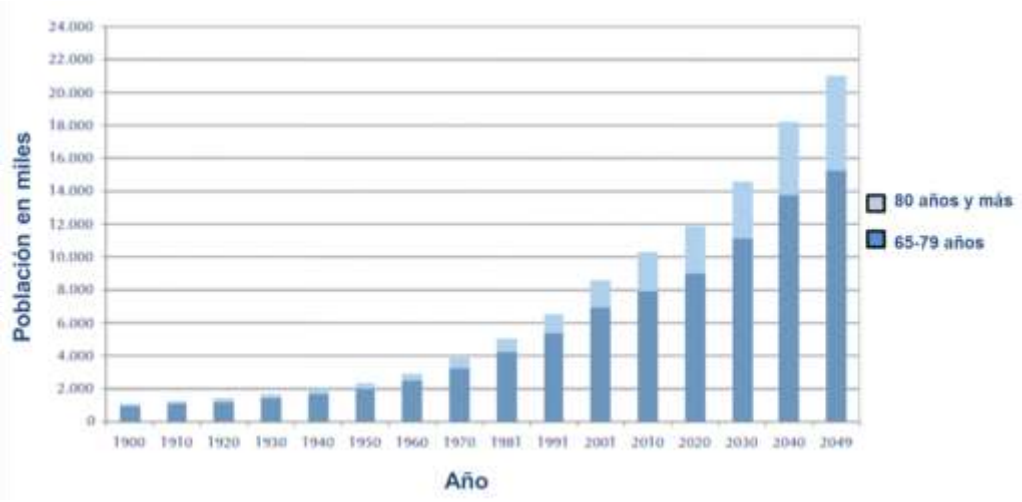


Figura 1. Cifras de población según la edad desde 1900 hasta 2049. Los datos desde 2010 son proyecciones de población a largo plazo (fuente: INE, INEBASE, 2014).

Una de las consecuencias de este cambio demográfico es el incremento de la incidencia de patologías relacionadas con el envejecimiento, como es el caso de las enfermedades neurodegenerativas (Bishop y cols., 2010; O´Callaghan y cols., 2009). Con el aumento de la edad se observa un cierto declive en algunas funciones cognitivas y aunque no es un hecho generalizado, existe un porcentaje relativamente alto de personas que experimentan déficits cognitivos, especialmente problemas de memoria (Deary y cols., 2009). Así, en torno a un 20% de la población mayor sufre deterioro cognitivo leve (DCL) (Petersen, 2011) y entre un 5-10% desarrolla demencia (Plassman y cols., 2008). Incluso, datos más alarmantes ofrecidos por la Organización Mundial de la Salud (OMS) y la Asociación Internacional de Alzheimer (2013) revelan que cada año aparecen 7,7 millones de nuevos casos de demencia, lo que equivale a un caso nuevo cada cuatro segundos.

Por tanto, toda investigación que tenga como objetivo comprender el proceso de envejecimiento, incluyendo cómo las capacidades de aprendizaje y memoria cambian con la edad, resulta necesaria con el fin de proporcionar las bases sobre las que se desarrollen estrategias preventivas (Fratiglioni y Qiu, 2011).

1.2. ENVEJECIMIENTO Y ALTERACIONES COGNITIVAS

El proceso de envejecimiento provoca un declive natural de ciertas funciones cognitivas como la memoria declarativa, las habilidades visuoespaciales, la velocidad de procesamiento o las funciones ejecutivas (Brickman y Stern, 2009; Froufe y cols., 2009; Ofen y Shing, 2013; Subirana-Mirete y cols., 2014); sin embargo otras, como la memoria semántica, la

capacidad de comprensión o el vocabulario, pueden permanecer intactas o incluso mejorar con los años (Ballesteros y Reales, 2004; Fernández-Ballesteros y cols., 2013; Park, 2000).

Respecto a la percepción, pese a que muchas veces es entendida como todos aquellos procesos que ocurren previos a la cognición, cada vez más los límites entre percepción y cognición son menos claros y se habla de una interacción entre ambas. La evidencia indica que las funciones perceptivas, por ejemplo de tipo visual o auditivo, están reducidas en la mayoría de las personas mayores por lo que se les debe de prestar especial atención cuando se llevan a cabo experimentos sobre el rendimiento cognitivo en estas edades (Chan y cols., 2014; Mondon y cols., 2014).

Dentro del ámbito cognitivo, y en referencia a las funciones atencionales, las personas mayores muestran dificultades en pruebas en las que se debe de prestar atención a varios estímulos simultáneamente (atención dividida) pero en cambio, a pesar de su menor velocidad de procesamiento, son capaces de mantener la atención durante un período relativamente largo de tiempo (McDowd y Shaw, 2000; McGaughy y Eichenbaum, 2002; Verhaeghen y Cerella, 2002). Por otro lado, con el paso de los años también se observan déficits significativos en pruebas que requieren una manipulación activa de la información, inhibición de estímulos irrelevantes así como, reorganización e integración de los contenidos de nuestra memoria (Reuter-Lorenz y Sylvester, 2005; Salthouse y cols., 2003; Verhaeghen y Cerella, 2002). En estas últimas funciones está implicada la memoria de trabajo, relacionada con el control activo de la información durante un período corto de tiempo (Baddeley y Hitch, 1974). La memoria de trabajo forma parte de las funciones ejecutivas,

conocidas por ser dependientes del lóbulo frontal y claves en actividades de nuestra vida diaria como la toma de decisiones, el lenguaje, la planificación o la organización, las cuales se alteran también durante el proceso de envejecimiento (Binotti y cols., 2009).

En cuanto a los déficits de memoria, hace unas décadas se pensaba que la mayoría de sus componentes se veían, en algún grado, afectados por la edad (Junqué y Jurado, 1994). Sin embargo, revisiones actuales indican que la afectación de la memoria durante el envejecimiento normal no se da de forma generalizada, sino que las dificultades aparecen de manera específica en algunos sistemas o subcomponentes de la misma (Erickson y Barnes, 2003; Luo y Craik, 2008; Nilsson, 2003). Así, el envejecimiento afecta principalmente a la memoria de tipo episódico es decir, a la memoria de los acontecimientos o experiencias concretas que se produjeron en el pasado. Aunque muchos mayores creen que sus recuerdos de situaciones remotas son mejores que los recuerdos de los acontecimientos recientes, es probable que sólo conserven la información básica y general de ellos, pero carezcan de los detalles, en particular, de su contexto espacial y temporal. Igualmente, también existen muchas dificultades a la hora de recordar la fuente de la información (memoria de la fuente) (Henry y cols., 2012; Lyons y cols., 2014; Schacter y cols., 2013), de acceder a pistas o claves que permitan recuperar la información adquirida así como, de recordar actividades que hemos planeado llevar a cabo en el futuro. Este último tipo de memoria, conocido como memoria prospectiva, quizás se basa en ciertos aspectos de la memoria de trabajo al tener que mantener las futuras intenciones con el paso del tiempo, a la vez que también

implica a la atención dividida, estando ambas afectadas durante el envejecimiento (Craik y cols., 2010; West, 2005).

Respecto a la memoria semántica, que es la encargada de los conocimientos formales, se ha visto que se conserva en gran parte en la vejez. El hecho de que la información a la que se accede desde la memoria semántica se refiere a conocimientos generales, y no a detalles específicos, puede contribuir a la ausencia de diferencias entre jóvenes y mayores en pruebas que evalúan este tipo de memoria (Bataller, 2006; Piolino y cols., 2010). Otras memorias relativamente conservadas son la memoria procedimental y la memoria implícita. La primera de ellas se refiere al conocimiento de habilidades y procedimientos, y la segunda, a los cambios conductuales que aparecen como consecuencia de la experiencia previa (Ballesteros y Reales, 2004; Dew y Giovanello, 2010; Light y cols., 2000; Mitchell y Schmitt, 2006).

Por último, varios estudios han sugerido que las dificultades de memoria espacial que surgen durante el envejecimiento requieren de especial atención y evaluación al considerarse un déficit característico de una fase temprana de demencia de Alzheimer. De este modo, una detección precoz, junto con una intervención adecuada y personalizada, podrían retrasar el avance hacia estadios más severos (Benke y cols., 2014; Gazova y cols., 2012; Iachini y cols., 2009; Vlcek y Laczó, 2014).

1.2.1. Memoria espacial y envejecimiento

La memoria espacial ha mostrado ser una de las más afectadas con el paso de los años (Adamo y cols., 2012; Harris et al., 2012; Rodgers y cols., 2010) y sus déficits tienen repercusiones importantes sobre actividades de la

vida diaria, tales como recordar la localización de objetos u orientarse en ambientes nuevos (Bates y Wolbers, 2014; Lithfous y cols., 2013; Moffat y cols., 2006).

Haciendo una revisión breve sobre los trabajos acerca de memoria espacial y envejecimiento, ya en la década de los 90, Uttl y Graf (1993) observaron que las personas mayores tienen dificultades para recordar la posición de una serie de estímulos que habían visto mientras caminaban a lo largo de un museo. Posteriormente, en un estudio de Wilkniss y cols. (1997), los participantes debían de recordar una ruta previamente recorrida, reconocer las pistas con las que se habían encontrado y ordenarlas también temporalmente. Como resultado, las personas mayores fueron capaces de reconocer las pistas, pero no su orden ni el trayecto recorrido. Además, cuando se les pidió que aprendieran una ruta usando un mapa, mostraron dificultades tanto en la adquisición como en la navegación a partir de su recuerdo. Más adelante, Newman y Kaszniak (2000) evaluaron a un grupo de sujetos en una versión virtual del laberinto acuático de Morris (MWM) en la que debían de aprender a llegar a una posición determinada mediante el uso de pistas distales dispuestas alrededor del entorno. De nuevo, las personas mayores mostraron un rendimiento peor en comparación con los jóvenes. De forma similar, Moffat y cols. (2001, 2007) evaluaron a un grupo de sujetos en una prueba virtual de navegación espacial, encontrando que las personas mayores cometían más errores, a la vez que necesitaban más tiempo para completar la tarea en cada ensayo. Este peor rendimiento se relacionó con una dificultad a la hora de establecer relaciones entre las distintas pistas dispuestas en el ambiente, como fue observado posteriormente a través de otros estudios en los que también se

empleó realidad virtual (Head y Isom, 2010; Iaria y cols. 2009; Jansen y cols., 2010; Wiener y cols., 2012). En relación a este tipo de pruebas virtuales, se ha observado que aunque todos los grupos de edad muestran capacidad para adquirir la tarea, sujetos de mediana edad (40-60 años) y mayores (> 60 años) fueron más lentos en el aprendizaje (Driscoll y cols., 2005; Jansen y cols., 2010), lo cual parece indicar que este tipo de pruebas son sensibles a déficits tempranos. Además, el rendimiento en ambientes virtuales correlaciona positivamente con la ejecución de otras pruebas dependientes del hipocampo como medidas de memoria episódica y habilidades espaciales (Driscoll y cols., 2005; Moffat y cols., 2001).

Junto a lo anterior, existe una gran cantidad de evidencia científica que muestra que con el envejecimiento la memoria espacial, al igual que otras funciones relacionadas con ella, como la percepción visuoespacial, la rotación mental o el escaneo de imágenes, se alteran en todos los mamíferos. La memoria espacial ha sido objeto de estudio de diversos laboratorios durante décadas, ya que no requiere uso del lenguaje, y por lo tanto, representa un excelente ejemplo de una conducta que es fácilmente evaluable tanto en humanos como en animales de laboratorio. La metodología más aplicada en roedores viejos para evaluar la memoria espacial ha sido y es el laberinto acuático de Morris (Morris Water Maze, Morris y cols., 1982). Consiste en una piscina circular llena de agua en la que se sitúa una plataforma que debe ser localizada por el animal y cuya temperatura oscila entre 18 y 27°C, según se utilicen ratas o ratones. En el procedimiento tradicional, el agua se vuelve opaca con leche o alguna sustancia no tóxica, aunque se ha demostrado que no es necesario, ya que el animal nada con la cabeza por encima del agua, lo

que le impide ver la plataforma. La versión tradicional del laberinto es una tarea espacial en la que los animales nadan desde diferentes puntos de salida situados en el perímetro de la piscina hasta encontrar la plataforma sumergida en el agua. Con esta prueba es posible valorar la memoria de referencia, manteniendo la plataforma en la misma posición durante todos los ensayos y por otro lado, la memoria de trabajo, cuando se cambia la posición de la plataforma en cada ensayo. Así, mientras la memoria de referencia es independiente de los ensayos y permite aprender el procedimiento general para la ejecución de la tarea, la memoria de trabajo es una memoria de carácter temporal que depende de cada ensayo. Mediante variaciones metodológicas, el laberinto acuático de Morris también se utiliza como tarea no espacial (aprendizaje de guía). En este procedimiento la plataforma es visible y puede llevar acoplada alguna señal, por lo que los animales resuelven la tarea sin hacer uso, necesariamente, de la información espacial distal (ver revisiones de D´Hooge y De Deyn, 2001; Vicens y cols., 2003).

Generalmente, este tipo de aprendizajes en laberintos acuáticos comienzan con un período de habituación en el que se acostumbra al animal a las contingencias y requerimientos de la prueba. Posteriormente, en la fase de adquisición se introduce al animal con el hocico apuntando hacia las paredes de la piscina para que busque la plataforma durante 60s. En el caso de no encontrarla se le coloca 15s en la plataforma. Después se retira al animal de la plataforma y se le deja descansar brevemente (30s) antes de iniciar el siguiente ensayo. Este procedimiento se repite en los distintos ensayos y a lo largo de las sesiones de entrenamiento. La capacidad del animal para localizar con éxito la plataforma depende de la utilización de las pistas que rodean a la piscina,

empleando una estrategia de navegación conocida con el nombre de *alocéntrica* (O'Keefe y Nadel, 1978). Dicha estrategia consiste en establecer una relación espacial entre las pistas que rodean al laberinto configurando una especie de mapa que permite al animal aprender eficazmente, reduciendo la latencia así como, la distancia recorrida a lo largo de los ensayos. La mayoría de los estudios que emplearon esta prueba encontraron que los roedores viejos necesitan más tiempo para encontrar la plataforma, recorren una mayor distancia y requieren de más ensayos hasta alcanzar un rendimiento similar al de los jóvenes (Bizon y cols., 2004; Nicholson y cols., 2004; Tombaugh y cols., 2005). En contraste, cuando la plataforma es visible y los roedores viejos no tienen que hacer uso de pistas distales, su rendimiento es similar al de los adultos (Barnes y cols., 1997).

Además del MWM, otros laberintos, tales como el laberinto de Barnes, el laberinto en T, o el laberinto radial de Olton han sido también frecuentemente empleados para investigar el aprendizaje espacial en roedores (Dudchenko, 2004). En el caso del laberinto de Barnes, las ratas son colocadas en una plataforma circular muy luminosa y mediante el empleo de pistas visuales dispuestas en la habitación deben de localizar cual es el agujero, de los 18 disponibles, que les permite pasar a un túnel oscuro en el que se sienten más seguras. En esta prueba, las ratas viejas tienen problemas para aprender la localización del agujero que lleva al túnel de escape así como, nuevas localizaciones del mismo (Barnes, 1979; Barnes y cols., 1980). Por otro lado, gracias a los estudios en el laberinto en T se pudo observar que los roedores viejos tienden a emplear con mayor frecuencia una *estrategia de respuesta* en la que aprenden a girar hacia una dirección en particular para alcanzar el

refuerzo (izquierda o derecha) y no una *estrategia de lugar o alocéntrica* en la que deben de establecer una asociación entre el brazo que conduce al refuerzo y las pistas visuales del ambiente (Bates y Wolbers, 2014; Begega y cols., 2012). Por último, con el empleo del laberinto radial (Olton y Samuelson, 1976), se ha observado que los roedores viejos suelen cometer más errores que los jóvenes tanto de memoria de referencia, es decir, entradas en brazos que no contienen el refuerzo, como de memoria de trabajo, reentradas a brazos incorrectos previamente visitados (Grandchamp y Schenk, 2006; Shukitt-Hale y cols., 2004) (Figura 2).

En general, los resultados de todos estos estudios, tanto en humanos como en modelos animales, son consistentes y apuntan a un déficit de memoria espacial en el envejecimiento (Foster y cols., 2012).

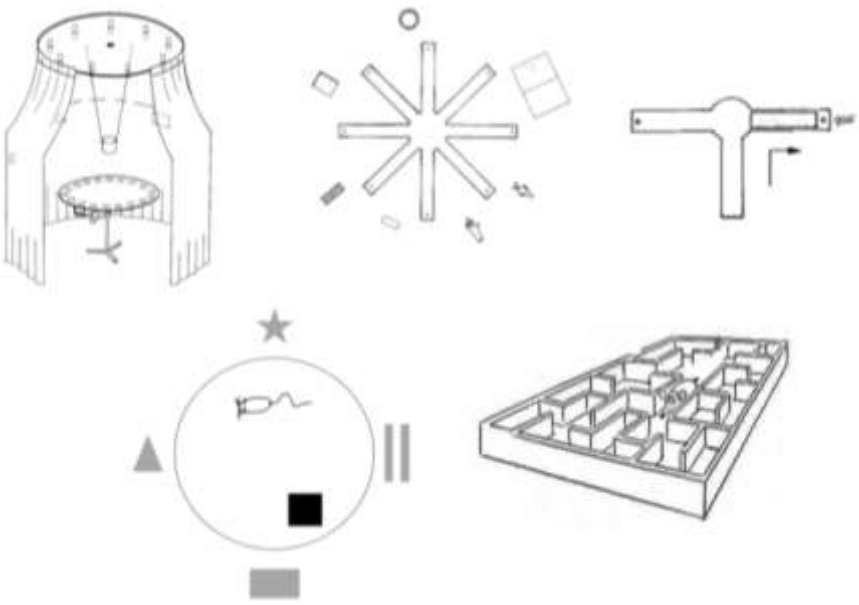


Figura 2. En esta figura se muestran algunos de los laberintos más empleados en la evaluación de la memoria espacial en roedores. De izquierda a derecha, y comenzando por la parte superior: laberinto de Barnes, laberinto radial de Olton, laberinto en T, laberinto acuático de Morris y laberinto de Hebb-Williams.

1.3. ENVEJECIMIENTO Y CAMBIOS CEREBRALES

Las principales regiones cerebrales que han mostrado estar implicadas en la navegación espacial son el hipocampo, las regiones parahipocampales, la red frontoparietal, el cerebelo y la corteza retrosplenial (Grön y cols., 2000; Moffat, 2009). De todas ellas, el hipocampo ha recibido especial atención y la mayoría de los estudios se han centrado en analizar los cambios fisiológicos y morfológicos que aparecen en él durante el envejecimiento (Luine y cols., 2011; Rosenzweig y Barnes, 2003). Por ejemplo, Gallagher y Nicolle (1993), ya a principios de los 90, apuntaron que la severidad de los problemas de memoria espacial de los roedores viejos estaba relacionada con el grado de alteración del sistema septohipocampal colinérgico. En relación a este sistema, se describió una reducción en el número y tamaño de sus neuronas. Esta vulnerabilidad se relacionó también con un declive de factores tróficos, como el factor de crecimiento nervioso (nerve growth factor, NGF), encargados de velar por el mantenimiento y la supervivencia neuronal (Deiana y cols., 2011; Veng y cols., 2003). Además de estas alteraciones, otros cambios en la circuitería hipocampal se han vinculado con los déficits de memoria espacial observados en el envejecimiento. Entre ellos, encontramos una reducida densidad sináptica (Mostany y cols., 2013), alteraciones en la plasticidad sináptica que conllevan a déficits en el mantenimiento e inducción de la potenciación a largo plazo (PLP, mecanismo fisiológico que subyace al aprendizaje y memoria) (Chapman y cols., 2010; Petralia y cols., 2014) y una reducción del umbral de inducción de la depresión a largo plazo (DLP) (Rosenzweig y Barnes, 2003). Finalmente, también se ha descrito un descenso de la densidad de neuronas piramidales en ratas viejas con problemas de memoria espacial (Oh y cols., 2013).

Por otro lado, debemos de tener en cuenta el papel modulador del estrés sobre el aprendizaje y la memoria espacial a la hora de entender los cambios que ocurren con el paso de los años en esta función cognitiva (Aguilera, 2011; Garrido, 2011; Pedersen y cols., 2001; Sindi y cols., 2013). Concretamente, durante el envejecimiento existe un alterado funcionamiento del eje hipotálamo-pituitario adrenal (HPA) caracterizado por una secreción excesiva de glucocorticoides (GCs)) y una reducida retroalimentación negativa, mediada a través de receptores específicos (RGs), que impide al eje HPA volver a sus niveles de actividad basal (Eichenbaum y cols., 2007; Prenderville y cols., 2014; Tasker y Herman, 2011).

En la década de los 80 y 90, varios estudios mostraron que las lesiones del hipocampo provocaban un aumento de la secreción de GCs bajo condiciones de estrés agudo (Jacobson y Sapolsky, 1991; Kant y cols., 1984), sugiriéndose así, un rol inhibitorio de esta región cerebral sobre la actividad del eje HPA. Esta inhibición es mediada por RGs (receptores para glucocorticoides) los cuales se encuentran reducidos en roedores viejos en comparación con los jóvenes (Bizon y cols., 2001; Garrido, 2011). Añadido a esto, también se observó que un aumento de los niveles de GCs provoca una reducción del número de neuronas en el hipocampo junto con un aumento de su vulnerabilidad (Jöels y cols., 2008; Sandi y Pinelo-Nava, 2007). Estos resultados dieron lugar a la *hipótesis de la cascada de GCs* (Sapolsky y cols., 1986), según la cual hay una relación entre una exposición a elevados niveles de GCs y la aparición de atrofia hipocampal. Esta hipótesis fue recientemente revisada y en la actualidad se conoce como *hipótesis de la neurotoxicidad* (Gilbertson y cols., 2002), ya que la exposición prolongada a las hormonas del

estrés reduce la capacidad de las neuronas para hacer frente al daño cerebral y aumenta la velocidad a la que son dañadas (Hibberd y cols., 2000; Miller y O´Callaghan, 2005) (Figura 3).

En roedores viejos, el estrés prolongado provoca también una reducción del flujo de información a través del circuito trisináptico hipocampal (Pavlides y cols., 2002). Este efecto es resultado de una supresión en la inducción de la PLP, principalmente en la vía perforante y comisural, (Pavlides y cols., 2002) junto con una facilitación de la DLP (Jöels y Krugers, 2007). El mecanismo exacto por el que los estresores prolongados afectan a la inducción de la PLP es todavía desconocido, pero se ha sugerido que el exceso de GCs produce un incremento del período de hiperpolarización de la neurona, de los niveles de calcio así como, cambios en la transmisión glutamatérgica, lo cual quizás interfiere con el potencial para inducir este mecanismo fisiológico implicado en el aprendizaje y memoria (Diamond y cols., 2005; Karst y cols., 2000; Weiss y cols., 2005).

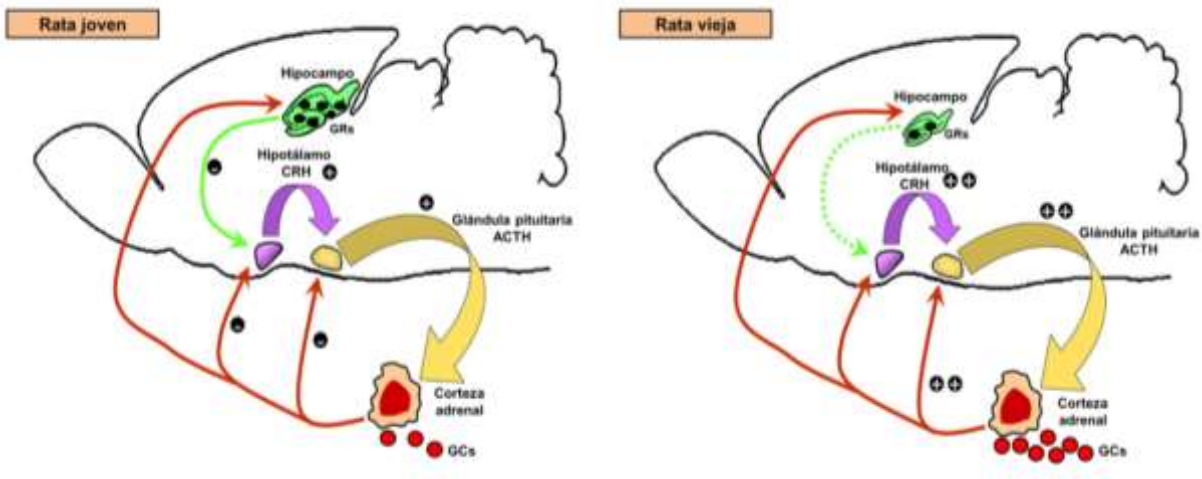


Figura 3. La presencia de un estresor dispara la liberación de GCs debido a la activación del eje HPA. Los GCs tienen la capacidad de atravesar la barrera hematoencefálica y unirse a RGs distribuidos abundantemente en regiones como el hipocampo. En roedores jóvenes, el hipocampo es capaz de ejercer un preciso control inhibitorio de la actividad del eje HPA permitiendo volver a niveles basales de activación, mientras que en roedores viejos existe una falta de control inhibitorio sobre la actividad de este eje.

De este modo, las perspectivas actuales consideran que los déficits de memoria que aparecen durante el envejecimiento no son debidos exclusivamente a las alteraciones anatómicas o neuroquímicas observadas en las regiones cerebrales implicadas en esta función, sino que cambios en sistemas moduladores, como el neuroendocrino, quizás contribuyen sustancialmente a los olvidos característicos de la edad.

ENVEJECIMIENTO ACTIVO

2.1. CONCEPTO

Hoy en día, las personas mayores son, en buena parte, activas, sanas y se cuidan para ser independientes y autónomas el mayor tiempo posible.

La Organización Mundial de la Salud definió el envejecimiento activo como *el proceso de optimización de oportunidades de salud, participación y seguridad con el objetivo de mejorar la calidad de vida a medida que las personas envejecen* (OMS, 2001). Es por tanto un concepto en el que se resalta la idea de potenciar las capacidades de la persona cuando envejece permitiéndole preservar su autonomía, dignidad y ofreciéndole igualdad de oportunidades.

2.2. RESERVA COGNITIVA Y CEREBRAL

Estudios recientes han mostrado que la participación en un estilo de vida activo, a través de actividades que promuevan la estimulación mental, puede ayudar a reducir el riesgo de demencia y constituir una medida prometedora de salud pública (LoGiudice y Watson, 2014; Navarro-González y cols., 2008; Valenzuela y Sachdev, 2006, 2009). El nivel educativo es uno de los factores

más estudiados (Brayne y cols., 2010; James y cols., 2014) así, aunque se ha demostrado que la educación no previene el inicio de Alzheimer, quizás proporciona protección contra la manifestación clínica de sus síntomas (Snowdon y cols., 1996). En sujetos con alto nivel educativo el grado de atrofia cerebral que acompaña a una demencia como la enfermedad de Alzheimer puede ser mucho más severo que en casos de bajo nivel educativo, pero curiosamente las alteraciones cognitivas que presentan puede que sean mucho más leves. Por ejemplo, enfermos de Alzheimer con alto nivel educativo tuvieron un déficit de perfusión parietotemporal más severo, indicando que la enfermedad a nivel cerebral era más avanzada, a pesar de que sus síntomas clínicos pareciesen indicar lo contrario (Barulli y Stern, 2013; Stern, 2009). De este modo, y de acuerdo a esta evidencia, dos sujetos con un similar deterioro cerebral pueden presentar un rendimiento cognitivo totalmente diferente (Bennet y cols., 2003; Kemppainen y cols., 2008). Los estudios *post-mortem* también confirman la hipótesis de la *reserva* al encontrar signos neuropatológicos propios de la demencia de Alzheimer, como ovillos neurofibrilares o placas seniles, en cerebros de personas mayores que nunca tuvieron deterioro cognitivo (Neuropathology Group, 2001). Sin embargo, en el momento en el que los síntomas clínicos aparecen, los sujetos con alto nivel educativo muestran un declive mucho más rápido, probablemente debido a que el grado de patología cerebral ya se encuentra muy avanzado. Todos estos datos proporcionan la base de la hipótesis conocida como *reserva cognitiva* y *cerebral* que se explica a continuación (Stern, 2002, 2009).

El constructo de *reserva* ha sido propuesto para dar cuenta de la falta de relación entre el grado de daño cerebral y la manifestación clínica del paciente.

Dentro del concepto de *reserva* podemos distinguir entre *reserva cognitiva* y *reserva cerebral*, siendo ambas no excluyentes y participando por igual en proporcionarnos protección contra enfermedades neurodegenerativas. La principal diferencia entre ambas es el carácter activo o pasivo que las constituye. La *reserva cerebral* es un ejemplo de reserva pasiva en donde diferencias individuales en el tamaño cerebral, número de neuronas, sinapsis, o de ramificaciones dendríticas permite al sujeto enfrentarse mejor a la patología cerebral. Varios estudios encontraron que la prevalencia de demencia fue menor en sujetos con cerebros mayores que en aquellos con cerebros más pequeños, quizás porque los primeros son capaces de soportar durante más tiempo la patología antes de que los síntomas aparezcan (Bigler y Tate, 2001; Drachman, 2002). Por el contrario, la *reserva cognitiva* tiene un carácter activo al ser mucho más importante el funcionamiento cerebral que su estructura. De este modo, nuestro cerebro intentaría enfrentarse contra la patología cerebral mediante la puesta de marcha de mecanismos compensatorios o de formas de procesamiento cognitivo más eficientes (Stern, 2002). El desarrollo de estas *reservas* va a depender de nuestra experiencia a lo largo de la vida, incluyendo, como hemos señalado anteriormente, nuestro nivel educativo, pero también, el apoyo emocional, el tipo de trabajo profesional desempeñado, las actividades de ocio, o la realización frecuente de ejercicio aeróbico moderado (Richards y Deary, 2005; Scarmeas y Stern, 2003; Smart y cols., 2014). Por ejemplo, un estudio reciente ha observado que aquellas personas que han desempeñado durante su vida profesiones en las que se requiere supervisar, organizar o dirigir presentan una reducción de la atrofia hipocampal que suele acompañar al envejecimiento normal (Suo y cols., 2012). Este hallazgo muestra

la importancia de nuestro estilo de vida sobre la plasticidad cerebral a largo plazo y más concretamente sobre la estructura e integridad hipocampal (Figura 4).

Por otro lado, estudios recientes han propuesto el concepto de *mantenimiento cerebral* en contraposición al de *reserva* descrito previamente (Nyberg y cols., 2012). Este nuevo concepto se basa en la observación de que existe un grupo de personas mayores que no muestran signos de deterioro cognitivo debido a la ausencia de patología cerebral y no a que sean capaces de compensar el daño existente mediante técnicas más eficientes de manejo de la información o determinadas características cerebrales (Barulli y Stern, 2013; Raz y Lindenberger, 2013). Desde esta nueva perspectiva, mantener un cerebro joven, más que compensar los déficits que aparecen durante el envejecimiento, sería la clave para un exitoso funcionamiento de nuestra memoria.

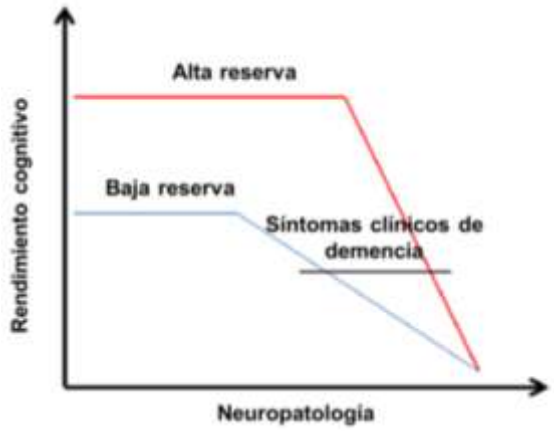


Figura 4. Ilustración de cómo en casos de alta *reserva cognitiva*, el diagnóstico clínico de demencia aparece más tarde cuando ya la patología cerebral es muy severa. En el momento en que en personas con alta reserva cognitiva aparecen los síntomas clínicos, la progresión de la enfermedad será mucho más rápida (fuente: Sampedro-Piquero y Begega, 2013).

Una de las principales limitaciones de la teoría de la *reserva cognitiva* es la laguna que existe entre el concepto y su sustrato neurobiológico (Richards y

Deary, 2005). De este modo, la investigación en modelos animales resulta útil para comprender la influencia de una vida activa sobre el deterioro cognitivo relacionado con el envejecimiento, a la vez que puede aportar información acerca de los mecanismos cerebrales implicados en el efecto beneficioso de vivir en un ambiente estimulante (Petrosini y cols., 2009). Los estudios acerca de cómo el cerebro responde a experiencias estimulantes han empleado con frecuencia el paradigma experimental del *Enriquecimiento ambiental* (EAM), e investigadores como Daffner (2010), han enfatizado la importancia de la investigación básica en animales para identificar qué factores promueven un envejecimiento saludable. En general, los animales mantenidos en ambientes enriquecidos muestran una menor velocidad de progresión de alteraciones cognitivas así como, cambios neurobiológicos que indican la presencia de mayor plasticidad cerebral (Nithianantharajah y Hannan, 2006).

ENRIQUECIMIENTO AMBIENTAL

3.1. CONCEPTO

El término *Enriquecimiento ambiental* (EAM) se refiere a una mejora de las condiciones de estabulación de los animales de laboratorio en comparación con las empleadas de forma estándar (Baumans y Van Loo, 2013). Estas condiciones incluyen jaulas de mayor tamaño que contienen objetos y espacios diversos que facilitan el ejercicio, el juego, la exploración, a la vez que permiten a los animales tener un mayor control sobre su ambiente. En algunos paradigmas experimentales, el EAM también puede incluir estimulación social mediante el aumento del número de animales por jaula favoreciendo así, constantes interacciones dinámicas e impredecibles (Stewart y Bayne, 2004).

Incluso, estudios recientes han optado por el empleo de espejos con el fin de simular estimulación social, observando un efecto positivo sobre los niveles de exploración y ansiedad de los roedores (Fuss y cols., 2013).

Con todo ello, se garantiza un mayor bienestar a los animales, fundamentalmente roedores, a través de la estimulación social, cognitiva, motora y sensorial ofrecida (Nithianantharajah y Hannan, 2006) (Figura 5).

Figura 5. Típico ambiente enriquecido en el que se ofrece estimulación motora, sensorial, cognitiva y física a los roedores. Los objetos son frecuentemente cambiados para motivar las conductas exploratorias en los animales (fuente: Petrosini y cols., 2009).

Todavía no existe un consenso sobre qué paradigma de EAM es el más beneficioso ya que los protocolos difieren ampliamente entre laboratorios (Abou-Ismail, 2011; Simpson y Kelly, 2011). Por ejemplo, en el caso de los objetos utilizados, estos varían en cuanto a composición, forma, tamaño, textura, olor y color entre los distintos estudios. Otra variable importante es el tiempo diario de exposición a EAM que también puede influir sobre los resultados. Referente a esto, varios estudios han mostrado que incluso exposiciones diarias durante períodos cortos (3h) tienen efectos positivos sobre

la cognición en ratas viejas (Leal-Galicia y cols., 2008; Sampedro-Piquero y cols., 2013; Widman y cols., 1992). Por el contrario, trabajos como el de Bennet y cols (2006) observaron que sólo exposiciones durante 24h a las condiciones de EAM conseguían reducir los déficits de memoria espacial en ratas viejas. En cuanto a la duración del protocolo de EAM, aunque una exposición breve (unas pocas semanas) ha mostrado ser capaz de reducir los déficits en aprendizaje y memoria en roedores viejos (Bennet y cols., 2006; Harburger, y cols., 2007), otros han encontrado que una exposición durante toda la vida puede tener un efecto más potente (Kobayashi y cols., 2002). Además, existen diferencias relativas a si el EAM implica tener o no acceso a ruedas en las que poder realizar ejercicio de forma voluntaria, ya que el ejercicio aeróbico, por sí solo, promueve la proliferación de nuevas neuronas en el Giro dentado (GD), la formación de nuevos vasos sanguíneos a partir de los existentes (Berggren y cols., 2014; Kempermann y cols., 2010; Klaus y Amrein, 2012) así como, mejoras en la cognición (Ang y cols., 2006; Hatchard y cols., 2014; Pietropaolo y cols., 2008).

A pesar de esta variabilidad de protocolos, algunos de los aspectos clave del EAM parecen ser la *complejidad ambiental*, con objetos que proporcionen una amplia gama de oportunidades para la estimulación visual, somato-sensorial y olfativa y la *novedad ambiental*, lograda a través del cambio de los objetos y de su posición.

3.2. HISTORIA

Tal y como indica Rosenzweig (1979), las primeras investigaciones sobre el EAM fueron realizadas por el italiano Malacarne (1744-1816). Malacarne encontró que pájaros que habían recibido EAM mostraban cerebros

de mayor tamaño (hecho especialmente evidente en el cerebelo) que aquellos no enriquecidos mantenidos en condiciones de aislamiento y provenientes de la misma nidada de huevos (Renner y Rosenzweig, 1987). Posteriormente, Charles Darwin, en 1874, también otorgó una gran importancia al papel de la estimulación ambiental sobre el tamaño cerebral al señalar que los cerebros de conejos domésticos eran menores que los de los salvajes, postulando que esas diferencias podrían atribuirse al confinamiento y al relativo empobrecimiento que comporta la vida doméstica. Ya en el siglo XX, Santiago Ramón y Cajal (1913) sugirió que la estimulación cerebral podía establecer nuevas y más numerosas conexiones entre las neuronas. Más adelante, Hebb, en 1949 hipotetizó que los animales criados en ambientes enriquecidos durante la infancia podían desarrollar cambios permanentes en el cerebro relacionados con el aumento de las capacidades de solución de problemas. Para ello se basó en el hecho de que las ratas utilizadas como mascotas, y que han experimentado condiciones de vida más estimulantes, eran mejores en la ejecución de laberintos que las ratas de laboratorio. Sin embargo, no fue hasta la década de los 60 cuando el EAM comenzó a ser considerado un paradigma científico. En este momento, en el Laboratorio de Psicología de Berkeley (Krech y cols., 1962) se realizaron los primeros estudios donde se demostraban claramente los primeros efectos neuroanatómicos del EAM. En ellos, un grupo de 12 ratas macho de 25 días de edad fue colocado en una jaula grande (64 x 64 x 46 cm) donde había un pequeño laberinto de madera que podían utilizar de nido y en la que se introducían diariamente juguetes de madera. Además, las ratas exploraban durante 30 minutos al día un laberinto con diferentes configuraciones que variaban diariamente. Simultáneamente, ratas de la misma

camada y edad que las expuestas a EAM fueron colocadas en una condición de empobrecimiento, en jaulas individuales (28 x 20 x 20 cm) con acceso sólamente a comida y agua *ad libitum*. Los análisis histológicos del cerebro de estos animales mostraron que estas manipulaciones ambientales provocaban cambios neuroquímicos y de peso cerebral. El resultado más sorprendente fue el incremento de peso de la corteza visual (8%) y de la corteza somato-sensorial (3%) en los animales enriquecidos respecto a los aislados.

A partir de estos trabajos pioneros, distintos autores han ido mostrando otros efectos neuroanatómicos y conductuales del EAM en animales de distintas edades y durante períodos de enriquecimiento que oscilaban desde varios días, hasta semanas o meses (Ramírez-Rodríguez y cols., 2014; Rampon y cols., 2000; Van Praag y cols., 2000). A continuación se describirán los resultados más interesantes acerca del potencial del EAM.

3.3. BENEFICIOS EN ROEDORES VIEJOS DEL EAM

3.3.1. Efecto sobre la cognición: memoria espacial

El EAM ha mostrado capacidad para minimizar las alteraciones que aparecen con la edad en varios tipos de memoria. Por ejemplo, en pruebas en las que se evalúa la memoria de tipo no espacial, como la prueba de reconocimiento de objetos o el condicionamiento contextual al miedo, los animales enriquecidos tienen un mejor rendimiento que aquellos que fueron estabulados en condiciones estándar (Duffy y cols., 2001; Lima y cols., 2014; Rampon y cols., 2000; Tang y cols., 2001). En el caso de la memoria espacial, el EAM consiguió mejorar significativamente el rendimiento de ratas y ratones de mediana edad en una prueba de memoria de referencia espacial en el

MWM, (Frick y Fernández, 2003; Pham y cols., 2002) así como, el aprendizaje en el laberinto de Hebb-Williams (Kobayashi y cols., 2002). Es en esta mediana edad cuando se considera que comienza el declive cognitivo y los animales empiezan a tener dificultades en resolver pruebas de memoria espacial en las que se han de utilizar pistas alocéntricas para encontrar la plataforma (Begega y cols., 2012; Bizon y cols., 2009; Jacobson y cols., 2008). De este modo, experiencias estimulantes como el EAM puede que tengan un alto efecto positivo en estas edades cuando las alteraciones relacionadas con el paso de los años no son todavía muy severas. Sin embargo, los resultados han sido a veces contradictorios. Por ejemplo, Freret y cols. (2012) apuntan que el EAM necesita ser aplicado antes de esta edad para tener un efecto positivo sobre la cognición, mientras que Kempermann y cols. (1998) observaron que el EAM, aún en este período, produce beneficios sobre el rendimiento en el MWM.

De forma similar, en ratas y ratones viejos, también se observó que el EAM es capaz de mejorar el rendimiento en una prueba de memoria de referencia espacial en el MWM (Frick y Fernández, 2003; Kumar y cols., 2012). Algunos estudios han sugerido que esa mejora es debida a una rápida adquisición y a un flexible uso de la información espacial (Speisman y cols., 2013), mientras que otros consideran que el EAM puede tener un mayor impacto sobre los procesos de consolidación, al encontrar que los animales enriquecidos muestran un mejor mantenimiento de la información espacial 24h después de haber finalizado el entrenamiento (Harati y cols., 2011).

Por otro lado, también durante la vejez, el EAM provoca mejoría en pruebas de emparejamiento demorado a la muestra en la que se valora la capacidad y funcionamiento de la memoria a corto plazo (Soffié y cols., 1999),

en la alternancia espontánea (Van Waas y Soffié, 1996) y en el aprendizaje incidental (Warren y cols., 1982).

3.3.2. Efecto sobre los niveles de ansiedad y exploración

Los efectos del EAM sobre la respuesta de estrés no son todavía del todo comprendidos (Benaroya-Milshtein y cols., 2004; Roy y cols., 2001). Sin embargo, la evidencia sobre el tema indica que el EAM puede proteger contra, o incluso revertir, el efecto negativo de la exposición a estresores intensos e incontrolables (Iwata y cols., 2007; Roy y cols., 2001). Con la excepción de un solo trabajo, en el que roedores fueron expuestos a un tipo especial de EAM que consistía en un incremento gradual de la dificultad para encontrar la comida (Martínez-Cue y cols., 2002), la mayoría de los estudios revelaron que el EAM es capaz de reducir la reactividad emocional con un descenso de los niveles de *freezing* y defecación (Fox y cols., 2006; Larsson y cols., 2002). Los animales enriquecidos se muestran también más relajados, más fáciles de manejar, menos impulsivos (Kirkpatrick y cols., 2014; Van de Weerd y cols., 2002) y con una mayor tendencia al juego (Marashi y cols., 2003).

Un tipo de estresor frecuentemente utilizado es la exposición de los animales a ambientes nuevos, puesto que la novedad crea un conflicto entre el deseo instintivo de explorar y el miedo a la condición nueva o neofobia. En relación a esto, cuando los roedores que recibieron EAM son expuestos a pruebas de ansiedad incondicionada como el laberinto en cruz, muestran frecuentemente un aumento de entradas a los brazos abiertos, indicando reducidos niveles de ansiedad y aumento de la exploración (Ravenelle y cols., 2014; Takahashi y cols., 2014). Es interesante destacar que el efecto ansiolítico del EAM parece ser más notable cuando la prueba de evaluación es altamente

desafiante para el animal (Fernández-Teruel y cols., 2002). Por ejemplo, el EAM mejoró la exploración sólo cuando en el laberinto radial estaban disponibles 8 brazos, pero no 4 (Janus y cols., 1995). Este último dato parece sugerir que el EAM llega a producir efectos ansiolíticos sólo cuando el nivel de estrés que conlleva la nueva situación es relativamente alto como para causar pronunciada ansiedad.

Incluso, estudios recientes como los de Goes y cols. (2014) y Ravenelle y cols. (2014) ha mostrado que el EAM es capaz de reducir los niveles de *ansiedad rasgo* en ratas adultas, entendida como aquellas características individuales relativamente estables en el tiempo, que hacen al sujeto más vulnerable a problemas de ansiedad (Kennedy y cols., 2001).

Generalmente, se asume que las conductas exploratorias, tales como el grado de locomoción o *rearing*, son reflejo de bajos niveles de ansiedad (Casarrubea y cols., 2013). Así, los animales enriquecidos suelen presentar un patrón de exploración diferente, tanto cuantitativa como cualitativamente respecto a roedores no enriquecidos, a la vez que invierten más tiempo en la búsqueda de estímulos no familiares (Lambert y cols., 2001; Schrijver y cols., 2002). Estos niveles superiores de exploración parecen ser mayores durante los primeros minutos de pruebas conductuales como el campo abierto, mientras que posteriormente se suele reducir la actividad debido a una habituación más rápida al nuevo ambiente (Hughes y Collins., 2010; Kempermann y cols., 2002; Schrijver y cols., 2002). Algunos autores sostienen que esta rápida habituación a la novedad puede ser entendida como una mejor capacidad de aprendizaje y de memoria a corto plazo, o bien una constante

tendencia hacia la búsqueda de novedad (Barnes y cols., 1991; Thiel y cols., 1998).

Recientemente, Harris y cols., (2009) llegaron incluso a sugerir que la reducción de los niveles de ansiedad que provoca el EAM cuando los roedores se encuentran en una situación de evaluación, ya sea en el MWM u otro tipo de laberinto, es clave para entender el mejor rendimiento de estos animales en las pruebas de evaluación cognitiva.

3.3.3. Reserva cerebral

Todos estos beneficios conductuales se encuentran relacionados con cambios en la estructura y función cerebral. A nivel morfológico, los primeros estudios sobre el tema describieron un aumento del peso y volumen cortical, fundamentalmente de la corteza visual, somato-sensorial y frontal (Bennet y cols., 1964). Del mismo modo, se vio también que el EAM era capaz de incrementar el grosor de la corteza occipital (Diamond y cols., 1964), el volumen del hipocampo (Diamond y cols., 1976), e incluso el de algunas áreas subcorticales (Greenough y cols., 1973).

Asimismo, los efectos que han despertado un mayor interés durante los últimos años son los referentes al impacto del EAM sobre la neurogénesis. Se ha visto que el EAM produce un aumento de la supervivencia de las nuevas neuronas granulares que proliferan en el GD del hipocampo del ratón (Castilla-Ortega y cols., 2011; Kempermann y cols., 2002) y de rata adulta y vieja (Segovia y cols., 2006; van Praag y cols., 2000). En la actualidad, hay numerosos estudios que sugieren que la neurogénesis adulta está implicada en funciones tanto cognitivas como emocionales dependientes del hipocampo (Kempermann, 2008). De este modo, aquellos roedores que muestran una

mayor capacidad neurogénica presentan también un mejor rendimiento en pruebas de memoria espacial, llegando a encontrarse una relación entre el nivel de aprendizaje alcanzado y el número de neuronas nuevas (Sisti, y cols., 2007).

Por otro lado, el EAM no sólo produce cambios en el número neuronal, sino también en la propia morfología de las células nerviosas, incrementando el número de ramificaciones y el de espinas dendríticas (Rampon y cols., 2000). Dado que las espinas dendríticas son los lugares donde se establecen las conexiones sinápticas, este incremento no es más que una indicación indirecta de un aumento en la actividad sináptica de las neuronas, lo cual podría tener consecuencias funcionales sobre la capacidad de procesamiento de información de los animales enriquecidos.

Estos cambios en el número y morfología de las neuronas se entienden mejor si se tienen en cuenta los efectos que el EAM tiene sobre la expresión génica y de factores neurotróficos. El aumento de la expresión de ciertos genes posiblemente está mediado por factores de transcripción como CREB (Camp response element-binding) cuyos niveles incrementan tras EAM (Williams y cols., 2001). Por ejemplo, los roedores enriquecidos muestran cambios en la expresión de genes relacionados con la formación de nuevas sinapsis y la reorganización y fortalecimiento de las existentes (Rampon y cols., 2000). Además, el EAM, también incrementa la expresión de proteínas pre y postsinápticas, como Sinaptofisina y Postsynaptic density-95 (PSD-95) respectivamente, que participan en el proceso de sinaptogénesis (Frick y cols., 2003; Lambert y cols., 2005; Nithianantharajah y cols., 2004).

Otro tipo de moléculas que aumentan su expresión como consecuencia del EAM son los factores neurotróficos, como por ejemplo NGF, o el factor neurotrófico derivado del cerebro (Brain Derived Neurotrophic Factor, BDNF), la neurotrofina-3 (NT-3) y la neurotrofina-4/5 (NT-4/5) (Mohammed y cols., 2002; Mora y cols, 2007; Pham y cols., 1999). Estos factores neurotróficos son proteínas que promueven la supervivencia, la división, el crecimiento así como, la diferenciación y la plasticidad morfológica de las neuronas, a la vez que se encargan de nutrir a estas células nerviosas durante todo el ciclo vital.

Además, el EAM induce alteraciones en la expresión de los receptores N-methyl-D-aspartate (NMDA) y α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), que forman parte de la transmisión de señales glutamatérgicas (Naka y cols., 2005; Tang y cols., 2001). En este sentido, en el estudio de Segovia y cols. (2006) se halló un incremento en los niveles de glutamato en la región CA3 del hipocampo en ratas viejas, lo cual supondría un aumento de los potenciales sinápticos excitatorios (Mohammed y cols., 2002) y también de la PLP (Van Praag y cols., 2000).

Igualmente, en el cerebro de roedores enriquecidos también ha sido descrito un aumento del número de células gliales, fenómeno conocido como gliogénesis (Theodosis y cols., 2008; Williamson y cols., 2012) así como, un cambio en su morfología. Este cambio morfológico está probablemente implicado en mediar la interacción entre las nuevas espinas dendríticas y las ramificaciones astrocíticas (Haber y cols., 2006; Ullian y cols., 2001). Evidencias recientes sobre el tema sugieren que los astrocitos forman parte activa de la transmisión sináptica, de la plasticidad y de la neurotransmisión (Auld y Robitaille, 2003; Haydon, 2001). Esto ha llevado a algunos autores a

formular el concepto de *sinapsis tripartita*, entendida como el conjunto formado por las neuronas pre y postsináptica y los procesos astrocíticos que modulan la neurotransmisión a través de la liberación de gliotransmisores (Panatier y cols., 2011; Parpura y cols., 2010).

Por último, en relación a la reducción de respuestas ansiosas en los roedores viejos enriquecidos, varios estudios han apuntado que gracias a este protocolo de estimulación se consigue una reducción en sangre de los niveles de hormonas implicadas en la activación del eje HPA, como las hormonas adenocorticotropina, secretada por el núcleo paraventricular del hipotálamo, y los GCs, secretados por la corteza de las glándulas adrenales, cuando los animales son expuestos a diferentes estresores (Garrido, 2011; Moncek y cols., 2004; Sztainberg y cols., 2010). También, el EAM aumenta los niveles de RGs en el hipocampo, lo cual facilita la inhibición de la actividad del eje HPA y un restablecimiento de los niveles basales de GCs (Fox y cols., 2006; Olsson y cols., 1994). Por último, el aumento que el EAM provoca sobre la expresión del factor de crecimiento BDNF en el cerebro envejecido, se ha relacionado con una mayor capacidad de resiliencia en estos animales (Segovia y cols., 2008; Wolf y cols., 2006) (Figura 6).

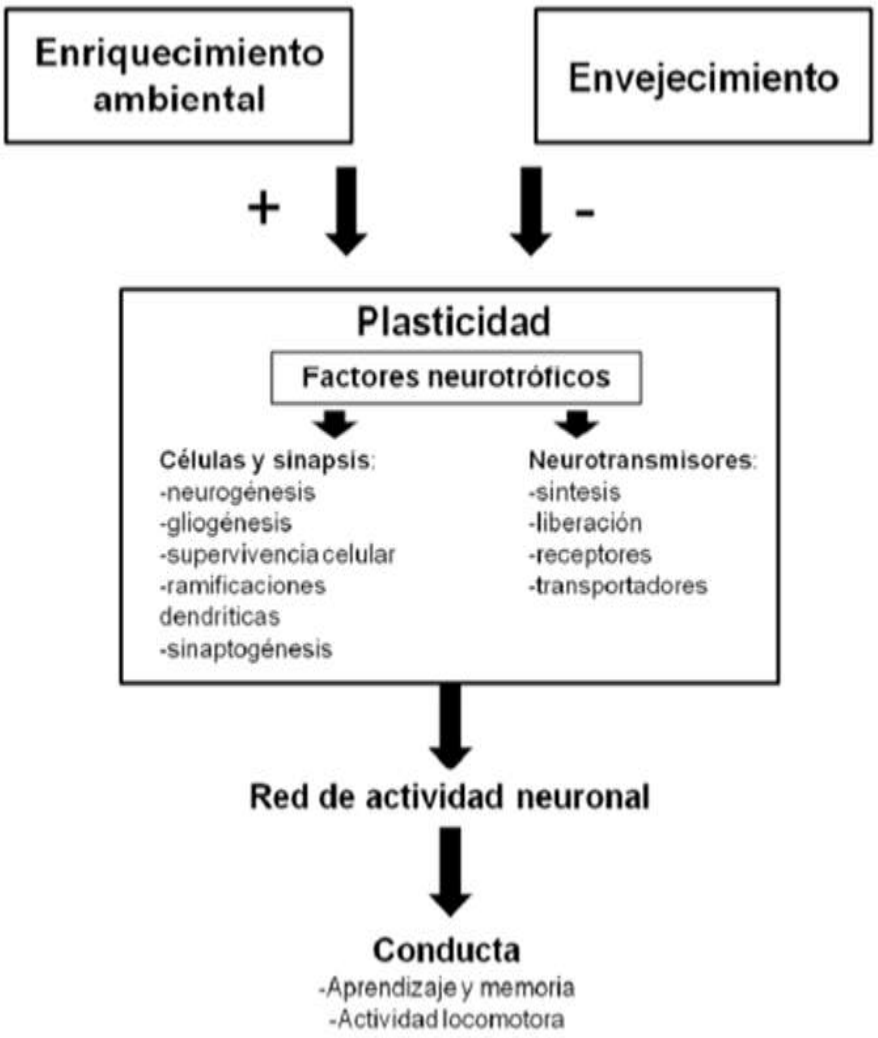
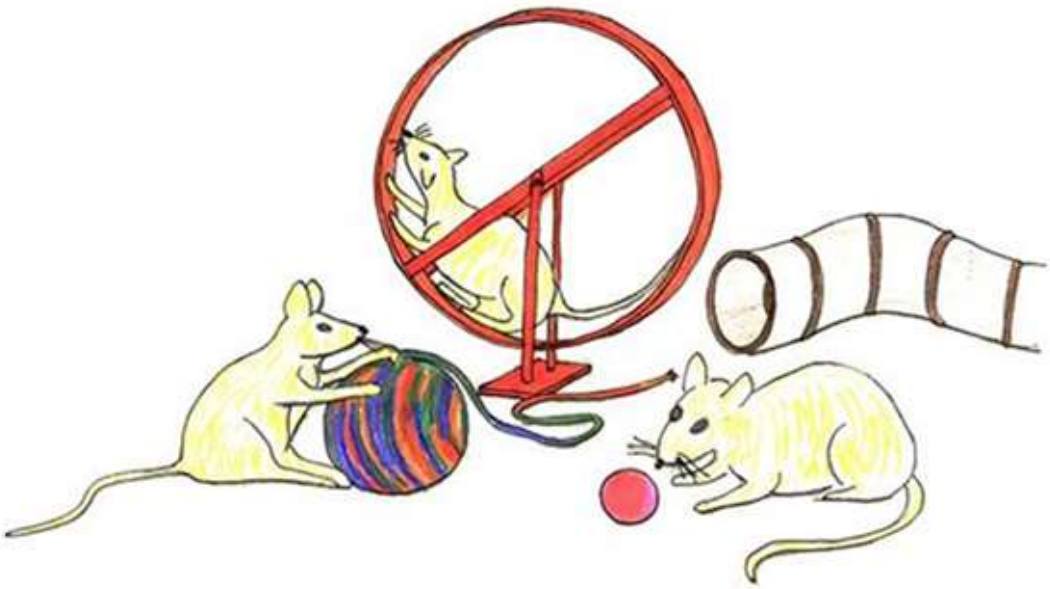


Figura 6. Esquema que muestra el efecto del EAM y del proceso de envejecimiento sobre la plasticidad cerebral. La interacción entre los cambios positivos inducidos por el EAM y los negativos del envejecimiento se manifestaría en el funcionamiento cognitivo y conductual a causa de los cambios que se producen en las redes neuronales (fuente: Sampedro-Piquero y Begega, 2013).

Teniendo en cuenta todo lo anterior, podría ser que los cambios descritos tras un protocolo de EAM estén relacionados con la formación de una *reserva cerebral* que permita el establecimiento de nuevas conexiones neuronales necesarias para un procesamiento más eficiente de la información y una mayor capacidad de resolución de problemas, además de compensar el funcionamiento de redes alteradas durante el envejecimiento.

PLANTEAMIENTO
Y
OBJETIVOS



PLANTEAMIENTO Y OBJETIVOS

Tal y como se ha descrito en el marco teórico, los programas de EAM constituyen una de las experiencias con más efectos positivos descritos sobre el sistema nervioso y con más repercusiones en el ámbito cognitivo y emocional de los animales de laboratorio (Duffy y cols., 2001; Hutchinson y cols., 2012; Larsson y cols., 2002; Leggio y cols., 2005; Moncek y cols., 2004; Petrosini y cols., 2009). No obstante, aunque es extensa la literatura publicada sobre el tema, todavía existen varias cuestiones que han sido poco investigadas. Una de ellas, y que constituye uno de los objetivos de esta Tesis doctoral, es analizar el posible efecto diferencial del EAM sobre la conducta dependiendo de la edad de los animales. En relación a esto, es posible que el EAM provoque mayores beneficios en roedores jóvenes debido a su mayor plasticidad cerebral así como, a un mejor aprovechamiento de la estimulación ofrecida. En el caso de roedores viejos, los resultados acerca del efecto del EAM son más heterogéneos. Así, aunque existen estudios que han mostrado un efecto positivo sobre la cognición (Frick y cols., 2003; Harburger y cols., 2007; Speisman y cols., 2013), otros sugieren que alcanzado un cierto grado de deterioro cognitivo, el EAM no sería suficiente para revertir los déficits (Freret y cols., 2012). Esta variabilidad de resultados puede que sea debida también a los diferentes protocolos de EAM que han sido aplicados en los distintos estudios (diferente duración, número de animales, tipo de objetos) (Simpson y Kelly, 2011). De este modo, consideramos que el mejor diseño experimental es aquel en el que se comparan grupos de diferente edad bajo un mismo protocolo de EAM.

Otro de los objetivos de esta Tesis ha sido el estudio de los cambios que ocurren en diferentes procesos implicados en la plasticidad cerebral tras la experiencia de EAM o de condiciones estándar de laboratorio. Así, entre los marcadores estudiados se encuentra la expresión de ciertas proteínas implicadas en la plasticidad sináptica, el análisis de la actividad metabólica neuronal, e incluso el estudio morfológico de las células astrocíticas, recientemente implicadas en la transmisión sináptica (Pérez-Álvarez y cols., 2014; Verkhratsky y cols., 2014). Como factor modulador de la actividad sináptica y regulador del eje HPA, también se analizó la expresión de los RGs en el hipocampo. Estos mecanismos cerebrales han sido relacionados también con procesos de aprendizaje y memoria y de este modo, otro de nuestros objetivos fue evaluar cómo el aprendizaje de una prueba de memoria espacial puede modularlos de forma diferente dependiendo de la condición previa de estabulación de los animales. Como ya se ha descrito previamente, el EAM mejora el rendimiento en pruebas de memoria, fundamentalmente de tipo espacial, lo cual podría entenderse en parte por la modulación que ejerce sobre diferentes mecanismos implicados en los procesos de aprendizaje y memoria (van Praag y cols., 2000).

Por tanto, y considerando todo lo anterior, los objetivos de esta Tesis doctoral son los siguientes:

Primer objetivo: Estudiar la conducta de ratas Wistar tras un programa de EAM y de aquellas que vivieron en condiciones estándar de laboratorio.

a) Estudiar el efecto sobre los niveles basales de ansiedad y de exploración en el laberinto elevado en zero y si este es dependiente de la edad.

b) Estudiar el impacto de este programa de estimulación sobre la memoria de referencia espacial en el laberinto radial acuático y si su efecto es dependiente de la edad.

Segundo objetivo: Analizar el impacto de las condiciones experimentales grupo control (CO); grupo enriquecimiento ambiental (EAM); grupo aprendizaje espacial (AP); grupo enriquecimiento ambiental + aprendizaje espacial (EAM+AP) sobre diferentes mecanismos subyacentes a los procesos de aprendizaje y memoria:

a) La actividad metabólica neuronal (actividad citocromo c oxidasa) y la red de activación asociada con cada condición experimental.

b) La plasticidad astrocítica (número y morfología de células positivas a la proteína ácida fibrilar glial, GFAP).

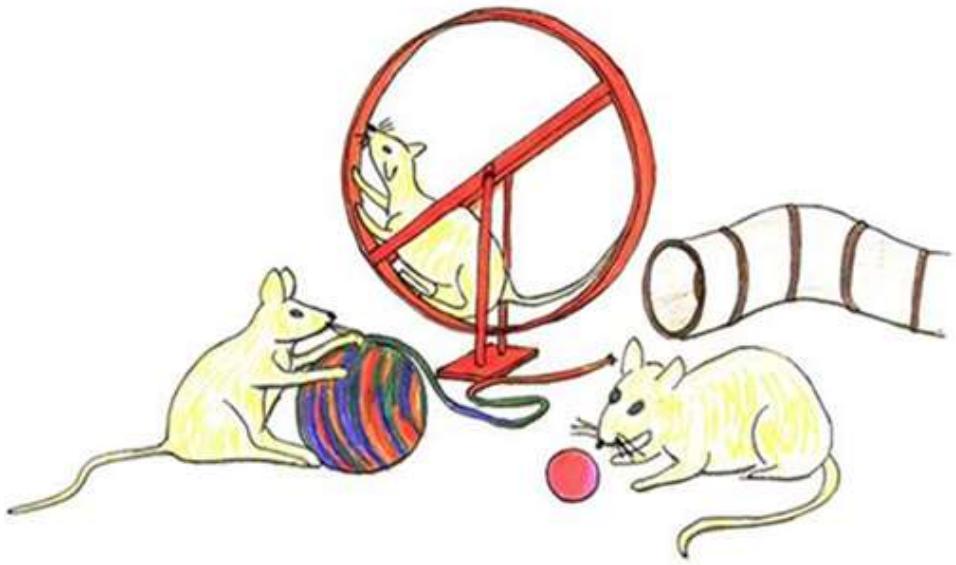
c) Los niveles de Sinapsina I en el hipocampo dorsal y ventral.

d) La expresión de RGs en el hipocampo dorsal y ventral.

A continuación, se muestra un esquema donde se representan las condiciones experimentales utilizadas en los trabajos que componen esta Tesis doctoral (Figura 7).

Figura 7. Esquema que muestra las condiciones experimentales planteadas en esta Tesis doctoral (CO: grupo control mantenido en condiciones de estabulación estándar; EAM: grupo estabulado 3h/día durante 2 meses en condiciones de enriquecimiento ambiental; AP: grupo mantenido en condiciones de estabulación estándar y sometido a una prueba de memoria espacial; EAM+AP: grupo estabulado 3h/día durante 2 meses en condiciones de enriquecimiento ambiental y sometido a una prueba de memoria espacial).

EXPERIMENTO I



Age-dependent effects of environmental enrichment and spatial memory in Wistar rats

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AGE-DEPENDENT EFFECTS OF ENVIRONMENTAL ENRICHMENT ON BRAIN NETWORKS AND SPATIAL MEMORY IN WISTAR RATS

P. SAMPEDRO-PIQUERO,[a]* A. BEGEGA,[a] C. ZANCADA-MENENDEZ,[a] M. CUESTA[b] AND J. L. ARIAS[a]

[a] *Laboratorio de Neurociencias, Departamento de Psicología, Universidad de Oviedo, Plaza Feijoo s/n, 33003 Oviedo, Spain*

[b] *Área de Metodología, Departamento de Psicología, Universidad de Oviedo, Plaza Feijoo s/n, 33003 Oviedo, Spain*

Abstract—We assessed the effect of 3 h of environmental enrichment (EE) exposure per day started at different ages (3 and 18 months old) on the performance in a spatial memory task and on brain regions involved in the spatial learning (SPL) process using the principal component analysis (PCA). The animals were tested in the four-arm radial water maze (4-RAWM) for 4 days, with six daily trials. We used cytochrome c oxidase (COx) histochemistry to determine the brain oxidative metabolic changes related to age, SPL and EE. Behavioural results showed that the enriched groups, regardless of their age, achieved better performance in the spatial task. Interestingly, in the case of the distance travelled in the 4-RAWM, the effect of the EE was dependent on the age, so the young enriched group travelled a shorter distance compared to the aged enriched group. Respect to COx histochemistry results, we found that different brain mechanisms are triggered in aged rats to solve the spatial task, compared to young rats. PCA revealed the same brain functional network in both age groups, but the contribution of the brain regions involved in this network was slightly different depending on the age of the rats. Thus, in the aged group, brain regions involved in anxiety-like behaviour, such as the amygdala or the bed nucleus of the stria terminalis had more relevance; whereas in the young enriched group the frontal and the hippocampal subregions had more contribution.

Key words: environmental enrichment, spatial learning, cytochrome c oxidase, principal component analysis, ageing, Wistar rat.

*Corresponding author. Tel: +34-985104192.
E-mail addresses: uo179677@uniovi.es (P. Sampedro-Piquero), begega@uniovi.es (A. Begega), zancadaclara.uo@uniovi.es (C. Zancada-Menendez), mcuesta@ uniovi.es (M. Cuesta), jarias@ uniovi.es (J. L. Arias).

Abbreviations: Acb, accumbens nucleus; ANOVA, analysis of variance; BlA, basolateral amygdala; BST, bed nucleus of the stria terminalis; CA1, CA3, hippocampal cornu ammonis; CeA, central amygdala; Cg, cingulate cortex; COx, cytochrome c oxidase; DG, dentate gyrus; EE, environmental enrichment; HPA, hypothalamic–pituitary–adrenal axis; IL, infralimbic cortex; LS, lateral septal nucleus; LTP, long-term potentiation; MANOVA, multivariate ANOVA; MM, medial mammillary nucleus; PC, parietal cortex; PCA, principal component analysis; PL, prelimbic cortex; RAWM, radial water maze; RM ANOVA, ANOVA of repeated measures; RSC, retrosplenial cortex; SPL, spatial learning; SuM, supramammillary nucleus.

INTRODUCTION

Environmental enrichment (EE) is an experimental condition that consists of a combination of enhanced social relations, physical exercise and interactions with stimulating objects (van Praag et al., 2000; Würbel, 2001; Simpson and Kelly, 2011). EE is known to improve spatial memory and learning abilities (Leggio et al., 2005; Petrosini et al., 2009). For example, spatial reference memory is improved in adult rats (Nilsson et al., 1999) and mice (Kempermann et al., 1997; Williams et al., 2001) exposed to 1–3 months of EE relative to isolated or social controls. Other studies have also shown that non-spatial memories, such as object recognition and contextual fear conditioning are enhanced by EE (Rampon et al., 2000; Duffy et al., 2001; Tang et al., 2001). In aged rodents, EE also provides mnemonic benefits reducing the age-related deficits in numerous types of spatial memory tasks (Kempermann et al., 1998; Frick and Fernandez, 2003). These effects are probably due to a wide range of changes in critical brain regions for spatial memory (Leggio et al., 2005; Sale et al., 2009). According to this, EE has been shown to enhance the long-term potentiation (LTP), neurogenesis, dendritic spine growth and neurotrophin expression in the hippocampus (Moser et al., 1994; Ickes et al., 2000; Kempermann, 2002; Landers et al., 2011). Also, neural changes, such as dendritic branching, presynaptic vesicle number and density of dendritic spines are increased in neocortical regions by EE (Greenough et al., 1973; Nakamura et al., 1999; Leggio et al., 2005).

An important factor in the magnitude of these benefits is the age at which the EE condition is applied to the rodents; however, few researches have been carried out to study the effectiveness of EE at different ages. Recent studies suggest that the positive effects of EE on memory and neural function may start at some point in the lifespan (Frick and Fernandez, 2003; Bennett et al., 2006; Rosenzweig and Bennett, 1996). Thus, some studies found that the EE condition improved the spatial memory acquisition in aged rats (Speisman et al., 2013), whereas others did not find these positive effects, suggesting the existence of a critical period for the benefits of EE on cognitive ageing (Freret et al., 2012). On the other hand, it is possible that young animals benefit more from the EE condition because they interact more with the environment and they have

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more brain plasticity (Bouet et al., 2011). This variability of results may be due to the variability of the EE protocols, so the best design is to try to compare the different age groups within the same EE protocol (Simpson and Kelly, 2011).

We consider middle-age as a critical period in which the cognitive decline begins (Bizon et al., 2009) and the animals find it more difficult to solve a spatial allocentric associated task (Begega et al., 2012). Stimulating experiences, such as EE or aerobic exercise (Sampedro-Piquero et al., 2013) could have a high benefit in this age group compared to young rats. The results about the effect of EE during middle-age are contradictory. Freret et al. (2012) point out that EE needs to be initiated before middle-age in order to have a positive effect on cognition. In contrast, Kempermann et al. (1998) found that EE had a positive effect on the performance in the Morris water maze (MWM) in aged animals when EE was initiated during middle-age.

This study was designed to assess the effects of the age at which the animals start an EE protocol (3 and 18 months old) on the performance in a spatial memory task radial water maze (RAWM) and on brain functional networks involved in the spatial memory process. Cytochrome c oxidase (COx) histochemistry (Gonzalez-Lima and Cada, 1994; Gonzalez-Lima and Jones, 1994) was used to map sustained regional changes in neuronal energy metabolism in the different experimental conditions. We used quantitative analysis of COx histochemistry as a reliable marker of neuronal metabolic capacity, because COx activity represents an index of the energy demands after prolonged stimulation of neurons (Villarreal et al., 2002; Mendez-Lopez et al., 2009, 2013; Rubio et al., 2012; Sampedro-Piquero et al., 2013). To understand and differentiate brain networks establishing functional correlations between the brain regions of interest we used principal component analysis (PCA) applied recently in different types of studies, both in humans and animals (Cracchiolo et al., 2007; Salmon et al., 2009; Castilla-Ortega et al., 2010; Meunier et al., 2010; Begega et al., 2012).

EXPERIMENTAL PROCEDURES

Animals

A total of 37 18-month-old (593.5–700.3 g) and 41 3-month-old male Wistar rats (268.7–350.3 g) from the vivarium of the University of Oviedo were used. Subjects were housed in groups. All the animals had access *ad libitum* to food and tap water and were maintained at constant room temperature (20–21 °C), with a relative humidity of 65–70% and artificial light–dark cycle of 12 h (08:00–20:00 h. light/20:00–08:00 h. dark). The procedures and manipulation of the animals used in this study were carried out according to the Directive 86/609/EEC (The Council Directive of the European Community) concerning the protection of animals used for experimental and other scientific purposes. The National legislation, in agreement with this Directive, is defined in Royal Decree N°. 1201/2005. The young rats were 3 months old at the start of the experiment and 5 months old at the beginning of the spatial testing. The aged rats were 18 months old at the start of the experiment and 20 months old at the beginning of the spatial memory testing. All the animals were randomly assigned to eight groups: Young control group (5/CO, $n = 11$), Aged control group (20/CO, $n = 11$), Young spatial learning (SPL) group (5/SPL, $n = 10$), Aged SPL group (20/SPL, $n = 10$), Young EE group (5/EE, $n = 10$), Aged EE group (20/EE, $n = 8$), Young EE + SPL group (5/EE + SPL, $n = 10$) and Aged EE + SPL group (20/EE + SPL, $n = 8$). The CO groups were used as a reference of basal COx activity and consisted of animals without any learning or EE experience. The SPL groups were handled, habituated and trained in the 4-RAWM, like the EE + SPL groups. Finally, the EE groups performed the same EE protocol as the EE + SPL groups.

EE

Young and aged animals were housed separately in large cages of 100 cm × 95 cm × 54 cm (eight aged rats and 10 young rats per cage) for three hours every day (10:00 am/13:00 pm). It has been shown that even a restricted daily exposure to EE condition already produces a positive effect on stress response and cognition (Widman and Rosellini, 1990; Widman et al., 1992; Pereira et al., 2007). We ensured that we always put together in the EE cages the same group of rats and the stimulating objects were similar in the different cages. The rest of the day, the animals submitted to EE were housed in groups of five (young rats), or four (aged rats) rats in standard cages without stimulating objects. In this case, the distribution of the rats in the standard cages was random to ensure that all rats of the same age and experimental condition had lived together and so avoid possible fighting between them in the EE cage. EE cages contained a variety of objects, such as toys, running wheels, ropes, plastic tubes of different diameters, platforms, wooden houses, odorous and sound objects and nesting materials (Diamond, 2001). The configuration of the cages was changed once a week over a period of 2 months and the cages were cleaned twice a week to ensure the welfare of the animals. Video records were not taken throughout the EE condition; however, we observed the animals at different moments for 10 min (at the beginning of the condition, at the middle and at the end) to ensure that all the animals made the same use of the EE elements.

Behavioural procedures

Animals were handled daily for 5 days during 10 min (even the CO and EE groups that did not perform the spatial memory task) in order to avoid stress reactions to subsequent manipulation. One day prior to the spatial task, the SPL and EE + SPL groups received a habituation session in which they were given three trials with the platform using different starting positions in a small square water tank (47 × 75 × 38 cm). We applied a habituation session in order to habituate them to test

contingencies. The next day, the SPL and EE + SPL groups were trained in a black fibreglass 4-RAWM (each arm: 80 cm × 12 cm) that was placed 50 cm above the floor level. The maze had four arms in the shape of a cross. The maze was filled with tap water to a height of 32 cm and a black escape platform was placed 2 cm beneath the water surface. The water temperature was kept at 22 ± 1 °C during the task period. The maze was placed in a room with dimmed lights, and there were several extra-maze cues on the walls that the rats could use to navigate. The 4-RAWM testing was performed for 4 days, six trials/day with a 30-seconds (s) inter-trial interval. At the beginning of each trial, the rat was immersed in the water, facing the wall, at one of three start positions. Start locations were randomized, but the same starting location was never used twice in a row. The platform was in the same arm throughout the entire task (Arm A), and the animals were never released into the water from that arm. Each rat was allowed 60 s to reach the platform and if the rat failed, it was guided. Once the rat reached the platform, it remained there for 15 s. Between trials, the animal was placed in a small square tank for 30 s. At the end of each day, the rats were dried and returned to their home cage. Total escape latency (s), total distance swum (cm), total velocity (cm/s) and escape latency and distance travelled in the first trial on each day of training were monitored and assessed. Each trial was recorded and analysed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

COx histochemistry

COx histochemistry was performed as described earlier (Rubio et al., 2012).

Densitometry

COx densitometry was performed as described earlier (Sampedro-Piquero et al., 2013). Fig. 1 shows the overall scheme of EE and cut off points to assess spatial memory and COx activity. The regions of interest were anatomically defined according to Paxinos and Watson's atlas (2005). The stained coronal sections, from anterior to posterior, corresponded to Bregma levels 3.24 mm cingulate cortex (Cg), Prelimbic cortex (PL) and infralimbic cortex (IL); 2.20 accumbens nucleus (Acb); −0.26 bed nucleus of the stria terminalis (BST) and lateral septal nucleus (LS); −3.84 mm hippocampal cornu ammonis (CA1, CA3), dentate gyrus (DG), retrosplenial cortex (RSC) and parietal cortex (PC); −3.12 mm central amygdala (CeA) and basolateral amygdala (BlA); −4.8 mm supramammillary nucleus (SuM) and medial mammillary nucleus (MM) (Fig. 2).

Statistical analysis

Data were analysed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and were expressed as mean ± SEM. The results were considered statistically significant if $p < 0.05$ and they were represented graphically with SigmaPlot 8.0 (Systat, Richmond, EEUU).

Behavioural analysis. Days of training were considered as a within-factor and age (young/aged) and EE (present/absent) between-factors with two levels each. Therefore, a two-way analysis of variance of repeated measures (RM ANOVA) was performed for all the measures taken throughout the study (total escape latency, total distance travelled, total velocity, and latency and distance in the first trial of each day of training). Appropriate post hoc comparisons were conducted when significant differences were found (Bonferroni test).

COx activity analysis. Changes in the total COx activity were analysed with a three-way multivariate ANOVA (MANOVA), in which age (young/aged), EE (present/absent), SPL (present/absent) were the three factors with two levels each. The Bonferroni post hoc test was used when significant differences were found. We also carried out a two-way MANOVA for each age group separately, with EE and SPL as factors, with the aim to know the differences in the total COx activity between the different experimental conditions. Again, Bonferroni was used as a post hoc test.

On the other hand, we aimed to determine the brain functional networks underlying the different brain regions studied. Hence, changes in neuronal metabolic activity were analysed by PCA with SPSS 19.0 and the program FACTOR (Lorenzo-Seva and Ferrando, 2006). Subsequently, we calculated the factor scores by the regression method for each animal with SPSS19.0 and

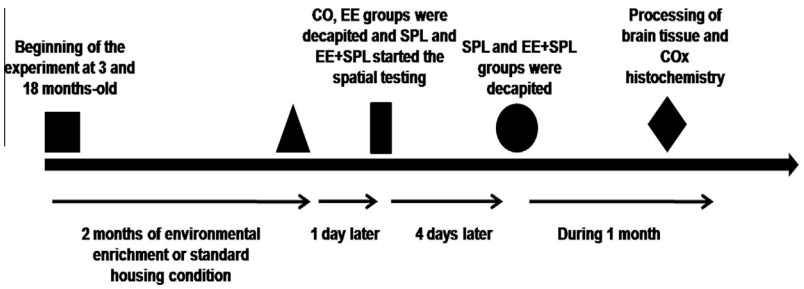

Fig. 1. The scheme is illustrating the behavioural protocol of the experiment from its beginning at 3 and 18 months old. Observe the distribution of cut off point for behavioural testing and cytochrome c oxidase histochemistry.

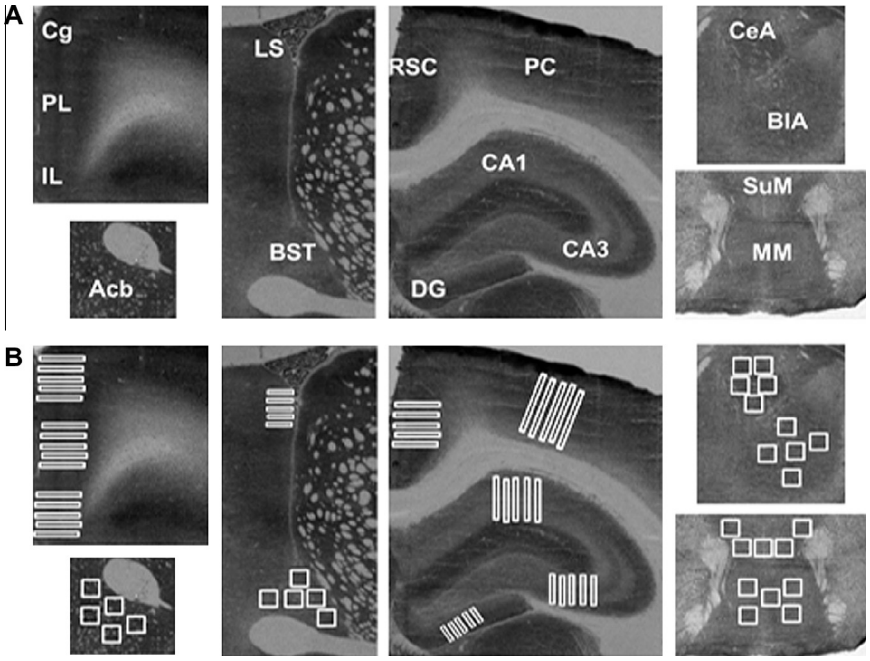

Fig. 2. Photographs of coronal sections stained with COx histochemistry: (A) cingulate cortex (Cg), prelimbic cortex (PL), Infralimbic cortex (IL), accumbens nucleus (Acb), bed nucleus of the stria terminalis (BST), lateral septum nucleus (LS), retrosplenial cortex (RSC), CA1, CA3, dentate gyrus (DG), parietal cortex (PC), central amygdala (CeA), basolateral amygdala (BIA), medial mammillary nucleus (MM) and supramammillary nucleus (SuM). (B) The relative optical density (OD) of each region was measured, taking five non-overlapping readings in each section using a square-shaped sampling window that was adjusted for each region size.

then we applied an ANOVA to test if there were significant differences between groups in the functional component obtained. When significant differences were found, Tukey post hoc or C de Dunnet (when variances are not homogeneous) tests were applied. PCA allows us to understand and differentiate brain networks by establishing functional correlations between brain regions of interest. It is a bottom-up inferential statistical method (from data to concepts) that makes a dimensionality reduction of the sample by extracting a few artificial variables, called factors, which can be more or less correlated with each original variable (Wolfer and Lipp, 2000; Graziano et al., 2002).

RESULTS

Behavioural results

Latency. A two-way RM ANOVA revealed significant days ($F_{3,102} = 38.96$, $p = 0.001$), EE ($F_{1,34} = 13.01$, $p = 0.001$) and age ($F_{1,34} = 8.12$, $p = 0.007$) effects. The interactions EE × days, age × days and EE × age, were not significant ($F_{3,102} = 1.97$, $p = 0.12$; $F_{3,102} = 1.24$, $p = 0.30$; $F_{1,34} = 0.07$, $p = 0.79$ respectively). The interaction EE × age × days was not significant ($F_{3,102} = 0.87$, $p = 0.46$). The enriched groups, regardless of the age or day of training, and the young rats, regardless of the housing condition or day of training, had lesser latency to reach the platform (Fig. 3A).

Distance. A two-way RM ANOVA revealed significant days ($F_{3,102} = 12.50$, $p = 0.001$), and EE ($F_{1,34} = 22.09$, $p = 0.001$) effects. The age factor was not significant ($F_{1,34} = 0.62$, $p = 0.44$). The interactions age × days and EE × age were significant ($F_{3,102} = 5.32$, $p = 0.002$; $F_{1,34} = 5.44$, $p = 0.03$, respectively). The interactions EE × days and EE × age × days were not significant ($F_{3,102} = 0.83$, $p = 0.48$; $F_{3,102} = 0.87$, $p = 0.46$ respectively). In the case of the distance travelled we have found a dependence of the effect of EE upon age, so young enriched rats had shorter distance travelled with regard to to enriched aged rats ($p = 0.04$) (Fig. 3B).

Velocity. A two-way RM ANOVA revealed significant days ($F_{3,102} = 4.89$, $p = 0.003$), EE ($F_{1,34} = 17.96$, $p = 0.001$) and age ($F_{1,34} = 56.20$, $p = 0.001$) effects. The interactions EE × days, age × days and EE × age were not significant ($F_{3,102} = 0.96$, $p = 0.39$; $F_{3,102} = 1.10$, $p = 0.35$; $F_{1,34} = 0.13$, $p = 0.72$). The interaction EE × age × days was not significant ($F_{3,102} = 0.89$, $p = 0.45$). The enriched groups, regardless of the age or day of training, had lesser velocity in the task and the young rats, regardless of the housing condition or day of training, were faster than aged rats (Fig. 3C).

Latency of the first trial. A two-way RM ANOVA revealed significant days ($F_{3,102} = 12.89$, $p = 0.001$) effect. However, the age and EE effects were not significant ($F_{1,34} = 1.17$, $p = 0.29$ and $F_{1,34} = 0.20$, $p = 0.66$ respectively). The interaction EE × days was significant ($F_{3,102} = 4.68$, $p = 0.004$), but the interactions age × days and EE × age were not

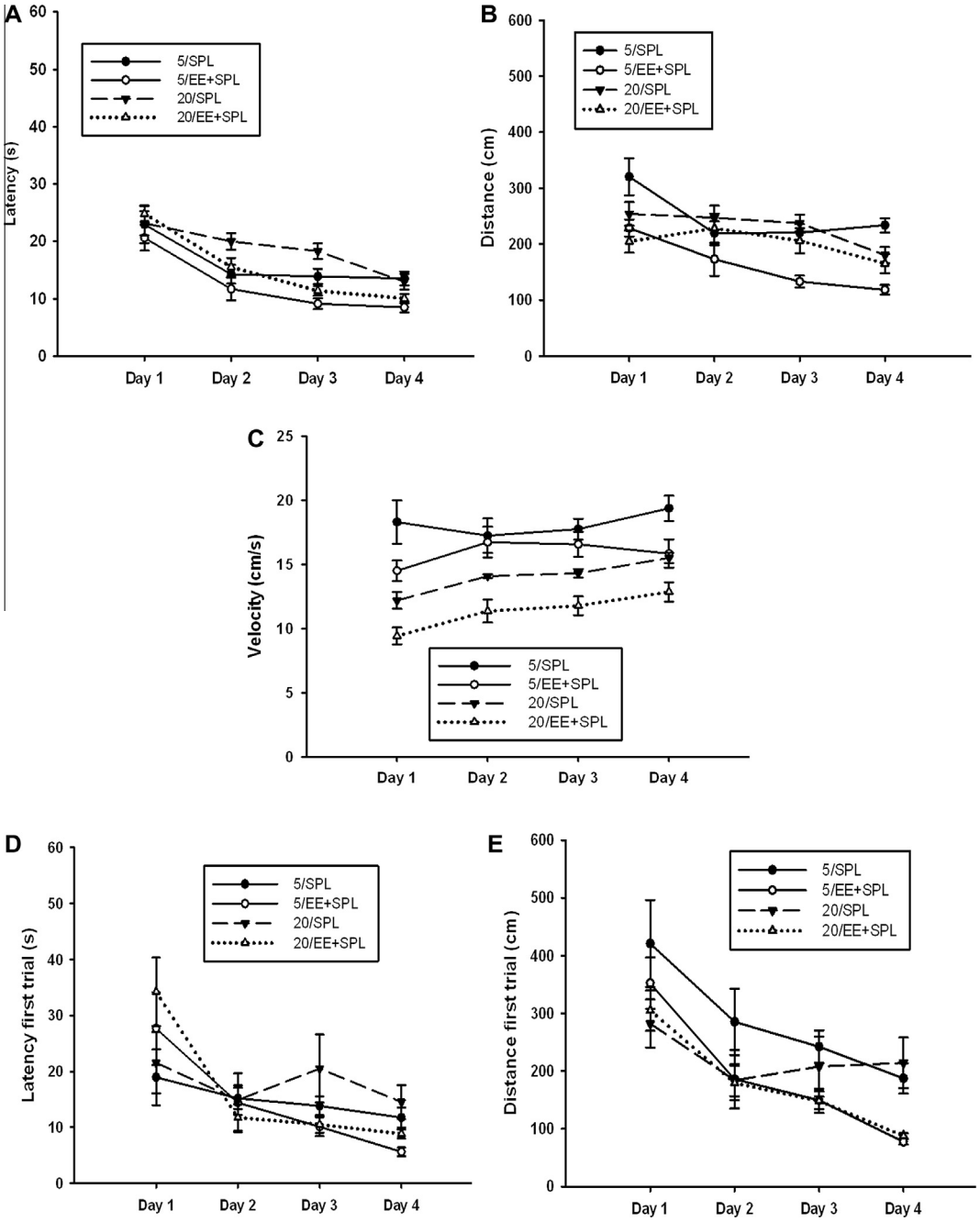

Fig. 3. Escape latency to the platform (A), total distance travelled (B), total velocity (C) and escape latency (D) and distance swam in the first trial of each day of training (E) (mean ± SEM). A two-way ANOVA showed significant EE and age effects in the total latency and velocity ($p < 0.05$), a significant effect of EE in the distance travelled the first trials of each day of training ($p < 0.05$) and a significant EE × age effect in the total distance travelled ($p < 0.05$).

significant ($F_{3,102} = 0.52$, $p = 0.67$ and $F_{1,34} = 0.06$, $p = 0.81$, respectively). The interaction EE × age × days was not significant ($F_{3,102} = 0.34$, $p = 0.80$) (Fig. 3D).

Distance of the first trial. A two-way RM ANOVA revealed significant days and EE effects ($F_{3,102} = 17.57$, $p = 0.001$ and $F_{1,34} = 10.14$, $p = 0.003$, respectively), but age was not significant ($F_{1,34} = 2.99$, $p = 0.09$). The interactions EE × days, age × days and EE × age were not significant ($F_{3,102} = 0.98$, $p = 0.41$; $F_{3,102} = 1.39$, $p = 0.25$; $F_{1,34} = 1.43$, $p = 0.24$ respectively). The interaction EE × age × days was not significant ($F_{3,102} = 0.42$, $p = 0.52$). The enriched groups, regardless of the age or day of training, travelled a shorter distance the first trials of each day of training with regard to non-enriched animals (Fig. 3E).

COx results

MANOVA. The three-way MANOVA showed that the factors EE, age and SPL and the interactions EE × age,

EE × SPL and age × SPL were significant ($F_{15,56}$ = 4.26, p = 0.001; $F_{15,56}$ = 10.41, p = 0.001; $F_{15,56}$ = 1.88, p = 0.04; $F_{15,56}$ = 1.88, p = 0.04; $F_{15,56}$ = 3.41, p = 0.001; $F_{15,56}$ = 2.66, p = 0.004 respectively). The interaction, EE × age × SPL was also significant ($F_{15,56}$ = 3.76, p = 0.001). We analysed the second level interaction with Bonferroni post hoc test and the mean values of each experimental condition and their comparisons in the different brain regions studied are presented in Table 1.

The two-way MANOVA in the young group showed that the factors EE, SPL and the interaction EE × SPL were significant ($F_{15,23}$ = 2.98, p = 0.009; $F_{15,23}$ = 2.59, p = 0.02; $F_{15,23}$ = 2.82, p = 0.01 respectively). The two-way MANOVA in the aged group showed that the factor EE was significant ($F_{15,19}$ = 3.92, p = 0.003), while the SPL effect did not reach statistical significance ($F_{15,19}$ = 0.86, p = 0.001). The interaction EE × SPL was significant ($F_{15,19}$ = 5.11, p = 0.001). We analysed the interactions EE × SPL with Bonferroni post hoc test and the comparisons in the total COx activity between the different experimental conditions within each age group are represented in Table 1.

PCA. Owing to the fact that significant differences were found between ages (young/aged) in the total COx activity of different brain regions (MANOVA section, factor age: $F_{15,56}$ = 10.41, p = 0.001), we decided to apply the PCA analysis for each age group separately, considering all treatments for each age (CO, EE, SPL, EE + SPL), to check if different functional networks were obtained. Firstly, we verified if the data of each age group were adequate for the model of principal components (Aged group: KMO = 0.82, Bartlett's test = 615.5, p = 0.00001; Young group: KMO = 0.85, Bartlett's test = 940.8, p = 0.00001), and the result was positive. Secondly, we determined the number of brain functional networks (components) obtained. For this purpose, we used the Scree-Test provided by the SPSS program and Horn's Analysis implemented by FACTOR (Lorenzo-Seva and Ferrando, 2006). Both analyses showed that our data corresponded to the same component or brain functional network in young and aged groups (Table 2). The components obtained in each group had a high correlation (r = 0.88) and congruence (0.95) (Wrigley and Neuhaus, 1955) between them, which suggests that it was the same brain functional network, but the contribution of the brain regions was slightly different depending on the age of the animals (Table 2). We did not consider significant brain regions with a load below or equal to 0.4 (PC). The brain network obtained in the young group is made up of RSC, prefrontal cortex (Cg, PL, IL), dorsal hippocampus (CA1, CA3 and DG), amygdala (BlA and CeA), Acb, LS, MM, SuM and BST. This component explained 71.62% of the total variance. On the other hand, the component obtained in the aged group is made up of the same brain regions as the young group. This component explained 60.24% of the total variance. Finally, we assessed the relevance of the component between the different experimental conditions within the same age group. Hence, we calculated the factor scores by regression method (SPSS 19.0) in each age group and then we carried out an ANOVA to analyse the differences between experimental conditions. Regarding the young group, the results showed significant differences between groups in the component ($F_{3,40}$ = 6.65, p = 0.001). The post hoc analysis C de Dunnett showed significant differences between 5/EE

Table 1. COx activity values in the measured regions of the different groups

	5/CO	20/CO	5/SPL	20/SPL	5/EE	20/EE	5/EE + SPL	20/ EE + SPL
Cg	25.33 ± 1.29	26.28 ± 1.45	28.13 ± 2.36[@,^]	40.63 ± 1.94[%]	24.02 ± 1.12[$]	33.40 ± 2.56	38.27 ± 4.33[%]	26.16 ± 0.88
PL	25.62 ± 1.22	27.41 ± 1.37	28.92 ± 2.40[@,^]	40.71 ± 3.12[%]	22.82 ± 1.03[$]	32.64 ± 1.20	37.35 ± 4.14[%]	22.91 ± 0.59
IL	25.88 ± 1.19	25.79 ± 1.46	29.51 ± 2.35[@,^]	36.65 ± 2.13[%]	24.09 ± 1.21	28.71 ± 1.08	37.95 ± 4.80[%]	20.74 ± 0.85
Acb	24.64 ± 0.81	25.44 ± 1.71	34.57 ± 1.89	32.64 ± 2.58[%]	29.50 ± 1.49	28.01 ± 2.56	40.01 ± 4.01[%]	22.04 ± 0.97
BST	24.97 ± 2.50[/]	23.42 ± 1.83	27.47 ± 0.94[@]	29.36 ± 1.24[%]	18.83 ± 0.73	20.22 ± 1.76	29.25 ± 2.70[%]	18.27 ± 0.56
LS	19.47 ± 0.89[&]	24.94 ± 2.13	24.91 ± 1.63	29.49 ± 2.37[%]	20.01 ± 1.23	23.45 ± 1.75	28.72 ± 3.11[%]	22.49 ± 0.79
BlA	27.06 ± 1.38	30.16 ± 1.38	37.57 ± 1.29	37.11 ± 2.36[%]	26.75 ± 1.80	27.22 ± 1.37	40.11 ± 3.69[%]	23.78 ± 1.23
CeA	27.44 ± 1.25	30.46 ± 1.41	29.48 ± 1.37[@]	38.86 ± 2.68[%]	24.32 ± 1.30[$]	30.27 ± 1.59	33.89 ± 2.38[%]	23.35 ± 0.55
CA1	20.94 ± 1.33	21.83 ± 1.74	22.67 ± 1.17	26.14 ± 1.19[%]	20.56 ± 0.93	19.80 ± 1.15	25.71 ± 2.56[%]	18.34 ± 0.54
CA3	20.73 ± 1.37	20.13 ± 1.65[$]	22.05 ± 0.94	23.25 ± 1.06[%]	18.61 ± 0.78	15.64 ± 1.52	24.61 ± 2.53[%]	15.91 ± 0.99
DG	28.16 ± 1.74	28.92 ± 1.67	33.06 ± 1.77	35.18 ± 2.40[%]	26.73 ± 0.82	27.26 ± 2.60	38.39 ± 5.22[%]	23.46 ± 0.86
RSC	27.96 ± 1.56	32.56 ± 1.43	37.02 ± 1.71	40.42 ± 2.60[%]	30.62 ± 1.41	35.54 ± 2.12	43.75 ± 4.54[%]	27.39 ± 0.59
MM	22.46 ± 0.84	20.17 ± 1.42	23.44 ± 1.54	26.77 ± 1.96[%]	19.86 ± 1.13	19.50 ± 1.72	26.31 ± 2.67[%]	17.30 ± 0.77
SuM	23.42 ± 1.25	22.45 ± 1.81	27.61 ± 2.01	29.85 ± 1.59[%]	21.71 ± 1.21	24.70 ± 2.37	31.71 ± 3.76[%]	19.41 ± 1.15
PC	24.36 ± 1.30	24.98 ± 0.74	26.95 ± 2.30	25.98 ± 1.40	23.37 ± 0.96	22.94 ± 1.45	26.69 ± 2.13	25.12 ± 0.89

Data represent mean ± SEM values.
[&] $p < 0.05$ as compared to the 20/CO group.
[@] $p < 0.05$ as compared to the 20/SPL group.
[/] $p < 0.05$ as compared to the 5/EE group.
[$] $p < 0.05$ as compared to the 20/EE group.
[^] $p < 0.05$ as compared to the 5/EE + SPL group.
[%] $p < 0.05$ as compared to the 20/EE + SPL group.

Table 2. Contributions of each brain region to the component 1 in the two different age groups, revealed by PCA

Component 1			
Young group		Aged group	
RSC	.944	BST	.923
PL	.934	MM	.888
IL	.933	BlA	.870
DG	.931	RSC	.865
BlA	.929	CeA	.853
CA1	.907	DG	.837
Cg	.904	IL	.834
CA3	.889	SuM	.827
CeA	.866	Cg	.816
Acb	.861	LS	.804
LS	.854	PL	.792
SuM	.828	CA1	.788
BST	.783	CA3	.713
MM	.602	Acb	.675
PC	.412	PC	−.309

% Variance explained (component 1 adult group): 71.62%.
% Variance explained (component 1 aged group): 60.24%.

and 5/EE + SPL ($p = 0.001$) and 5/SPL ($p = 0.03$). The 5/EE group had the lowest score in the component, whereas the 5/EE + SPL group had the highest (Fig. 4). With regard to the aged group, the results also showed significant differences between groups ($F_{3,36} = 15.34$, $p = 0.001$). C de Dunnett showed that 20/SPL had the highest score in the component and this group had significant differences with the rest of the groups (20/CO, $p = 0.001$; 20/EE, $p = 0.001$; 20/EE + SPL, $p = 0.001$). The 20/EE + SPL group had the lowest score in the component and it had significant differences with 20/EE ($p = 0.01$) (Fig. 4). Due to the fact that it is the same functional component in both age groups, it would be possible to merge all the groups and calculate a single PCA solution. However, we consider it clearer and more easily understandable to present the data separated by age.

DISCUSSION

This study assessed if the age at which the animals start an EE protocol, 3 or 18 months old, affects the performance in a spatial memory task. Also, we included the novelty of studying the effects of age, spatial memory and EE condition on brain functional networks.

With respect to behavioural results, both the young and aged enriched groups (5/EE + SPL and 20/EE + SPL) outperformed their respective age control groups (5/SPL and 20/SPL). The better performance in enriched animals could be due to the changes in brain neurochemistry and physiology. Several aspects of hippocampal and neocortical functioning, such as LTP, neurogenesis, dendritic branching, presynaptic vesicle number and synaptophysin levels, dendritic spine growth and neurotrophins mRNA expression are reported to be enhanced by EE (Diamond et al., 1967; Falkenberg et al., 1992; Foster et al., 1996; Nakamura et al., 1999; Van Praag et al., 2000; Duffy et al., 2001; Kempermann, 2002; Frick and Fernandez, 2003; Leggio et al., 2005). The memory consolidation is related to LTP phenomenon and the measure of the distance travelled in the first trial of each day of training allows us to test the ability of consolidation of the animals. The 20/SPL and 5/SPL groups showed mild memory consolidation deficits, whereas the 20/EE + SPL and 5/EE + SPL groups showed an improvement in their performance as the days progressed (Fig. 3E). EE is known to increase memory consolidation, perhaps due to an improvement in the susceptibility of LTP induction (Kumar et al., 2012). The absence of differences in this variable according to the age, showed that the 20/EE + SPL group performed at the same level as the 5/EE + SPL group, which could suggest a rejuvenation of senescent physiology by EE. However, in the case of the total distance travelled, we found that the effect of EE is dependent on the age of the animals, so young enriched rats swam a shorter distance to reach the

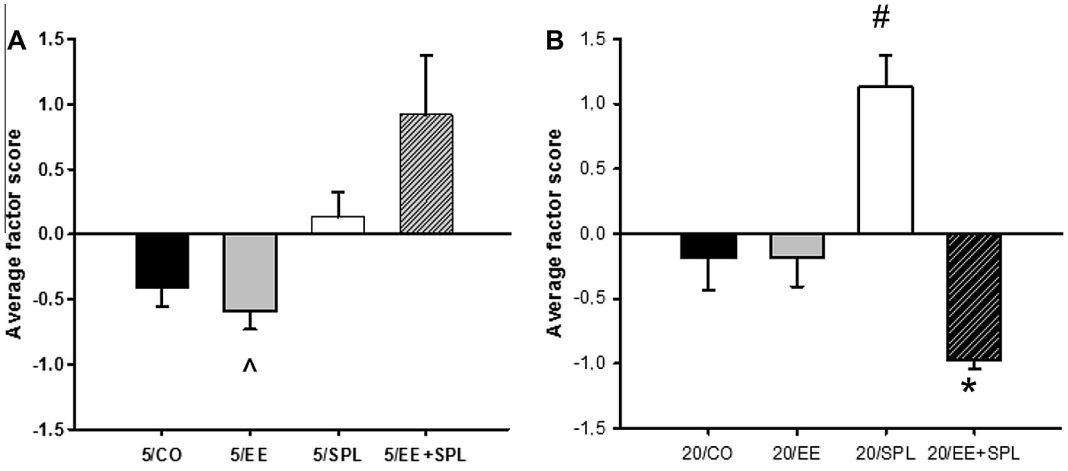

Fig. 4. Average scores of the two age groups for each of the four experimental conditions in the component obtained. Significant differences between 5/EE group and 5/EE + SPL group ($^{\wedge}p < 0.05$) and 5/SPL ($p < 0.05$). Significant differences between the 20/EE + SPL group and the 20/EE ($^{*}p < 0.05$). Significant differences between the 20/SPL and the rest of the groups ($^{\#}p < 0.05$).

platform compared to aged enriched rats. This result could be due to a limited plasticity in aged rats, even if they have received EE. For example, a reduction of neurogenesis in the hippocampus (Drapeau et al., 2003) synaptic connections or gene expression has been found in aged rodents (Baudry, 2009; Wagner et al., 2000). On the other hand, the duration of our EE protocol could have been too short to produce a higher positive effect in the aged group (Bell et al., 2009; Redolat and Mesa-Gresa, 2012). As with other researches (Zimmermann et al., 2001; Schrijver et al., 2002), in our study EE also decreased the velocity during the spatial task both in young and aged rats (Fig. 3C). It is known that EE accelerates habituation of the locomotor activity both within and between trials (Schrijver et al., 2002), which could be related to our results in this variable.

Therefore, the differential effect of EE, that allowed a larger improvement in the young rats compared to the aged, was specific for the acquisition of the spatial task and not for the memory consolidation.

With regard to the total COx activity, the major differences, both between age groups and in the different experimental conditions in each age group, were found when we introduced the experimental conditions of SPL and EE + SPL. These are expected results because COx activity reflects changes related to the consumption of neuronal energy after demands, for example a spatial memory task (Villarreal et al., 2002; Begega et al., 2012; Rubio et al., 2012). Regarding the differences between ages, we found that the 20/SPL group had higher COx activity in Cg, PL, and IL cortices, BST and CeA with respect to 5/SPL. The medial prefrontal cortex has a complex role in stress regulation, with different subregions having divergent actions on anxiety responses (Cerqueira et al., 2008; Blanco et al., 2009). The PL and Cg cortices may suppress the activity of the IL network through inhibitory actions on this cortex (Cerqueira et al., 2008). Thus, the higher neuronal oxidative metabolism in the Cg and PL cortices in the 20/SPL group could be explained by an attempt to inhibit circuits mediating stress responses. The high activation in BST and CeA, both involved in stress response and activation of hypothalamic–pituitary–adrenal axis (HPA) axis (Walker et al., 2003; Akirav and Richter-Levin, 2005), seems to reveal more activity of stress-related regions in aged animals during the spatial testing. When EE is present, we found that the 5/EE + SPL group had more neuronal metabolic activity in all brain regions, with the exception of the PC, regarding the 20/EE + SPL group. Recent research has shown that the PC is involved in egocentric strategy and not in allocentric spatial memory, as in our study (Weniger et al., 2009; Schindler and Bartels, 2013). An increase in the metabolic activity has been related to better behavioural performance (Leger et al., 2012). Also, the EE condition in young rats could have developed a brain reserve in these animals. This brain reserve is characterized by larger brains that contain more neurons and synaptic connections (Graves et al., 1996; Petrosini et al., 2009; Nithianantharajah and Hannan, 2009), which could produce higher neuronal metabolic activity when a challenging demand, such as the perform of a spatial memory task, is required. With regard to the differences between experimental conditions (SPL and EE + SPL conditions) within the same age group, we found that in the young group, EE increased the neuronal metabolic activity in the Cg, PL and IL cortices. Neocortical brain regions are very vulnerable to the EE effects (Kolb et al., 1998; Gelfo et al., 2009) and these brain subregions have been linked to the attentional process, decision-making, behavioural flexibility, integration of spatial information and control of stress response, which could partly explain the better behavioural performance (Vertes, 2006; Cerqueira et al., 2008; Robinson et al., 2011). In the aged group, we found that the EE condition reduced the COx activity of all brain regions, with the exception of the PC, during the spatial task regarding the 20/SPL group. The 20/EE + SPL group had better performance in the 4-RAWM than the 20/SPL group, so this metabolic reduction cannot be due to worse performance. In contrast, it is possible that our EE protocol produced a more accurate performance and it increased efficiency, reducing the neuronal metabolic needs in the regions involved in the spatial memory process.

PCA revealed the same brain functional network in young and aged groups, but the brain regions involved in this functional network had a slightly different contribution to the component depending on the age of the animals. In the young group, RSC, Cg, PL, IL, CA1, CA3, DG or BIA were the brain regions with slightly more contribution to the component. These brain regions have been linked to the attentional process, decision-making, behavioural flexibility, integration of spatial information, acquisition and consolidation of context-dependent spatial information and regulation of stress response (Vertes, 2006; Cerqueira et al., 2008; Robinson et al., 2011). We found that the 5/EE + SPL group had the highest score in this component and the lowest was found both in the 5/CO and 5/EE groups. COx activity is known to reflect long-term changes in neuronal metabolic activity after an energetic demand. According to this, it is not unusual that 5/EE and 5/CO had the lowest score, because these groups did not perform any spatial task. The highest score of the 5/EE + SPL group in this brain functional network may be due to the benefits of EE on spatial cognition. Several studies have shown that EE improves the ability to discriminate the spatial localization of a platform, allows the flexible use of spatial information (Speisman et al., 2013), and the quick acquisition of an accurate navigational strategy (Leggio et al., 2005) in adult rats. The hippocampus forms associations between stimuli and produces a cognitive map necessary to correctly perform a spatial allocentric task (Aggleton et al., 2000). Also, it is suggested that the newborn neurons of DG after EE play an important role in spatial memory and they are preferentially involved in the acquisition and retrieval of a spatial reference memory task (Kee et al., 2002; Goodman et al., 2010).

In contrast, the BST was the brain region with slightly more contribution to the aged component. It is involved in anticipatory anxiety (Straube et al., 2007) and in the activation and termination of the HPA axis response (Conrad et al., 2011). In this respect, several studies have found a hyperactivity of the HPA axis in aged rats (Scaccianoce et al., 1990). Moreover, we found that brain regions involved in the control and inhibition of stress, such as Cg and PL cortices, the hippocampus or LS (Cerqueira et al., 2008; Thomas and Gunton, 2011) had a slightly lower load than the activating brain regions of the HPA axis. Interestingly, both the 20/EE and 20/EE + SPL groups had the lowest score in the brain functional network obtained in the age group (Fig. 4), and therefore, in brain regions involved in anxiety, which seems to suggest a mild positive effect of EE on the control of anxiety responses. Hence, EE could be considered a eustressor that produces an elevated activity of the HPA axis at the beginning of this housing condition, whereas it makes this axis more adaptive to the future stressors reducing HPA axis activation and improving the performance (Larsson et al., 2002). The slightly low contribution of the dorsal hippocampus in the aged component contrasts with the findings in the young group. According to this result, several studies suggest that the alteration in the hippocampus may underline the behavioural deficits found with ageing (Barnes, 1988; Foster, 1999; Begega et al., 2012). Thus, in the aged group, it is possible that the positive effect of EE on the spatial performance is mediated by a reduction of anxiety during the cognitive testing (Harris et al., 2009), and not by an improvement in cognitive functions, as in the young group.

We must clarify that the differences between groups in the factor loadings of the component obtained were very slight (Table 2). However, we can observe a mild different tendency in the contribution of the brain regions to the component depending on the age of the animals.

CONCLUSIONS

Taking into account these results, we are able to suggest that EE is a good option to improve the performance in spatial memory tasks, both in young and aged rats. However, the benefits found were slightly higher in the young group than the aged group, maybe due to the limited duration of the EE protocol or the reduction of brain plasticity in aged animals. Our study found a diferential effect on the COx activity depending on the age of the animals, so different brain mechanisms are triggered in aged rats to solve the spatial task, compared to young rats. PCA showed that the same brain functional component underlies both age groups, but the contribution of the brain regions to the component was slightly different depending on the age of the animal. Future research is required to clarify this hypothesis. It would also have been interesting to have included more groups of rats of different ages to test the entire lifespan.

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EXPERIMENTO II

Effects of environmental enrichment on anxiety responses, spatial memory and cytochrome c oxidase in adult rats

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Research report

Effects of environmental enrichment on anxiety responses, spatial memory and cytochrome c oxidase activity in adult rats

P. Sampedro-Piquero[a,*], C. Zancada-Menendez[a], A. Begega[a], S. Rubio[b], J.L. Arias[a]

[a] Laboratorio de Neurociencias, Departamento de Psicología, Universidad de Oviedo, Plaza Feijoo s/n, 33003 Oviedo, Spain
[b] Área de Psicobiología, Departamento de Psicología Biológica y de la Salud, Facultad de Psicología, Universidad Autónoma de Madrid, Campus de Cantoblanco s/n, 28049 Madrid, Spain

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ABSTRACT

We have studied the effect of an environmental enrichment (EE) protocol in adult Wistar rats on the activity in the elevated zero-maze (EZM), performance in the radial-arm water maze (RAWM) and we have also examined the changes in the neuronal metabolic activity of several brain regions related to anxiety response and spatial memory through cytochrome c oxidase histochemistry (COx). Our EE protocol had anxiolytic effect in the EZM; the animals spent more time and made more entries into the open quadrants, they had lower latency to enter into the open quadrant and lower levels of defecation. Also, the EE group showed fewer working memory and reference memory errors, as well as lesser distance travelled in the first day of the spatial training. In relation to the neuronal metabolic activity, EE reduced the COx activity in brain regions related to anxiety response, such as the infralimbic cortex, the paraventricular thalamic and hypothalamic nucleus, the basolateral amygdala, and the ventral hippocampus. Interestingly, there were no significant differences between groups in the dorsal hippocampus, more related to spatial cognition. These results suggest a beneficial effect of EE on spatial memory as a result of reducing anxiety levels and the COx activity in brain regions involved in anxiety response. We also found a differential pattern of activation inside the hippocampus, suggesting that the dorsal hippocampus has a preferential involvement in spatial learning and memory, whereas the ventral hippocampus has a role in anxiety response.

1. Introduction

Anxiety response can be characterized as behavioural and neuroendocrine activation associated with exposure to threatening stimulus. Over a century of behavioural research has revealed a powerful influence of anxiety on learning and memory (James, 1890; Yerkes and Dodson, 1908; Hebb, 1955; McGaugh, 2000). The literature in this area lacks consistency, with studies reporting that high levels of anxiety can enhance, impair or have no effect on learning and memory (Conrad, 2005; Diamond, 2005; Lupien et al., 2005). The majority of studies examining anxiety effects on memory in rats use spatial learning tasks (Diamond and Rose, 1994; Luine et al., 1994). The relationship between anxiety and memory performance follows an inverted U-shaped curve, with better performance when anxiety levels are in an optimal level (Salehi et al., 2012). For example, Herrero et al. (2006) have found that rats with high and low levels of anxiety show different performance in the acquisition and in the retrieval of spatial information. Interestingly, high levels of anxiety influence differently the spatial reference and working memory domains in the radial-arm water maze (RAWM), so the spatial reference memory domain decays sooner than the spatial working memory domain (Hutchinson et al., 2012).

In this context, several studies have reported an anxiolytic effect and improvement of memory function after aerobic exercise and environmental enrichment (EE) protocols (Petrosini et al., 2009; Hutchinson et al., 2012; Sciolino and Holmes, 2012; Kennard and

Abbreviations: EE, environmental enrichment; EZM, elevated zero-maze; 4-RAWM, four-arm radial water maze; COx, cytochrome c oxidase histochemistry; HPA, hypothalamic–pituitary–adrenal axis; GC, glucocorticoids; LTP, long-term potentiation; EPM, elevated plus-maze; CO, control group; SPL, spatial learning group; EE+SPL, environmental enrichment + spatial learning group; EE, environmental enrichment group; TbE, time by entries; MO, medial orbital cortex; Cg, cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex; Acb, accumbens nucleus; BNST, bed nucleus stria terminalis; CeA, central amygdala; BlA, basolateral amygdala; PVNt, paraventricular thalamic nucleus; PVNh, paraventricular hypothalamic nucleus; dCA1 dCA3, dorsal hippocampal cornu ammonis; dDG, dorsal dentate gyrus; vCA1 vCA3, ventral hippocampal cornu ammonis; vDG, ventral dentate gyrus; RM ANOVA, ANOVA of repeated measures; MANOVA, multivariate analysis of variance; OD, optical density.

* Corresponding author. Tel.: +34 985103212; fax: +34 985104144.
E-mail addresses: uo179677@uniovi.es, patrisampiq@gmail.com (P. Sampedro-Piquero), uo179845@uniovi.es (C. Zancada-Menendez), begega@uniovi.es (A. Begega), sandra.rubio@uam.es (S. Rubio), jarias@uniovi.es (J.L. Arias).

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Woodruff-Pak, 2012; Pang and Hannan, 2013). Our research group has found for example, that a protocol of aerobic exercise applied over a period of 2 months in aged rats reduced the cytochrome c oxidase activity (COx) in brain regions traditionally involved in anxiety response such as the amygdala or the bed nucleus of the stria terminalis and this training slightly enhanced the memory performance in the RAWM (Sampedro-Piquero et al., 2013a,b). In the case of EE, this complex housing condition has shown to have important benefits on spatial cognition, maybe due to the effect on the brain plasticity in frontal and parietal cortex, hippocampus, striatum and cerebellum (Ekstrand et al., 2008; Leggio et al., 2005; Vazquez-Sanroman et al., 2013) and also, it reduces the anxiety in a novel situation giving the animals more control over their environment (Van de Weerd et al., 2002). This last positive effect can be explained if we consider the EE as a eustressor that makes the hypothalamic–pituitary–adrenal axis (HPA) more adaptive to the future stressors diminishing the emotional reactivity (Larsson et al., 2002). For example, in unconditioned tests such as the elevated plus-maze (EPM) or the elevated zero-maze (EZM), the animals show a reduction of anxiety with low levels of defecation, more entries into the open sections and lesser latency to enter into the open section (Friske and Gammie, 2005; Heredia et al., 2012). During the cognitive testing, Harris et al. (2009) conclude that the cognitive benefits of EE occur because rats are less anxious during the cognitive task.

It is unknown if the EE condition influences neuronal metabolic activation recruited during a spatial memory task and if this activation changes as a result of lower levels of anxiety. A common method for measuring the neuronal oxidative metabolism, after prolonged stimulation or training in behavioural tasks, is the Cytochrome c oxidase (COx) histochemistry (Begega et al., 2012; Riha et al., 2008; Rubio et al., 2012; Mendez-Lopez et al., 2009a). COx is a mitochondrial enzyme involved in the oxidative phosphorylation process in which ATP is generated for sustaining neuronal functions (Wong-Riley, 1989). This method is suitable to detect regional brain activity changes relative to control conditions (Gonzalez-Lima and Cada, 1994). COx histochemistry has been extensively used in studies of learning and memory in animal species (Begega et al., 2010; Bruchey and Gonzalez-Lima, 2008; Puga et al., 2007; Sakata et al., 2005) and some studies have related high COx activity in brain regions such as the amygdala or the medial mammillary body with high levels of anxiety during the cognitive testing (Sampedro-Piquero et al., 2013a,b; Leger et al., 2012; Conejo et al., 2004).

We assessed the effect of EE on anxiety-related behaviours in the EZM and its impact on working memory and reference memory in the RAWM. We chose to expose the animals only 3 h per day to the EE condition as other studies had already found a positive effect on anxiety response and cognition with restricted daily exposure (Widman et al., 1992; Widman and Rosellini, 1990). We also analyzed the possible changes in the neuronal metabolic activity of several brain regions related to anxiety response and spatial cognition.

2. Materials and methods

2.1. Subjects

Forty male 3-month-old Wistar rats from the vivarium of the University of Oviedo were used. The subjects were housed in groups of five animals. All the animals had ad libitum access to food and tap water and were maintained at constant room temperature (20–21 °C), with a relative humidity of 65–70% and artificial light–dark cycle of 12 h (08:00–20:00 h light/20:00–08:00 h dark). Since rats (Wistar strain) were used as experimental animals, the experimental procedures applied in this research were previously reviewed and accepted by a bioethics committee of the University of Oviedo. In addition, the procedures and manipulation of the animals used in this study were carried out according to the Directive 86/609/EEC (The Council Directive of the European Community) concerning the protection of animals used for experimental and other scientific purposes. The National legislation, in agreement with this Directive, is defined in Royal Decree no. 1201/2005.

Fig. 1. A typical enriched setting enhancing motor, sensory, cognitive and social stimulation in rats is illustrated in the Fig. 1.

The rats were randomly assigned to four groups: control group (CO: 300.5–346.7 g; $n = 10$), environmental enrichment group (EE: 284.5–342.6 g; $n = 10$), environmental enrichment + spatial learning group (EE + SPL: 298.7–327.2 g; $n = 10$) and spatial learning group (SPL: 302.6–350.3 g; $n = 10$). The CO group was used as a reference of basal COx activity and it consisted of two groups of five rats kept in standard cages without any learning or environmental enrichment experience. When evaluating the impact of EE, it is initially important to consider the use of appropriate control groups. In general, it is recommended that laboratory animals are housed in social groups, because rats reared in isolation display a behavioural pattern called "*social isolation syndrome*" which is associated with hyperactivity in novel environments and poor adaptability, as well as higher impulsivity compared to rats housed in groups (Simpson and Kelly, 2011).

2.2. Environmental enrichment

The EE and EE + SPL groups were housed in large cages of 100 cm × 95 cm × 54 cm (each experimental group in a different cage, ten rats per cage) for a period of 3 h every day (10:00 am/13:00 pm). We ensured that we always put the same group of ten rats together in the EE cages and the stimulating objects were similar in both cages. The rest of the day, the animals submitted to EE were housed in groups of five in standard cages without stimulating objects, as were the control group. In this case, the distribution of the ten rats of each group in the two standard cages was random to ensure that all rats had lived together and so, avoid possible fighting between them. The EE cages contained various objects like toys, running wheels, ropes, plastic tubes of different diameters, platforms, wooden houses, odorous and sound objects and nesting materials. To ensure novelty, the configuration of the cages was changed once a week and the cages were cleaned twice a week. EE rats were placed and maintained in this condition two months before the start of the behavioural testing. The animals were exposed to the EE condition for only 3 h per day (Fig. 1).

2.3. Elevated zero-maze (EZM)

The day before the spatial task, SPL and EE + SPL groups were assessed in the EZM. This maze was constructed of black acrylic in a circular track 10 cm wide, 81 cm in diameter, and elevated 82 cm from the floor (Noldus Information Technology). It was divided into four quadrants of equal lengths, two open quadrants and two closed quadrants with black acrylic walls 35 cm in height. The session consisted of five minutes, under the same lighting conditions, and the animal was placed in the center of a closed quadrant. When an animal had finished the test, the maze was cleaned with 70% ethanol in order to eliminate odor and start with the next rat. Behavioural measures taken included: (a) closed head dips (the number of times the rat looked over the edge of the maze while a portion of the body was in the closed sections); (b) open head dips (the number of times the rat looked over the edge of the maze while its body was completely in the open sections); (c) duration

Table 1
Activity in the elevated zero-maze for each group.

	EE+SPL	SPL
Closed head dips (mean frequency)	1.80 ± 0.51*	0.1 ± 0.1
Open head dips (mean frequency)	8.80 ± 1.00	10.00 ± 0.98
Duration in open quadrants (s)	110.40 ± 23.05*	10.90 ± 6.99
Latency (s)	25.08 ± 3.50*	233.69 ± 35.30
Open quadrant entries (mean frequency)	7.90 ± 1.41*	0.70 ± 0.40
Fecal boli (mean frequency)	0.40 ± 0.16*	1.70 ± 0.42
Rearing (mean frequency)	9.80 ± 1.43*	3.10 ± 0.57
Time by entries	37.88 ± 24.56*	6.64 ± 12.77
Distance travelled (cm)	2762.61 ± 16.43*	1112.89 ± 20.98

Data represent mean + SEM values.
* $p < 0.05$ significant differences.

in open quadrants (seconds in the open quadrants); (d) latency (the time before the first entry into the open quadrant, s); (e) the number of open quadrant entries; (f) fecal boli; (g) rearing (frequency of vertical standing of rat on two hind legs); (h) distance travelled in the maze. We also introduced the index, *Time by Entries* (*TbE*, ratio between the time spent in the open section and the square-root of the number of entries for each animal) (Heredia et al., 2012). This index adjusts the influence of the high levels of activity on the time in the open section. Heredia et al. (2012) interpret this parameter as a correction of the time spent in the open area of the EZM and for that, high *TbE* rating is indicative of low levels of anxiety. All the animals were tested at the same time of day, from 10:00 am to 12:00 pm. The movements of the rats were recorded with a camera connected to a computer running the EthoVision 3.1. software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA).

2.4. Behavioural procedures

The SPL and EE + SPL groups were trained in a black fiberglass 4-RAWM (each arm: 80 cm × 12 cm) that was placed 50 cm above the floor level. The maze had 4 arms in the shape of a cross. The maze was filled with tap water to a height of 32 cm and a black escape platform was placed 2 cm beneath the water surface. The water temperature was kept at 22 ± 1 °C during the task period. The maze was placed in a room with dimmed lights, and there were several extra-maze cues on the walls that the rat could use to navigate. Each trial was recorded and analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands). All the animals were handled daily for seven days, even the CO and EE group that did not perform the spatial memory task, so we ensure the same familiarity with humans. The day before the spatial task, the rats received a habituation session in which they were given three trials with the platform using different starting positions in a small square water tank (47 cm × 75 cm × 38 cm). The 4-RAWM testing was performed for four days, six trials/day with a 30 s inter-trial interval. At the beginning of each trial, the rat was immersed in the water, facing the wall, at one of three start positions. Start locations were randomized, but the same starting location was never used twice in a row. The platform was in the same arm throughout the entire task (Arm A), and the animals were never released into the water from that arm. Each rat was allowed 60 s to reach the platform and if the rat failed, it was guided to the platform. Once the rat reached the platform, it remained there for 15 s. Between trials, the animal was placed in a small square tank for 30 s. At the end of each day of training, the rats were dried and returned to their home cage. Working memory (reentries into an arm in the same test trial) reference memory errors (entries into an arm that does not contain the platform) and the distance travelled (cm) in the last trial of each day of training were registered.

2.5. COx histochemistry

COx histochemistry was performed as described earlier (Rubio et al., 2012).

2.6. Densitometry

COx densitometry was performed as described earlier (Gonzalez-Lima and Jones, 1994; Sampedro-Piquero et al., 2013a,b).

The regions of interest were anatomically defined according to Paxinos and Watson's atlas (2005). The stained coronal sections, from anterior to posterior, corresponded to bregma levels 4.20 mm medial orbital cortex (MO); 2.70 mm cingulate cortex (Cg), prelimbic cortex (PL) and infralimbic cortex (IL); 1.60 mm accumbens nucleus (Acb); −0.26 mm bed nucleus stria terminalis (BNST); −2.12 mm central amygdala (CeA) and basolateral amygdala (BlA); −1.80 mm paraventricular thalamic nucleus (PVNt) and paraventricular hypothalamic nucleus (PVNh); −3.84 mm dorsal hippocampal cornu ammonis (dCA1, dCA3), dorsal dentate gyrus (dDG); −5.80 mm ventral hippocampal cornu ammonis (vCA1, vCA3), ventral dentate gyrus (vDG).

Fig. 2 shows the overall scheme of EE and cut off points to assess activity in the EZM, spatial memory and cytochrome c oxidase activity.

2.7. Statistical analysis

Behavioural data were analysed with SPSS 19.0 (SPSS Inc., Chicago, USA) and were expressed as mean ± SEM. The results were considered statistically significant if $p < 0.05$ and they were represented graphically with SigmaPlot 8.0 (Systat, Richmond, EEUU).

2.7.1. Elevated zero-maze

Student's *t*-test for independent samples was carried out to assess the differences between EE+SPL and SPL groups in the selected variables of the EZM.

2.7.2. Four-arm radial water maze

Days of training were considered as a within-factor and *enrichment* between-factor. Therefore, an ANOVA of repeated measures (RM ANOVA) was performed considering all groups tested (SPL and EE+SPL groups) for all the measures taken throughout the study (working memory errors, reference memory errors and distance travelled in the last trial of each day of training). Appropriate post hoc comparisons were conducted when we found significant differences (Bonferroni test).

2.7.3. Pearson's correlation coefficients

To know if rats with less anxiety perform better on the 4-RAWM, *Pearson*'s correlation coefficients (*r*) were calculated between memory errors and the index *TbE*. These results do not allow to infer any causality relation between these parameters, because they are correlations.

2.7.4. COx activity results

The changes in COx activity were analyzed with Multivariate analysis of variance (MANOVA). Post hoc Tukey's tests were used when we found significant differences between groups.

3. Results

3.1. Elevated zero-maze

t-test for independent samples revealed significant differences between groups in closed head dips ($T_{18} = 3.26$, $p = 0.01$), duration in open quadrants ($T_{18} = 4.13$, $p = 0.002$), latency to enter into the open quadrant ($T_{18} = -5.88$, $p = 0.001$), entries in open quadrant ($T_{18} = 4.92$, $p = 0.001$), fecal boli ($T_{18} = -2.87$, $p = 0.02$), rearing ($T_{18} = 4.36$, $p = 0.001$), time by entries ($T_{18} = 3.56$, $p = 0.003$) and distance travelled ($T_{18} = 6.13$, $p = 0.03$). We did not find significant differences between groups in open head dips ($T_{18} = -0.85$, $p = 0.4$). Table 1 shows the mean value for each group in these variables.

3.2. Four-arm radial water maze

3.2.1. Spatial reference memory errors

RM ANOVA showed that the *enrichment* factor was significant ($F_{1,18} = 27.18$, $p = 0.001$). The Mauchly sphericity test was not significant ($p = 0.21$), so we used the Assumption of Sphericity to analyze the *days* factor and the interaction *enrichment* × *days*. The *days* factor and the interaction *enrichment* × *days* showed statistical significance ($F_{3,54} = 21.68$, $p = 0.001$; $F_{3,54} = 3.53$, $p = 0.02$ respectively). We analyzed the interaction to discover in which days there were significant differences between groups. To that end, we used pairwise comparisons with Bonferroni adjustment and we found

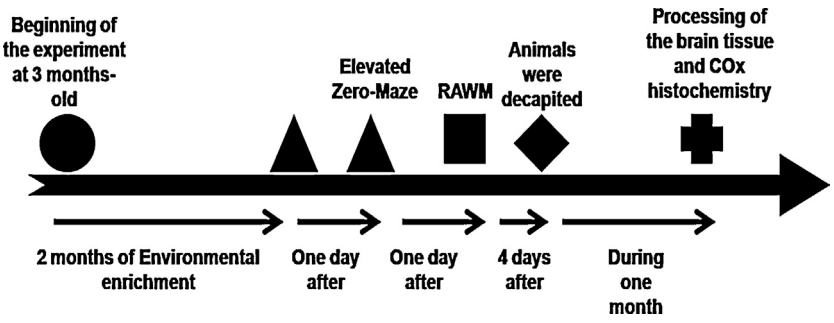

Fig. 2. The scheme is illustrating the behavioural protocol of the experiment from its beginning at 3 month-old. Observe the distribution of cut off point for behavioural testing and cytochrome c oxidase histochemistry.

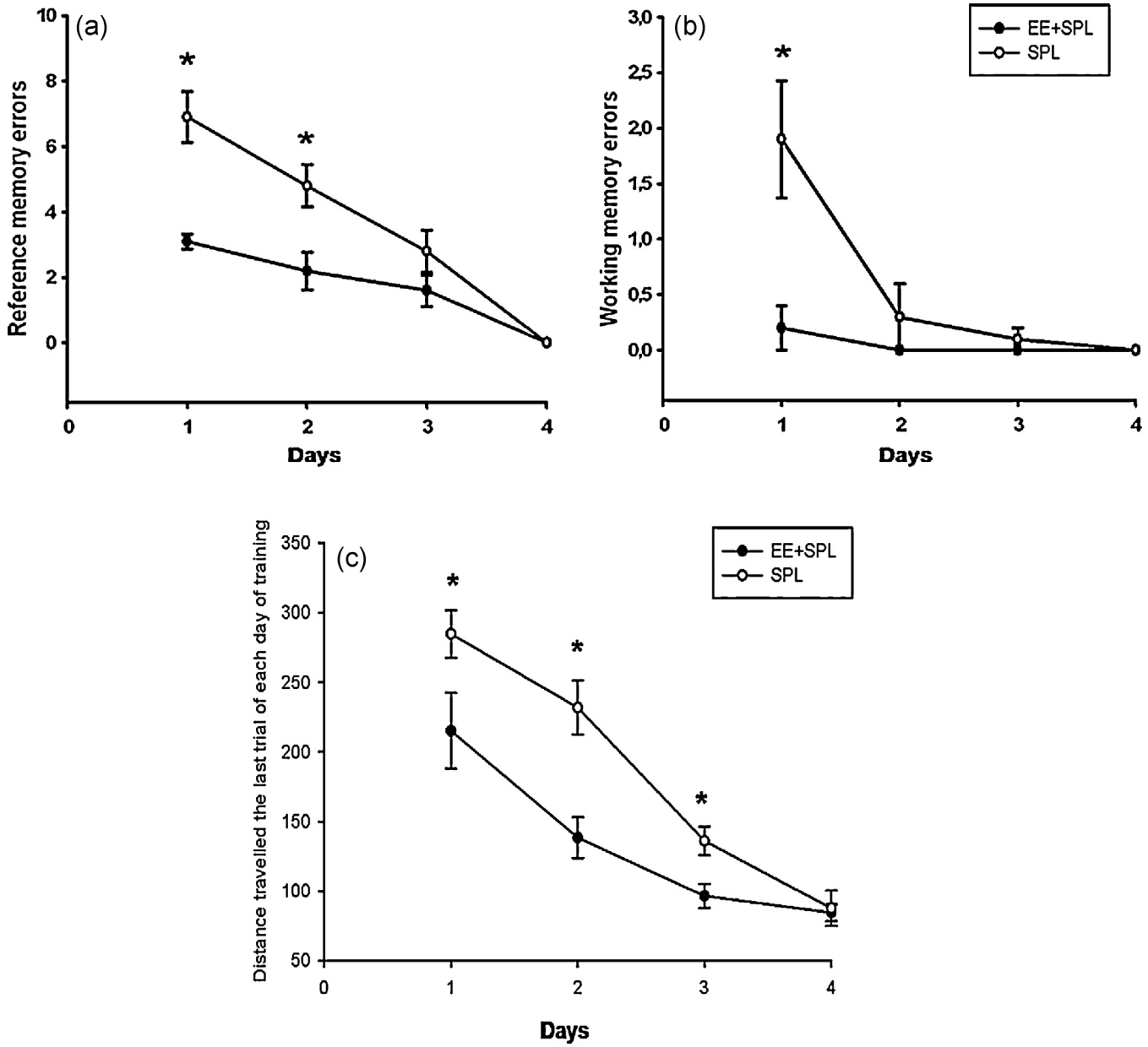

Fig. 3. Working memory and reference memory errors in the RAWM. (a) Average of reference memory errors in each group during the training (mean + SEM). (b) Average of working memory errors in each group during the training (mean + SEM). (c) Average of distance travelled in each group in the last trial of each day of training (mean + SEM).*(a) Significant differences between the EE + SPL and SPL groups in reference memory errors on days 1 and 2. The EE+SPL group had fewer reference memory errors than the SPL group ($p < 0.05$). *(b) Significant differences between the EE + SPL and the SPL groups in working memory errors on day 1. The EE + SPL group had fewer working memory errors than the SPL group ($p < 0.05$) on this day. *(c) Significant differences between the EE + SPL and the SPL groups in the distance travelled in the last trial of days 1, 2 and 3. The EE + SPL group had shorter distance travelled than SPL group ($p < 0.05$).

significant differences between groups on the first ($p = 0.001$) and second days ($p = 0.008$) of training, in where the EE + SPL group showed significantly fewer reference memory errors than the SPL group. In contrast, we did not find significant differences between groups on the third ($p = 0.15$) and fourth ($p = 0.18$) days of training (Fig. 3a).

3.2.2. Spatial working memory errors

RM ANOVA showed that the *enrichment* factor was significant ($F_{1,18} = 18.29$, $p = 0.001$). The Mauchly sphericity test was significant ($p = 0.001$), so we used the Greenhouse-Geisser test to analyze the *days* factor and the interaction *enrichment* × *days*. The *days* factor showed statistical significance ($F_{1.36,24.40} = 8.27$, $p = 0.001$). The interaction *enrichment* × *days* also showed statistical significance ($F_{1.36,24.40} = 5.30$, $p = 0.003$), thus we analyzed the interaction to know in which days there were significant differences between groups. For that, we used pairwise comparisons with Bonferroni adjustment and we found significant differences between groups on the first day of training ($p = 0.007$). The EE + SPL group showed fewer working memory errors than the SPL group on this day. We did not find significant differences between groups on the second ($p = 0.33$), third ($p = 0.33$) and fourth (without errors) days of training (Fig. 3b).

3.2.3. Distance travelled in the last trial of each day of training

We found that the *enrichment* factor was significant ($F_{1,18} = 12.28$, $p = 0.003$). The Mauchly sphericity test was significant ($p = 0.04$), so we used the Greenhouse-Geisser test to analyze the *days* factor and the interaction *enrichment* × *days*. The *days* factor showed statistical significance ($F_{2.04,36.77} = 56.33$, $p = 0.001$). The interaction *enrichment* × *days* also showed statistical significance ($F_{2.04,36.77} = 3.98$, $p = 0.01$). We analyzed the interaction with pairwise comparisons with Bonferroni adjustment and we found significant differences between groups on the first ($p = 0.04$), second ($p = 0.001$) and third ($p = 0.009$) days of training. The EE + SPL group showed shorter distance travelled than the SPL group on these days. We did not find significant differences between groups on the fourth day of training ($p = 0.83$) (Fig. 3c).

3.2.4. Pearson's correlation coefficients

Pearson's correlation coefficients showed that *TbE* index correlated negatively with memory errors in different days of training (reference memory errors of day 1 and *TbE* index: $r = -0.68$, $p = 0.01$; reference memory errors of day 2 and *TbE* index: $r = -0.50$, $p = 0.02$; working memory errors of day 1 and *TbE* index: $r = -0.53$, $p = 0.01$).

3.3. COx activity

MANOVA showed significant differences between groups in the Medial orbital cortex ($F_{3,36} = 11.11$, $p = 0.001$), the Prelimbic cortex ($F_{3,36} = 3.29$, $p = 0.03$), the Infralimbic cortex ($F_{3,36} = 6.05$, $p = 0.002$), the Accumbens nucleus ($F_{3,36} = 3.74$, $p = 0.02$), the Bed nucleus of the stria terminalis ($F_{3,36} = 3.34$, $p = 0.03$), the Basolateral amygdala ($F_{3,36} = 3.99$, $p = 0.015$), the Paraventricular thalamic nucleus ($F_{3,36} = 26.34$, $p = 0.001$), the Paraventricular hypothalamic nucleus ($F_{3,36} = 33.79$, $p = 0.001$), the Ventral CA1, CA3 and DG ($F_{3,36} = 15.94$, $p = 0.001$; $F_{3,36} = 18.57$, $p = 0.001$; $F_{3,36} = 29.93$, $p = 0.001$ respectively). In contrast, we did not find significant differences in the Dorsal CA1, CA3, DG ($F_{3,36} = 0.72$, $p = 0.55$; $F_{3,36} = 0.45$, $p = 0.72$ respectively), the cingulate cortex ($F_{3,36} = 1.82$, $p = 0.16$) and in the Central amygdala ($F_{3,36} = 2.74$, $p = 0.06$).

Tukey's post hoc tests showed significant differences between the EE + SPL and the SPL groups in the MO cortex ($p = 0.01$), PL cortex ($p = 0.03$), IL cortex ($p = 0.001$), BNST ($p = 0.03$), BlA ($p = 0.02$), PVNt nucleus ($p = 0.02$), PVNh nucleus ($p = 0.001$), vCA1 ($p = 0.01$), vCA3 ($p = 0.001$) and vDG ($p = 0.001$). SPL and CO groups had significant differences in the MO cortex ($p = 0.001$), IL cortex ($p = 0.02$), Acb ($p = 0.02$), BlA ($p = 0.04$), PVNt nucleus ($p = 0.001$), PVNh nucleus ($p = 0.001$), vCA3 ($p = 0.001$), vCA1 ($p = 0.001$) and vDG ($p = 0.001$). Also, we found significant differences between the SPL and EE groups in MO cortex ($p = 0.001$), IL cortex ($p = 0.03$), PVNt nucleus ($p = 0.02$), PVNh nucleus ($p = 0.001$), vCA1 ($p = 0.001$), vCA3 ($p = 0.002$), vDG ($p = 0.004$) and finally, between the EE and CO groups in vDG ($p = 0.001$) and between the EE + SPL and CO groups in vDG ($p = 0.001$).

Table 2 shows the average of COx activity values in these groups in the different brain regions studied and their comparisons between groups. Fig. 4 shows differences in COx staining intensity between groups.

4. Discussion

This study aims to assess the effects of EE on anxiety-related behaviours in the EZM and on the performance in the RAWM. We have also assessed the impact of the EE protocol on the cytochrome c oxidase activity of several anxiety-related brain regions after a spatial memory task.

4.1. Effects of EE on the activity in the EZM

To assess anxiety-related parameters we chose the EZM. We found that rats who were submitted to EE were less anxious, showing more head dips, higher time in the open section, lower latency to leave the closed section, as well as low levels of defecation. Also, we found high levels of rearing and frequent entries into the open sections. A disadvantage of unconditioned tests is that they do not allow us to discriminate between exploration of the novelty and anxiety levels. For that reason, we used the index *Time by entries* (Heredia et al., 2012) in order to minimize the effect of high levels of activity over the time spent in the open section. We found significant differences between groups in this parameter, which is indicative of reduced anxiety levels. In contrast, other studies suggest that high levels of activity and exploration are indicative of low levels of anxiety (Larsson et al., 2002). However, we consider these parameters independent. According to our results, we can suggest that our EE protocol showed anxiolytic effects against the exposure to the novelty and enhanced the locomotor activity (distance travelled) in the EZM. We cannot distinguish if the enhanced locomotor activity in the EE+SPL group is due to reduced anxiety levels or increased physical activity in the enriched environment. However, data of our research group showed that adult rats housed in the EE condition had lower velocity in the RAWM than non-enriched rats (Sampedro-Piquero et al., 2013a,b). However, other research has found that enriched animals are more rapid in spatial memory tasks and spend more time in the central sectors of the Open Field test, thus displaying thus lower levels of anxiety and higher exploratívity (Foti et al., 2011).

4.2. Effects of EE on the spatial memory in the RAWM

RAWM is a spatial memory task sensitive to the effects of anxiety (Shukitt-Hale et al., 2004). EE has shown to attenuate the deficits in the RAWM induced by anxiety and enhance spatial memory in adult rats (Leggio et al., 2005). This evidence coincides with our results, because animals submitted to EE showed fewer working memory errors and reference memory errors than the SPL group, above all in the first days of training, equaling the performance of the groups on the last day. Spatial reference errors were also higher than working memory errors from the first day of training in the EE + SPL and SPL groups, which suggests that this type of memory is more affected by anxiety. Interestingly, in the EE + SPL group, no

Table 2
COx activity values in the measured regions.

	CO	SPL	EE+SPL	EE
MO	21.38 + 0.64	29.23 + 1.53[@,=]	24.21 + 1.11[*]	22.66 + 0.53
Cg	23.83 + 0.79	28.56 + 1.47	25.61 + 1.39	24.25 + 1.05
PL	23.79 + 0.51	28.74 + 1.42	25.20 + 0.70[*]	23.50 + 1.02
IL	23.98 + 0.69	31.18 + 1.82[@,=]	24.01 + 0.78[*]	24.22 + 1.08
Acb	24.38 + 1.20	34.05 + 1.86[@]	29.38 + 1.10	30.04 + 2.07
BNST	21.58 + 1.22	24.77 + 1.39	21.39 + 0.84[*]	19.72 + 0.61
CeA	25.60 + 0.79	30.16 + 1.43	25.41 + 1.06	28.30 + 1.99
BlA	25.18 + 0.67	32.41 + 1.58[@]	26.60 + 1.16[*]	26.62 + 1.63
PVNt	18.62 + 1.07	31.52 + 1.22[@,=]	26.30 + 1.43[*,/,+]	19.78 + 1.02
PVNh	15.84 + 0.97	29.52 + 1.15[@,=]	17.40 + 0.60[*]	26.54 + 0.90
vCA1	22.34 + 1.19	31.80 + 1.47[@,=]	26.03 + 1.16[*]	22.06 + 0.34
vCA3	21.55 + 0.85	32.26 + 1.47[@,=]	24.72 + 0.81[*]	28.53 + 1.21
vDG	19.31 + 0.77	34.39 + 1.54[@,=]	26.70 + 0.86[*,/]	21.57 + 1.09[&]
dCA1	21.21 + 1.43	22.67 + 1.17	25.71 + 2.56	18.97 + 0.88
dCA3	20.76 + 1.51	22.05 + 0.94	24.62 + 2.53	26.50 + 1.05
dDG	24.04 + 0.60	33.06 + 1.77	38.39 + 5.23	18.85 + 0.98

Data represent mean ± SEM.
[*] $p < 0.05$ significant differences between EE+SPL and SPL groups.
[/] $p < 0.05$ significant differences between EE+SPL and CO groups.
[+] $p < 0.05$ significant differences between EE+SPL and EE groups.
[@] $p < 0.05$ significant differences between SPL and CO groups.
[=] $p < 0.05$ significant differences between SPL and EE groups.
[&] $p < 0.05$ significant differences between EE and CO groups.

animal had working memory errors after the second day of training, whereas in the SPL group, this was achieved on the last day, suggesting a facilitating effect of EE on the acquisition of the spatial task and a remarkable improvement of the working memory. The distance travelled during the last trial of each day of training also supports these data because the EE + SPL group had travelled lesser distance the first three days of learning with regard to the SPL group, equaling the performance the last day. Harris et al. (2009) concluded that EE reduces anxiety in a cognitive test situation, and therefore, the cognitive benefits of EE occur because enriched animals are less anxious during cognitive testing. Most of the tasks used to investigate learning and memory in rodents can be considered stressful and as a consequence of their aversive nature, they elicit the activation of stress systems (Aguilar-Valles et al., 2005). One possible explanation of the anxiolytic effect after EE condition is the increase of GC receptors, which exert negative feedback on the HPA axis and control the anxiety response (Vivinetto et al., 2013).

We must take into account possible alternative explanations for the better performance of the EE + SPL in the RAWM, owing to the fact that daily exposures to EE have shown to improve the navigation in complex environments (Leggio et al., 2005; Speisman et al., 2013), regardless of the level of anxiety. However, the negative correlation coefficients found between the index *TbE* and the memory errors seem to suggest that the good performance of the EE + SPL group is due to reduced levels of anxiety in the spatial task.

4.3. COx activity

COx activity reflects the neuronal functional activity occurring over long time periods lasting hours to weeks. Hence, this technique does not show the neuronal metabolic changes of the last training session or the initial short-term metabolic changes, but it shows a mapping of the neuronal metabolic activity throughout all the learning process. Hence, COx histochemistry provides insight into the metabolic history of brain regions (Gonzalez-Lima and Cada, 1994). In our study, we wanted to discover if the environmental enrichment condition influences the neuronal networks recruited during a spatial memory task, including both its learning, performance and retrieval. For that reason, we considered appropriated the use of COx histochemistry. If we had to study the COx activity during the process of learning or retrieval separately, we would have had to study it at different time points (Mendez-Lopez et al., 2013), but that was not our aim. In contrast, 2-deoxyglucose shows similar patterns of activity occurring over a few minutes and it would be more appropriate to reflect the neuronal metabolic activity of a training session.

Our COx results suggested an anxiolytic effect of EE, because regions traditionally related to anxiety response, such as the infralimbic cortex, the basolateral amygdala, the paraventricular nucleus or the ventral hippocampus showed lower neuronal oxidative activity with respect to the SPL group. On the other hand, we did not find significant differences between groups in the cingulate cortex, the central amygdala and the dorsal hippocampus. In relation to our results, Mällo et al. (2009) have seen that stress increased the COx activity in several limbic brain regions (the amygdala or the ventral hippocampus), which may indicate greater vulnerability to stress or anhedonia.

The medial prefrontal cortex has a complex role in stress regulation (Cerqueira et al., 2008; Blanco et al., 2009). We found that the EE + SPL and EE groups showed less activation in the Medial orbital cortex and infralimbic cortex than the SPL group, suggesting an anxiolytic effect of EE on anxiety-related brain regions. With regard to the prelimbic cortex, the higher neuronal metabolism in the SPL group could be explained by an attempt of this cortex to inhibit circuits mediating stress responses (Cerqueira et al., 2008). Recent data have shown that the stimulation of prelimbic cortex is sufficient to trigger inhibition of the HPA axis to the exposure to a novel environment (Jones et al., 2011). Finally, we do not find significant differences between the EE + SPL and SPL groups in the COx activity of the cingulate cortex. It plays an important role in the control of the performance, contributing to the processing of errors, response inhibition, working memory and attentional processes (van Veen et al., 2004; Osaka et al., 2004). All these functions are necessary for the correct performance in the RAWM, which may explain the similar COx activity in both groups. The CO and EE groups had the lowest activity in this cortex, perhaps due to the lack of learning experience.

High levels of amygdala activation may impair long-term storage of spatial information (Akirav and Richter-Levin, 2005), which could partly explain the worse performance of the SPL group in the RAWM. In our study, the COx activity in the central amygdala did

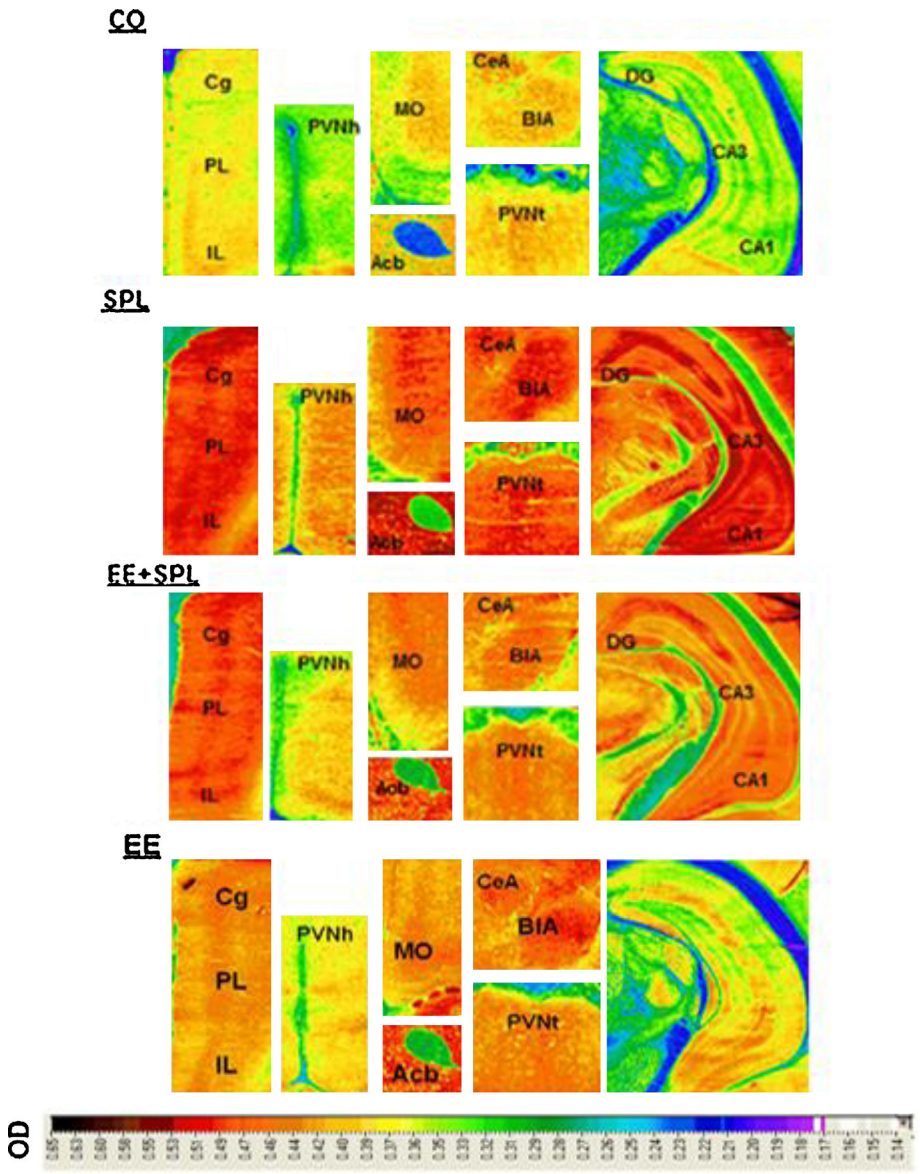

Fig. 4. Photographs of coronal stained sections (right) showing differences in COx expression between groups. Regions with statistical significant differences between groups. Color coding (values between 0 and 0.65): higher color intensity or optical density (OD) (black, red or yellow) represents higher COx activity, while lower color intensity or OD (blue or green) represents lower COx activity. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

not show significant differences between groups, but it also had low activation in the EE + SPL with a p value very near to the significance ($p = 0.054$). This result should be noted as a tendency because previous studies found that EE affected the COx expression in the amygdala (Leger et al., 2012). It is possible that the elevated activation of paraventricular thalamic nucleus in the SPL group, also involved in modulating stress responses, is inhibiting the central amygdala (Spencer et al., 2004).

Regarding the bed nucleus of the stria terminalis, we found that the EE and EE+SPL group had the lowest neuronal oxidative activity in this nucleus. Bed nucleus of the stria terminalis mediates long duration responses to stress (Walker et al., 2003), which links with the type of responses reflected by COx histochemistry. EE is known for decreasing the incentive value of stimuli associated with reward (Beckmann and Bardo, 2012) and this effect of EE could explain the lower COx activity found in the acumbens nucleus in the EE and EE + SPL groups compared to the SPL group. It is possible that this reduction of incentive salience may be produced by a faster habituation to the situation due to a continuous novelty-seeking orientation produced by the EE.

According to the topographic specificity of the hippocampus (Mendez-Lopez et al., 2009b; Bannerman et al., 2004), we found significant differences between groups in the ventral hippocampus, but not in its dorsal pole. In particular, the SPL group had higher COx activity in ventral hippocampus than the rest of the groups, which could be associated with an attempt to inhibit the elevated activity of the HPA axis, although it has been suggested that ventral hippocampus does not inhibit the HPA axis when an unconditioned stimuli is presented, because some level of reactivity is required in a novelty situation (Tuvnes et al., 2003). In contrast, we did not find significant differences between groups in the COx activity of dorsal hippocampus, mainly involved in the spatial performance. COx histochemistry is known for reflecting long-term changes in the regional brain activity, so at the end of the spatial training, the COx activity in the EE + SPL and SPL groups could be similar to the CO group because both groups had learned the task and therefore,

they required lower energy demand for the performance. In contrast, recent studies (Hawley et al., 2012) suggest that both the dorsal and ventral hippocampus play a different role in stressful experiences, with the dorsal part selectively involved in adaptative behaviours and the ventral subserving the emotional response.

The lack of significant differences in COx activity between the EE and EE + SPL groups in brain regions such as the dorsal hippocampus, cannot be interpreted as a lack of involvement of this region during the spatial task. In contrast, it is possible that the dorsal hippocampus was involved in the task, but its neuronal functional activity was not very high to reach statistically significant difference. As noted, COx histochemistry reflects sustained energy demands and after the fourth day of training, the behavioural performance became asymptotic, suggesting that the dorsal hippocampus does not require a lot of energy consumption. Besides, it is possible that our environmental enrichment protocol produced an accurate performance, as reflected the behavioural results, and it increased efficiency, reducing the neuronal metabolic needs in the regions involved in the spatial memory process. Previously, it has been found that an aerobic exercise protocol in aged rats also reduced the neuronal metabolic needs during a spatial task, which was related to a more accurate behavioural performance (Sampedro-Piquero et al., 2013a,b). Hence, the task difficulty is an important factor that affects to the oxidative metabolism of brain regions involved in spatial learning (Mendez-Lopez et al., 2010).

Finally, it would have been interesting to have determined the COx activity in brain regions related to anxiety response at different moments of the EE condition, because it is possible that COx would be higher at the beginning and much lower after 2 months, showing that EE really acts as a eustressor. Also, the measure of glucocorticoids response could have been adequate to support our results.

5. Conclusion

To conclude, our results show that EE reduces anxiety-related behaviour in the EZM; the EE + SPL animals spent more time and made more entries in the open areas, and also showed lower levels of defecation and lower latency to enter into the open area. This reduction of basal levels of anxiety could have positively affected the performance in the RAWM in the EE + SPL group, although we must take into account the benefits that the EE condition produces on the spatial cognition in complex environments. COx results suggested a reduction of anxiety levels after EE, as limbic brain regions traditionally related to anxiety response showed lower neuronal oxidative metabolism. Therefore, COx histochemistry seems to be appropriate to determine the effects of EE on different brain regions involved in the regulation of stress responses and in the spatial learning process.

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EXPERIMENTO III

Astrocytic plasticity as a possible mediator of the cognitive improvements after environmental enrichment in aged rats

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Astrocytic plasticity as a possible mediator of the cognitive improvements after environmental enrichment in aged rats

P. Sampedro-Piquero [a,*], Paola De Bartolo [b,c], Laura Petrosini [b,c], C. Zancada-Menendez [a], J.L. Arias [a], A. Begega [a]

[a] Laboratorio de Neurociencias, Departamento de Psicología, Universidad de Oviedo, Instituto de Neurociencias del Principado de Asturias, INEUROPA, Plaza Feijoo s/n, 33003 Oviedo, Spain
[b] Department of Psychology, University "Sapienza", via dei Marsi 78, 00185 Rome, Italy
[c] IRCCS S. Lucia Foundation, via del Fosso di Fiorano 64, 00143 Rome, Italy

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ABSTRACT

Currently, little is known about the effect of environmental enrichment (EE) on astrocytic plasticity, especially during aging. Given the newly discovered role of the astrocytes in regulating the synaptic transmission and thereby, the cognitive functions, we aimed to study the impact of EE on the performance in a spatial memory task and on the number and morphology of GFAP immunopositive cells in the dorsal hippocampus. After two months of EE (3 h/per day), the animals were tested in the Radial-Arm Water Maze (RAWM) for four days, with six daily trials. Next, we analyzed the changes in the GFAP immunopositive cells in CA1, CA3 and Dentate Gyrus (DG). Behavioral results showed that, even in advanced ages, EE improved the performance in a spatial memory task. Also, we found that aged rats submitted to EE had more GFAP immunopositive cells in the DG and more complex astrocytes, revealed by Sholl analysis, in all hippocampal subfields with respect to the other experimental conditions. Interestingly, the learning of a spatial memory task produced more morphological complexity and higher levels of GFAP immunopositive cells with regard to a standard control group, but not at the same level of the enriched groups. Thus, it is possible that the plastic changes found in the hippocampal astrocytes after EE are involved in a brain reserve to cope with age-related cognitive impairments.

1. Introduction

The aging process has long been associated with a decline in some physiological and cognitive functions, although these changes can be variable among subjects (Erickson & Barnes, 2003; Foster, 2006; Kelly et al., 2006). For example, the ability to perform new spatial memory tasks decreases with age (Smith, Gerrard, Barnes, & McNaughton, 2000), especially when allocentric spatial relationships have to be processed (Begega et al., 2001; Moffat, Elkins, & Resnick, 2006). This deficit has been attributed to changes in the dorsal hippocampus (Gonzalez-Ramirez, Velazquez-Zamora, Olvera-Cortes, & Gonzalez-Burgos, 2013; Maguire, Frackowiak, & Frith, 1996; Rosenbaum, Winocur, & Moscovitch, 2001). In the hippocampus of aging subjects altered synaptic plasticity, loss of functional synapses in CA1 and Dentate Gyrus (DG) (Rosenzweig & Barnes, 2003), reduction of neurogenesis in DG (Walter, Keiner, Witte, & Redecker, 2010), and deficits in the induction and the maintenance of long-term potentiation (LTP) (Rosenzweig & Barnes, 2003) have been described.

Interestingly, these age-related impairments seem to be alleviated by the exposure to complex environmental stimulations, as occurs in the environmental enrichment (EE) paradigm, a housing condition that combines social relations, physical exercise and interactions with stimulating objects (Bennet, McRae, Levy, & Frick, 2006; Petrosini et al., 2009; Simpson & Kelly, 2011; Van Praag, Kempermann, & Gage, 2000). Daily exposure to EE in aged rodents improved the acquisition and the flexible use of spatial information in the Morris water maze (Foster, 1999; Kumar, Rani, Tchigranova, Lee, & Foster, 2012; Rapp, Rosenberg, & Gallagher, 1987; Speisman et al., 2013). Furthermore, aged rats submitted to an intermittent condition of EE (3 h/day) from the median lifespan onwards showed better cognitive performance in the Radial-Arm Water Maze (RAWM) in comparison to non-enriched aged animals (Sampedro-Piquero, Begega,

* Corresponding author.
E-mail addresses: uo179677@uniovi.es (P. Sampedro-Piquero), paola.debartolo@uniroma1.it (P. De Bartolo), laura.petrosini@uniroma1.it (L. Petrosini), zancadaclara.uo@uniovi.es (C. Zancada-Menendez), jarias@uniovi.es (J.L. Arias), begega@uniovi.es (A. Begega).

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Zancada-Menendez, Cuesta, & Arias, 2013). EE also prevented some age-dependent alterations in a visuo-spatial attentional task (Harati et al., 2011). These cognitive benefits seem to be related with the formation of a brain reserve promoted by the EE condition. So, even despite aging, the brain appeared to remain responsive to EE, showing enhancement of cortical thickness (Mohammed et al., 2002), dendritic branching (Kolb, Gorny, Söderpalm, & Robinson, 2003), spine density (Kolb et al., 2003; Mohammed et al., 2002), LTP (Freret et al., 2012) and neurogenesis (Kempermann, Gast, & Gage, 2002). However, little is known about the EE effects on astrocytic response in aging.

Growing evidence suggests that, by enveloping synapses and forming tripartite synapses (Haydon, 2001), the astrocytes actively take part in synaptic transmission, plasticity and neuroprotection (Auld & Robitaille, 2003; Romero et al., 2013). Most studies about the EE effect on astrocytic plasticity were carried out on young rats and showed an increase of gliogenesis (Ehninger & Kempermann, 2003), alterations in glial morphology and antigen expression (Viola et al., 2009; Williamson, Chao, & Bilbo, 2012), as well as upregulation of the glial-derived neurotrophic factor (GDNF) expression (Young, Lawlor, Leone, Dragunow, & During, 1999). Conversely, the astrocytic changes in aged enriched rats have been scarcely studied, so their role in building brain and cognitive reserves remains unknown. Soffié, Hahn, Terao, and Eclancher (1999) found that prolonged EE (22 months, from weaning onwards) decreased the number and size of hippocampal astrocytes. Conversely, no studies have described the effect on the astrocytic plasticity of EE exposure from median lifespan, despite the fact that this period is considered a critical age for cognitive decline (Bizon et al., 2009). Besides, changes in the astrocytic morphology after EE have been described mainly at neocortical level, hippocampal data being scarce and controversial (Briones, Woods, Wadowska, Rogozinska, & Nguyen, 2006; Sirevaag & Greenough, 1991).

Thus, the present study assessed whether a non-continuous but prolonged EE condition was able to produce cognitive improvement in a spatial reference memory task in aged rats (20 months old) and if there was a relationship between the cognitive improvement and the changes in the number and morphology of Glial Fibrillary Acidic Protein immunopositive cells (GFAPs) in the dorsal hippocampus. We analyzed the dorsal hippocampus because of its remarkable degree of structural and functional plasticity induced by both EE and aging (Landfield, Rose, Sandles, Wohlstadter, & Lynch, 1977; Wimmer, Hernandez, Blackwell, & Abel, 2012).

2. Material and methods

2.1. Subjects

Thirty-eight 18 month-old male Wistar rats from the vivarium of the University of Sevilla were used. All the animals had *ad libitum* access to food and tap water and were maintained at constant room temperature (20–21 °C), with a relative humidity of 65–70% and artificial light-dark cycle of 12 h (08:00–20:00 h light/20:00–08:00 h dark). All the procedures complied with the European Communities Council Directive 2010/63/UE and RD 1201/2005, concerning the protection of animals used for experimental and other scientific purposes. All efforts were made to minimize the number of animals used and their suffering.

Rats were randomly assigned to four groups: Control group (CO: 731.8 g; $n = 10$), Radial-Arm Water Maze group (RAWM: 704.4 g; $n = 10$), environmental enrichment group (EE: 795.1 g; $n = 9$) and environmental enrichment + Radial-Arm Water Maze group (EE + RAWM: 689.1 g; $n = 9$). The weights were not significantly different ($p = 0.21$). The animals were kept in their respective experimental conditions until behavioral experiments were carried out two months later and also throughout the whole behavioral testing.

2.2. Housing conditions

Aged rats were housed in large cages of 100 cm × 95 cm × 54 cm during two months, 3 h/day (10:00 am/13:00 pm). We ensured that we always put together in the environmental enrichment cages the same group of rats (EE and EE + RAWM groups in a different cage each one) and the stimulating objects were similar in the two different cages. The cages contained a variety of objects, such as toys, running wheels, ropes, plastic tubes of different diameters, platforms, wooden houses, odorous and sound objects and nesting materials. The toys were chewable and bright. In each cage, we put a running wheel allowing to the rats made voluntary exercise and it was constant during all the period of environmental enrichment, as well as a yellow platform in which put some objects. The plastic tubes and the wooden houses allowed to the rats take cover and they made the cage more comfortable. Finally, we introduced odorous materials, such as pieces of orange, chocolate, scented soaps within small balls as well as, sound objects, such as little bells or little rattles. The rest of the day, the animals were housed in groups (four or five animal per cage) in standard cages (55 cm × 20 cm × 34 cm) without stimulating objects. The configuration of the cages was changed once a week and the cages were cleaned twice a week to ensure the welfare of the animals. The CO group was kept in two standard cages, five rats in each cage, without any learning or environmental enrichment experience. The RAWM group was kept in the same conditions as the CO group, but with learning experience.

2.3. Behavioral procedures

All the animals were handled daily for seven days. The day before the spatial memory task, the rats received a habituation session in which they were given three trials with the platform using different starting positions in a small square water tank (47 × 75 × 38 cm). The RAWM and EE + RAWM groups were trained in a black fiberglass RAWM (each arm: 80 cm × 12 cm) that was placed 50 cm above the floor level. The maze had four arms in the shape of a cross. The maze was filled with tap water to a height of 32 cm and a black escape platform was placed 2 cm beneath the water surface. The water temperature was kept at 22 ± 1 °C during the task period. The maze was placed in a room with dimmed lights, and there were several extra-maze cues on the walls that the rats could use to navigate. The RAWM testing was performed for four days, six trials/day with a 30 s inter-trial interval. At the beginning of each trial, the rat was immersed in the water, facing the wall, at one of three start positions. Start locations were randomized and the platform was in the same arm throughout the entire task (Arm A), and the animals were never released into the water from that arm. Each rat was allowed 60 s to reach the platform and if the rat failed, it was guided to the platform. Once the rat reached the platform, it remained there for 15 s. Between trials, the animal was placed in a small square tank for 30 s. At the end of each day of training, the rats were dried and returned to their home cage. Each trial was recorded and analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands). Working memory errors, reference memory errors, latency, distance travelled, velocity and time in the center of the maze of the last trial of each day of training were registered.

2.4. Tissue preparation

The day after the RAWM task, the animals were deeply anaesthetized (Ketamine 0.4 ml and Xylazine 0.2 ml) and perfused tran-

scardially with 0.9% saline (5 min), followed by 4% paraformaldehyde phosphate-buffer during 30 min (0.1 M; pH 7.4). The brains were postfixed with paraformaldehyde at 4% (0.1 M; pH 7.4) for 3 h, and then transferred into phosphate-buffer (0.1 M; pH 7.4) overnight. The brains were extracted and introduced in paraffin. Systematic cuts were performed with a microtome (Leica, RM2135, Germany) at 20 μm.

2.5. GFAP immunohistochemistry

After desparaffinizing the sections, they were permeabilized in Tris buffer saline (TBS) containing 0.1% Triton X-100, followed by a 30 min pre-incubation in 1% human serum (Sigma, USA) dissolved in TBS. After this blocking step, we carried out three washes with TBS containing 0.1% Triton X-100. Next, a polyclonal primary antibody, rabbit anti-GFAP, (Dako, Denmark) was applied at 1:800 dilution and the sections were incubated for 24 h at 4 °C. The following day, the sections were washed three times in TBS with Triton X-100, incubated in biotinylated secondary goat anti-rabbit IgG antibody (1:480 dilution; Pierce, USA) in 10% bovine serum for 30 min, and dipped three times in TBS with Triton X-100. Next, the sections were incubated with an avidin–biotin horseradish-peroxidase complex (Vectastain ABC-Ultrasensitive, Elite Kit; Pierce, USA) for 1 h at room temperature, washed twice in TBS with Triton X-100, rinsed in TBS, and then, visualized with DAB (Sigma, USA). Finally, the sections were dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, USA). For the GFAP immunohistochemistry, we randomly selected 10 animals/group for the CO and RAWM groups, nine animals for the EE group and six animals for the EE + RAWM group, for a total of 35 animals.

2.6. GFAP quantification

The dorsal hippocampus, Cornu Ammonis (CA1 and CA3) and Dentate Gyrus (DG) was identified at −3.84 mm posterior to bregma (Paxinos & Watson, 2005). Quantification of hippocampal GFAPs was assessed by using the Leica suite application (Version 2.5.0 R1, Leica Microsystems CMS, Switzerland). The microscope was connected to a computer. High image resolution was obtained using a numerical aperture × 20 objective for GFAP immunopositive cell counting. The count method began in the left part of the section and the microscope stage was moved stepwise in a meandering pattern over the entire section. Six sections per animal were analyzed. Representative samples were taken in each section, using an adapted square with a known area. GFAP immunopositive cells visualized inside the square were manually quantified, eliminating those cells that touch the boundary line. Results show immunopositive cells/area (100 mm^2).

We calculated the coefficient of error (CE) and the coefficient of variation (CV) of the number of GFAP immunopositive cells quantified, as described in Begega et al. (1998). These coefficients allow us to assess the accuracy of our sampling method. The acceptable level for the sampling method applied was defined as the ratio of the intrinsic error introduced by the methodology (CE^2) and the coefficient of variation (CV^2). Theoretically, this ratio should not be higher than 0.5.

2.7. Sholl analysis

To reconstruct the astrocytic morphology, a researcher blind of the identity of the groups performed the morphological analysis by using Neurolucida 64-bit software (v11, MicroBrightField, Williston, VT) connected to a light microscope (Axioskop; Carl Zeiss) with a 100 oil-immersion objective lens. To this aim, we used the Sholl analysis implemented in Neurolucida Explorer Software package (MicroBrightField, Williston, VT). This analysis is based on virtually including the cell in a set of concentric circles at a 10 μm interval (Sholl, 1953). The analyzed parameters were: branch length (in μm), calculated by adding up the lengths of all branches passing through each circle; number of nodes, calculated by adding up all points from which the branches arose; number of intersections of the branches with the overlaid concentric rings centered on the soma. GFAPs were selected according to these criteria: their labelling was uniform and without any precipitate of reaction, the astrocytic body and its branches were fully stained; the astrocyte was relatively isolated from surrounding astrocytes to obtain a clear image of the entire cell and the cell was located in the CA1, CA3 and DG of the dorsal hippocampus. For the Sholl analysis, we randomly selected eight animals/group for CO, RAWM and EE groups and six animals for EE + RAWM group, for a total of 30 animals. We analyzed the morphology of six astrocytes for each hippocampal subfield for a total of 18 astrocytes for each animal of the four experimental groups.

2.8. Statistical analysis

Behavioral and histological data were analyzed with SPSS 19.0 (SPSS Inc., Chicago, USA) and were expressed as mean ± SEM. The results were considered statistically significant if $p < 0.05$ and they were represented graphically with SigmaPlot 8.0 (Systat, Richmond, EEUU).

For the behavioral results, we carried out a two-way ANOVA of repeated measures (RM ANOVA) (within-factor: *days*; between-factor: *groups (EE present/EE absent)*. An appropriate post hoc test was used when we found significant differences (pairwise comparisons with Bonferroni adjustment). We used a one-way MANOVA to analyse the differences between groups in the number of GFAP immunopositive cells and in the different morphological parameters analyzed by Sholl analysis (intersections, length and nodes).

3. Results

3.1. Behavioral results

Working memory and reference memory errors

Working memory errors did not vary as the days progressed ($F_{3,54} = 1.37$, $p = 0.26$). The factor *groups* and the interaction *days* × *groups* did not show significant differences ($F_{1,17} = 1.05$, $p = 0.32$; $F_{3,54} = 1.67$, $p = 0.92$ respectively). Reference memory errors varied from day to day ($F_{3,54} = 3.76$, $p = 0.02$). The factor *groups* was significant ($F_{1,17} = 23.97$, $p < 0.001$) showing that the EE + RAWM group made fewer reference memory errors than the RAWM group, but the interaction *days* × *groups* was not significant ($F_{3,54} = 1.45$, $p = 0.24$) (Fig. 1).

Latency

The latency did not vary as the days went by ($F_{3,54} = 1.05$, $p = 0.38$). The factor *groups* and the interaction *days* × *groups* did not show statistical significance ($F_{1,17} = 0.06$, $p = 0.80$; $F_{3,54} = 1.03$, $p = 0.39$ respectively) (Fig. 1).

Distance

The latency did not vary as the days went by ($F_{3,54} = 0.72$, $p = 0.54$). The factor *groups* showed statistical significance ($F_{1,17} = 10.58$, $p = 0.004$), being the EE + RAWM which travelled shorter distance. The interaction *days* × *groups* was not significant ($F_{3,54} = 2.12$, $p = 0.10$) (Fig. 1).

Velocity

The velocity did not vary as the days went by ($F_{3,54} = 2.53$, $p = 0.07$). The factor *groups* and the interaction *days* × *groups* did

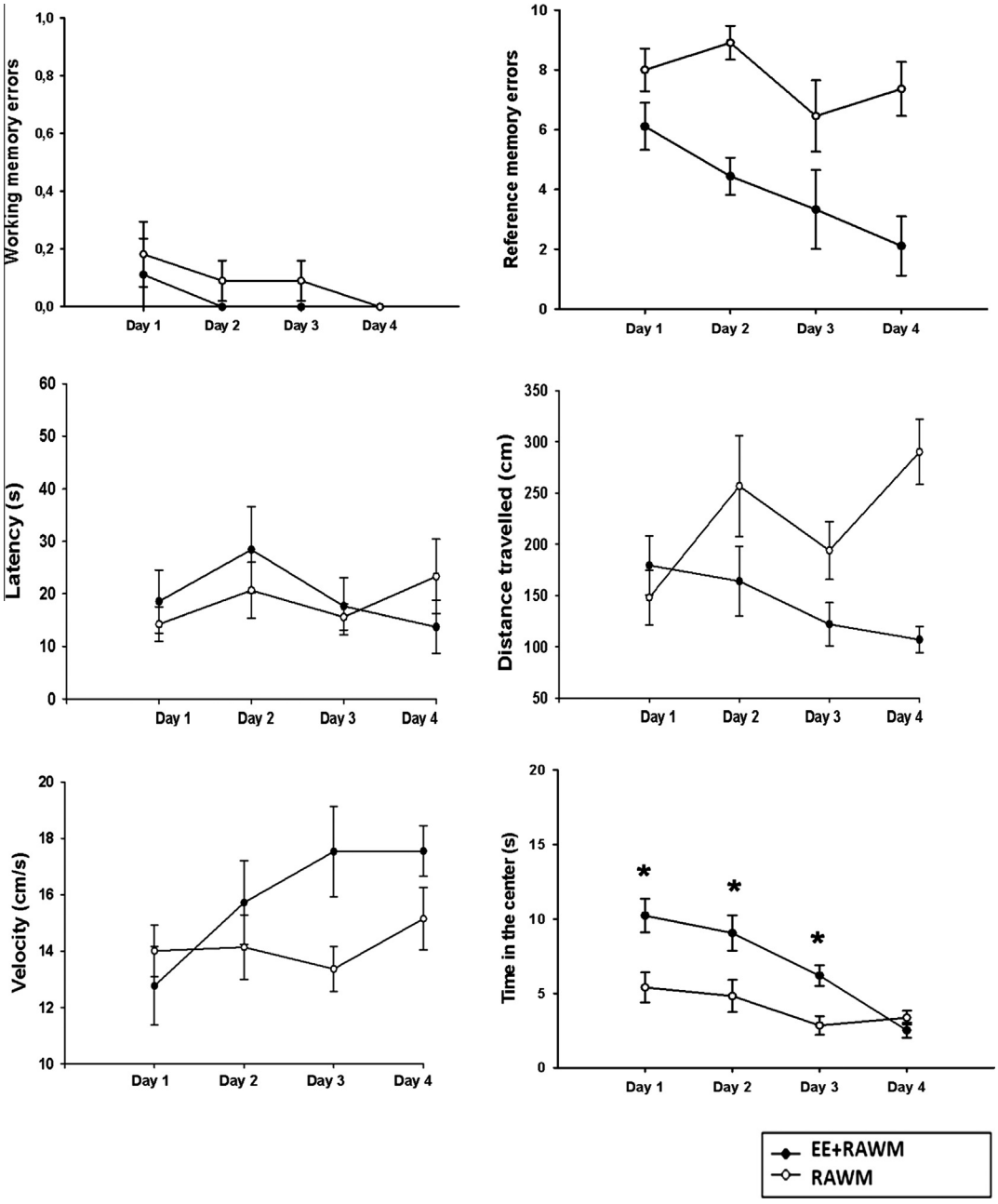

Fig. 1. Behavioral performance in the RAWM (mean ± SEM). The factor *groups* was significant in the reference memory errors and distance travelled, showing that the EE + RAWM group made fewer errors and travelled shorter distance than the RAWM group. In the variable *time in the center of the maze*, the interaction *groups* × *days* was significant, showing that the EE + RAWM group spent more time in the center in the last trial of the first, second and third days of training ($^{*}p < 0.05$).

not show statistical significance ($F_{1,17} = 3.12$, $p = 0.09$; $F_{3,54} = 2.08$, $p = 0.11$ respectively) (Fig. 1).

Time in the center

Finally, the time in the center of the maze varied as the days progressed ($F_{3,54} = 15.71$, $p < 0.001$). Also, the factor *groups* showed statistical significance ($F_{1,17} = 14.30$, $p < 0.001$), as well as the interaction *days* × *groups* ($F_{3,54} = 5.02$, $p = 0.004$). The Bonferroni adjustment test revealed us in which trials there were significant differences between groups, so we found that the EE + RAWM group spent more time in the center of the maze in the last trial of the first ($p = 0.006$), second ($p = 0.02$) and third ($p = 0.02$) days of training (Fig. 1).

3.2. GFAP results

3.2.1. Number of GFAP immunopositive cells

In the hippocampus, one-way MANOVA revealed significant differences between groups in the number of GFAP immunopositive cells in CA3 ($F_{3,31} = 78.77$, $p < 0.001$) and DG subfields ($F_{3,31} = 36.32$, $p < 0.001$), but not in CA1 ($F_{3,31} = 2.53$, $p = 0.15$). In CA3 and DG, Tukey's post hoc test showed significant differences between the CO group and the rest of experimental conditions (RAWM, $p < 0.001$; EE, $p < 0.001$; EE + RAWM, $p < 0.001$). In DG, we also found significant differences between the EE + RAWM and the RAWM groups ($p < 0.001$) and between the EE and the RAWM groups ($p < 0.001$) (Fig. 2).

Table 1 shows the CE and CV results and the ratio CE^2/CV^2.

3.2.2. GFAP morphological analysis

3.2.2.1. CA1. One-way MANOVA showed significant differences between groups in the number of intersections, nodes ($F_{3,26} = 75.26$, $p < 0.001$; $F_{3,26} = 270.28$, $p < 0.001$ respectively) and in the length of the astrocytic branches ($F_{3,26} = 70.86$, $p < 0.001$). Tukey's post hoc test revealed statistical significance between the CO group and the rest of the experimental conditions in all the morphological parameters analyzed ($p < 0.001$). Specifically, the CO group had fewer astrocytic intersections, nodes, and shorter branches in comparison to the other groups. Furthermore, the RAWM group exhibited significantly fewer intersections and nodes, as well as shorter branches in comparison to the EE and EE + RAWM groups ($p < 0.001$). Finally, the EE showed a higher number of nodes ($p < 0.001$) with respect to the EE + RAWM group (Fig. 3).

3.2.2.2. CA3. One-way MANOVA showed significant differences between groups in the number of intersections, nodes ($F_{3,26} = 56.08$, $p < 0.001$; $F_{3,26} = 164.10$, $p < 0.001$ respectively) and in the length of the branches ($F_{3,26} = 80.50$, $p < 0.001$). Tukey's post hoc test revealed that the CO group had fewer astrocytic intersections, nodes and shorter branches compared with the rest of the groups ($p < 0.001$). The RAWM group showed lesser astrocytic intersections, nodes and shorter branches than the EE and EE + RAWM groups ($p < 0.05$). As in CA1, we also found significant differences between the EE and EE + RAWM groups in the number of nodes ($p = 0.002$) (Fig. 3).

3.2.2.3. DG. One-way MANOVA showed significant differences between groups in the number of intersections, nodes ($F_{3,26} = 54.80$, $p < 0.001$; $F_{3,26} = 75.48$, $p < 0.001$ respectively) and in the length of the branches ($F_{3,26} = 55.78$, $p < 0.001$). Again, Tukey's post hoc test showed that the CO group had fewer number of intersections, nodes, and shorter astrocytic branches than the other experimental groups ($p < 0.001$). As in CA1 and CA3, the RAWM group had significant differences in comparison to the EE and EE + RAWM groups in all the morphological parameters ($p < 0.05$). In contrast to the other hippocampal subfields, in the DG, we did not find significant differences between the EE and EE + RAWM groups ($p > 0.05$) (Fig. 3).

Fig. 4 shows camera lucida drawings of representative GFAP immunopositive cells from CA1, CA3 and DG in each experimental condition.

Table 1
Estimation of the CE, CV and the ratio CE^2/CV^2 in the different experimental groups.

Groups	CE	CV	CE^2/CV^2
CA1			
CO	0.13	0.26	0.25
RAWM	0.10	0.17	0.35
EE	0.09	0.17	0.28
EE + RAWM	0.08	0.16	0.25
CA3			
CO	0.27	0.81	0.11
RAWM	0.10	0.23	0.19
EE	0.08	0.17	0.22
EE + RAWM	0.07	0.14	0.25
DG			
CO	0.15	0.66	0.05
RAWM	0.11	0.35	0.10
EE	0.08	0.18	0.20
EE + RAWM	0.07	0.15	0.22

4. Discussion

In the current study, we assessed the effects of non-continuous (3 h/day) but prolonged (two months) EE starting from the median lifespan on spatial memory and astrocytic plasticity in aged rats.

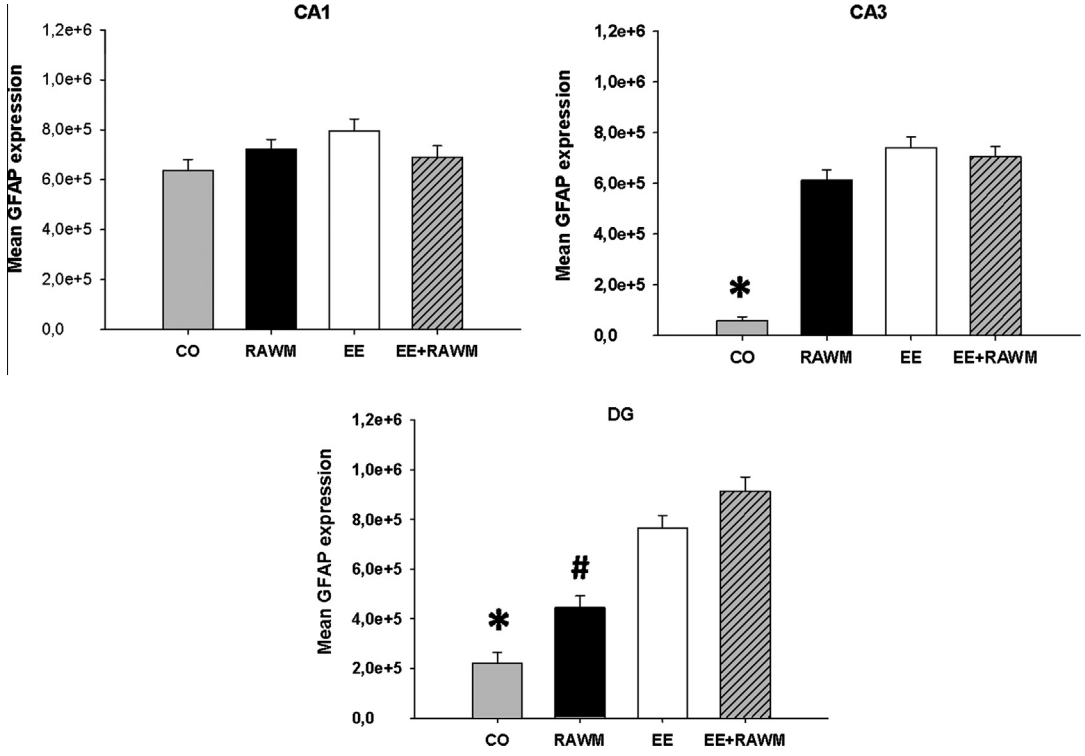

Fig. 2. Effects of the different experimental conditions on the number of GFAP expression; results show immunopositive cells/area (100 mm^2). The CO group had significantly lower levels of GFAP immunopositive cells in CA3 and DG with respect to the rest of experimental conditions ($^*p < 0.05$). The RAWM group showed lower levels of GFAP immunopositive cells in DG compared to the EE and EE + RAWM groups ($^\#p < 0.05$).

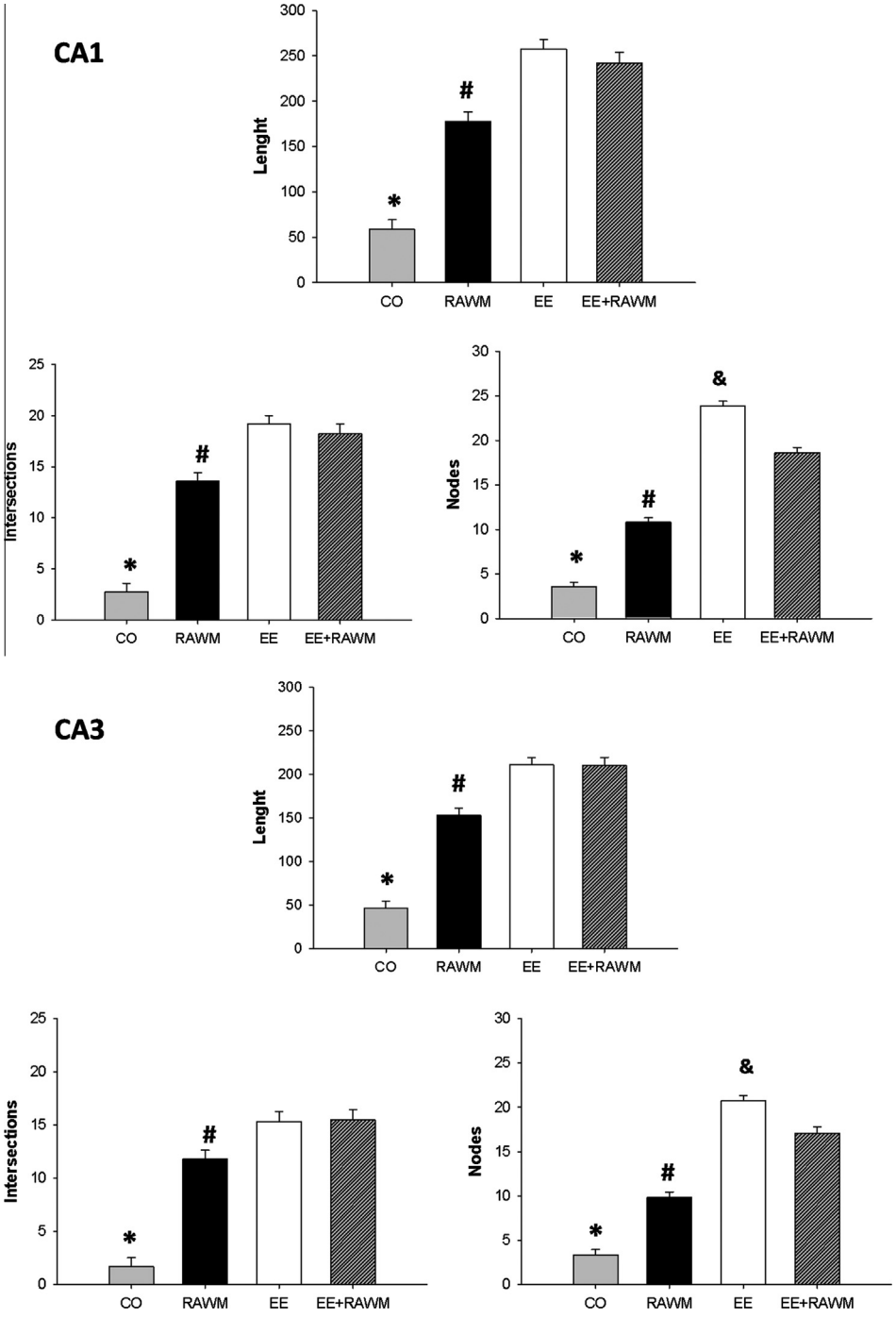

Fig. 3. Effects of the different experimental conditions on the astrocytic ramification by Sholl analysis in CA1, CA3 and DG (length, intersections and nodes). We found that the CO group had shorter branches with fewer intersections and nodes in all hippocampal subfields with regard to the other groups ($^{*}p < 0.05$). The RAWM group presented shorter branches with fewer intersections and nodes in all hippocampal subfields with respect to the EE and EE + RAWM groups ($^{\#}p < 0.05$). Finally, the EE group had branches with more nodes in CA1 and CA3 compared with the EE + RAWM group ($^{\&}p < 0.05$).

We found that EE was able to improve spatial memory and to induce the adoption of accurate spatial strategy. Aged rats exposed to EE made fewer spatial reference memory errors, travelled shorter distance and spent more time in the center of the maze in comparison to the RAWM group. These results indicate that enriched rats performed the task with a more accurate strategy, swam to the center of the maze, through the allocentric cues, localized the correct arm making fewer errors and travelling shorter distance. Furthermore, the lack of significant difference in the latency between groups indicated that both groups spent similar time to find the platform. However, latency may be an ambiguous measure, since it is affected by swimming velocity. In the present research, no significant differences in swimming velocity between groups were found. The EE + RAWM group spent most of its time in the center of the maze in order to localize the correct arm. Conversely, the RAWM group randomly searched for the platform, entered all arms to find it and, therefore, made a lot of reference memory errors. This result indicates that EE is able to improve the age-related spatial memory deficits when allocentric cues are required (Begega et al., 2001, 2012; Klencklen, Després, & Dufour,

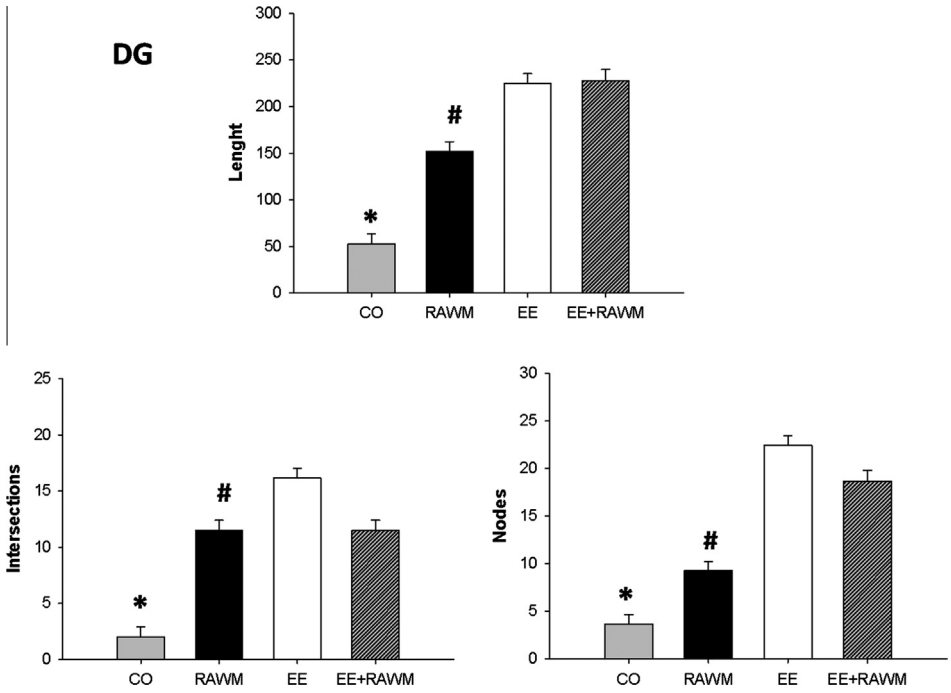

Fig. 3 (*continued*)

2012; Moffat et al., 2006). Allocentric strategy is demonstrated to be hippocampal-dependent. Not by chance, EE enhances hippocampal LTP, neurogenesis, dendritic spine growth and neurotrophin expression (Ickes et al., 2000; Kempermann, 2002; Landers, Knott, Lipp, Poletaeva, & Welker, 2011; Moser, Trommald, & Andersen, 1994).

The effects of the exposure to an enriched environment of middle-aged animals are controversial. While some studies point out that EE needs to be initiated before median lifespan to induce positive effects on cognition (Bouet, Freret, Dutar, Billard, & Boulouard, 2011; Freret et al., 2012), other studies found positive effects even when EE was initiated during this period of life (Kempermann, Kuhn, Winkler, & Gage, 1998). The beneficial effect of exposure to EE at advanced middle-age seems to indicate the existence of a sensitive period for its effectiveness in cognitive aging.

There are many open questions with respect to how astrocytic changes associated with EE and aging may impact cognitive functions. We found that the exposure to a spatial memory task and, even more powerfully, to an enriched environment increased the number and morphological complexity of astrocytes in all hippocampal subfields in comparison to the CO group. Strikingly, EE without exposure to the spatial task (EE group) enhanced the number of GFAPs in DG. The DG is shown to be a specific proliferative environment, particularly sensitive to age-related changes (Kempermann et al., 1998; Small, Chawla, Buonocore, Rapp, & Barnes, 2004; Wenzel, Lammert, Meyer, & Krug, 1991). In this light, it is interesting to discuss possible connections between the astrocytic changes and the cognitive EE-induced protection observed in the present study. Aged rats housed in a standard condition (CO group) exhibited the lowest number of GFAPs in CA3 and DG. This pattern has to be taken into account when considering the synaptic tuning in networks involved in cognitive processing and the control of the mossy fiber-to-CA3 synaptic input the astrocytes participate in (Jourdain et al., 2007). The functional integrity of the mossy fibers is essential in LTP as well as in storage and recall of spatial representations, which are compromised in aging (Ojo et al., 2013). Spatial learning elicits an increased astrocyte number in DG related to the length of the learning period (Jahanshahi, Sadegui, Hosseini, & Naghdi, 2007). This result is in accordance with our results, showing that the RAWM group had a higher number of astrocytes in DG in comparison to the CO group. The similarity of the astrocyte number in CA1 subfield in enriched and standard groups follows the same line of previous results described by Viola et al. (2009). RAWM and both enriched groups (EE and EE + RAWM) showed a similar number of GFAPs in CA1 and CA3, but not in DG where both enriched groups had a high number of GFAPs. The increase of astrocytes in DG could be related to the specific effects of EE on neurogenesis and synapse formation processes. Although a decreased hippocampal neurogenesis has been described in aging (Kuhn, Dickinson-Anson, & Gage, 1996; Nacher, Alonso-Llosa, Rosell, & McEwen, 2003), the maintenance of neurogenic potential and the capacity to generate new neurons by astrocytes has also been shown (Mori, Buffo, & Götz, 2005). The exposure to EE stimulates the survival of the neurons and their integration in hippocampal networks (Kempermann et al., 2002; Segovia, Yagüe, Garcia-Verdugo, & Mora, 2006). Specifically, GDNF, a neurotrophic factor secreted by glial cells, is shown to powerfully promote the survival of many types of neurons (Tomac et al., 1995).

With regard to the morphological data, the length of the branches, the number of intersections and nodes were increased by EE, leading to astrocytes with a complex morphology. The increased astrocytic ramification seems to be induced by BDNF, whose hippocampal levels are demonstrated to be enhanced by EE (Angelucci et al., 2009 Ickes et al., 2000; Ohira et al., 2007). The long and complex branches would allow the astrocytes to envelop the synaptic terminals and influence synaptic transmission through gliotransmitter release, acting in turn on pre- and post-synaptic neuronal receptors (Theodosis, Poulain, & Oliet, 2008; Wang & Bordey, 2008). The impact of BDNF on the astrocytic ramifications could be indirectly mediated by its action on the hippocampal neurogenesis (Kuzumaki et al., 2011). Thus, an enriched environment would increase the BDNF expression via sustained epigenetic modifications which could partly explain the hippocampal neurogenesis. These new neurons would secrete growth factors that stimulate the astrocytic complexity allowing a better control

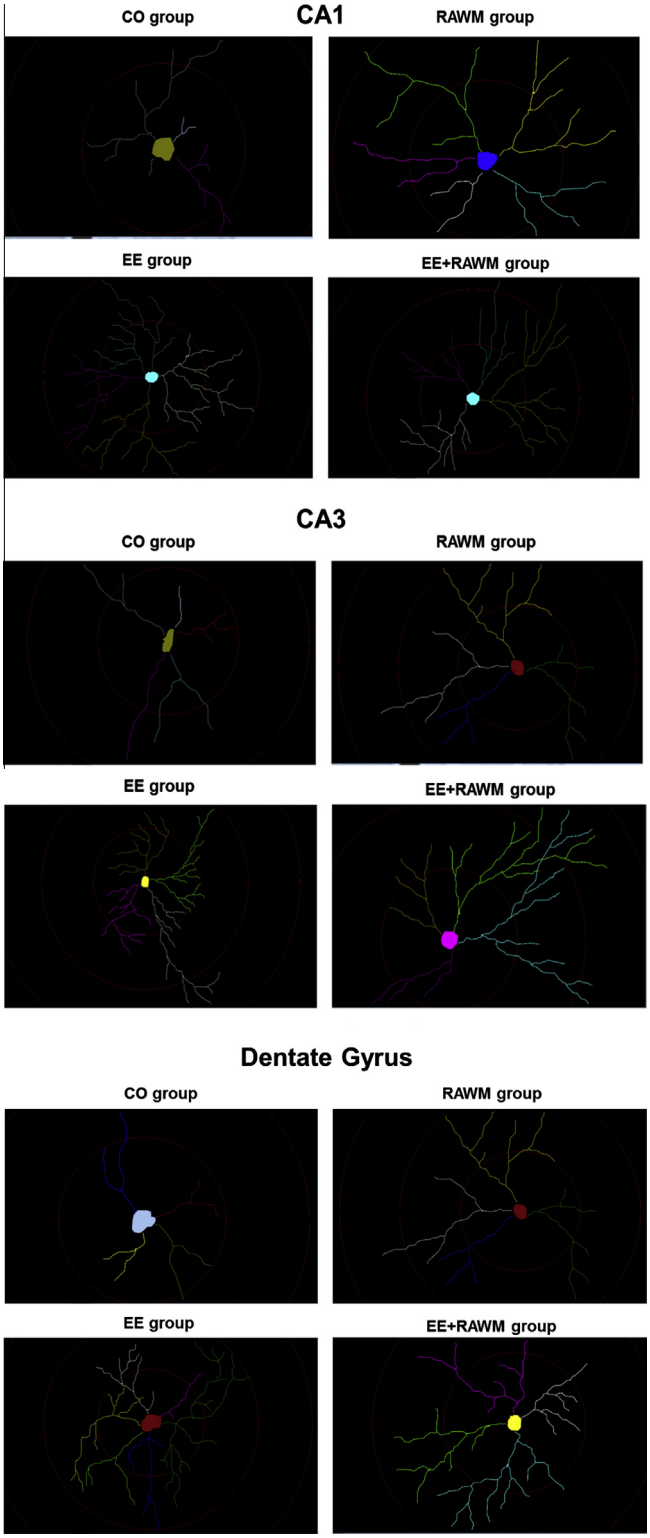

Fig. 4. Astrocytic reconstructions created by tracing the astrocyte in Neurolucida software (MicroBrightField) using concentric circles of increasing radii (10 μm) to measure the complexity of the ramifications. We can observe the differences in the astrocytic morphology between the different groups. The astrocytic reconstructions were made at different scales.

of extracellular ion homeostasis, metabolic supply to neurons, the maintenance of the blood–brain barrier (BBB), as well as the modulation of synaptic transmission (Parpura et al., 2012). Unlike our results, several studies described a higher degree of astrocytic shrinkage after EE in aged rodents (Diniz et al., 2010; Soffié et al., 1999). A likely explanation of this difference is linked to the applied EE protocols that induce different effects on neuronal and astroglial levels. In fact, while in the quoted experiments the animals grew up in an enriched environment, in the present study the EE was initiated at median lifespan. In this latter condition, astrocytes with long and complex branches could compensate the possible age-related neuronal and synaptic degeneration. In fact, during aging hippocampal synaptic transmission decreases in glutamatergic and cholinergic pathways (Billard, 2006), thus explaining the short and simple astrocytic branches found in the CO group. Interestingly, the RAWM group also showed a superior morphological complexity in the GFAPs in comparison to the CO group, but inferior in comparison to both enriched groups (EE and EE + RAWM). This result may be related to the astrocytic involvement in the hippocampal LTP demonstrated to be essential for spatial learning and memory (Allen & Barres, 2009). Following LTP, the astrocytes increase their ramifications to drive the neuronal plasticity (Zhang, Zhang, & Chen, 2009). The combination of EE and spatial learning did not produce additive effects on the astrocytic morphological changes, suggesting that the brain plasticity in aging, although present, is limited.

5. Conclusions

In summary, our findings showed that a non-continuous protocol of EE, initiated at advanced middle-aged, improved the cognitive performance in aged Wistar rats. Moreover, the enriched rats acquired a more accurate searching strategy making fewer reference memory errors. Interestingly, these cognitive improvements could be related in part with the astrocytic changes found in the dorsal hippocampus. The quantitative analysis showed that EE enhanced the GFAP immunopositive cells in the DG, which may be related to the proliferative characteristic of this hippocampal subfield and the synapse formation. EE also increased the morphological complexity of the hippocampal astrocytes. These astrocytes showed longer branches with more intersections and nodes compared to the other experimental conditions. We suggest that the astrocytic plasticity may represent at least a component of a brain reserve induced by EE that could account for the behavioral improvements found in aged rats.

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EXPERIMENTO IV

Behavioural testing-related changes in the expression of Synapsin I and glucocorticoid receptors in standard and enriched aged Wistar rats

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Behavioral testing-related changes in the expression of Synapsin I and glucocorticoid receptors in standard and enriched aged Wistar rats

P. Sampedro-Piquero *, J.L. Arias, A. Begega

Laboratorio de Neurociencias, Departamento de Psicología, Universidad de Oviedo, Plaza Feijoo s/n., 33003 Oviedo, Spain
Instituto de Neurociencias del Principado de Asturias, INEUROPA, Spain

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ABSTRACT

Our aim was to assess the changes in the Synapsin I and glucocorticoid receptor (GR) expression induced by behavioral testing in the dorsal and ventral hippocampi of standard and enriched aged Wistar rats. The environmental enrichment (EE) was carried out 3 h/day over a period of two months and then, the rats were tested in the elevated zero-maze (EZM) and radial-arm water maze (RAWM). Behavioral results showed that, even at an advanced age, EE was able to reduce anxiety-related behaviors and improve the performance in the RAWM. Regarding the neurobiological data, Synapsin I expression in the dorsal CA3, but not in the ventral, was enhanced both in enriched and standard rats when they performed the behavioral testing. Interestingly, the EE exposure was enough to increase Synapsin I in the ventral CA3. The analysis of GR in the dorsal hippocampus showed an increase of this receptor in the dDG both in enriched and standard rats when they performed the behavioral testing, whereas in the dCA1 and dCA3, the effect of the testing depended on the previous housing condition. In the ventral region, we found that the effects of EE were higher because on the one hand, the GR expression induced by the behavioral testing was enhanced in the dSUB, vCA1 and vCA3 when the rats were previously enriched and on the other hand, EE, regardless of the behavioral testing, increased the GR expression in the vDG and vSUB. Therefore, our results suggest that the effect of the behavioral testing on the neurobiological mechanisms studied is different depending on the previous housing condition of aged rats.

1. Introduction

Aging is considered as a risk factor to develop cognitive and neuroendocrine impairments which generally affect the quality of life (Garrido, 2011). Specifically, it has been shown that old age is associated with functional decrements in spatial cognition affecting daily activities, such as finding objects or navigating in new environments (Burger et al., 2007; Klencklen et al., 2012; Moffat et al., 2001). With regard to the neuroendocrine alterations, previous studies have revealed that aging is often linked to a difficulty in the inhibition of the hypothalamic–pituitary–adrenal axis (HPA), resulting in the hypersecretion of glucocorticoids (GCs) during times of stress and even during basal conditions (Aguilera, 2011; Bizon et al., 2001; Mizoguchi et al., 2009; Pedersen et al., 2001). Nowadays, a complex interaction between anxiety and cognition has been accepted, with high levels of anxiety generally promoting unsuccessful cognitive aging (Ulrich-Lai and Ryan, 2013). Thus, several studies have described that older people suffering from depression, as well as anxiety diseases show a high degree of dysregulation of HPA axis activity, which impacts negatively on spatial memory functions (Lupien et al., 2009; Zahodne et al., 2014).

The hippocampus, due to its established role in the spatial memory and in the control of anxiety levels (Lupien and Lepage, 2001; Sandi and Pinelo-Nava, 2007) is one of the brain regions that has been most studied in aging research. On the one hand, a decrease in the number and affinity of the glucocorticoid receptors, which are involved in the feedback inhibition of the HPA axis, has been described in the aged dorsal hippocampus (de Kloet et al., 2005; Sapolsky, 1999) leading to a long-term GC overexposure and in consequence, accelerating some aspects of brain aging. For example, aged rats exhibiting impaired spatial memory show a decrease of GR density and their mRNA in the hippocampus (Issa et al., 1990). On the other hand, the aged hippocampus is also characterized by an altered synaptic plasticity with the loss of synapses and deficits in the induction of the long-term potentiation (LTP), a cellular correlate of learning and memory (Petralia et al., 2014). One of the processes involved in the LTP is the increase in the neurotransmitter release which is mediated in part by the activity of presynaptic proteins (Hilfiker et al., 1999). Hence, it is possible that presynaptic proteins, such as Synapsin I, considered as a marker of synaptic activation during the LTP, are reduced in aging. In accordance with this, it has been found that aged rodents have deficits in the phosphorylation of Synapsin I (Eckles et al., 1997).

* Corresponding author at: Laboratorio de Neurociencias, Departamento de Psicología, Plaza Feijoo s/n., 33003 Oviedo, Spain.
E-mail addresses: uo179677@uniovi.es (P. Sampedro-Piquero), jarias@uniovi.es (J.L. Arias), begega@uniovi.es (A. Begega).

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At present, behavioral interventions, such as aerobic exercise and cognitive enrichment, have shown to be particularly beneficial during aging in contrast to aged people with a sedentary and non-stimulating lifestyle (Alves et al., 2013; Rizzuto and Fratiglioni, 2014). Rodent studies in which social, motor, cognitive and physical stimulation is provided have revealed that environmental enrichment (EE) in aged rats improves the spatial memory performance, increasing the ability to rapidly acquire a spatial memory task, along with a flexible use of spatial information (Baraldi et al., 2013; Harati et al., 2011; Speisman et al., 2013). Also, EE, in contrast to standard housing conditions, produces better indices of emotionality and faster adaptation to novel situations (Larsson et al., 2002; Zimmerman et al., 2001). In relation to the impact of EE on the behavior and brain, neurotransmitters, growth factors, synaptic plasticity, neurogenesis or HPA activity changes have all been investigated for their possible involvement in these improvements (Frick and Benoit, 2010; Sale et al., 2014; Simpson and Kelly, 2011).

Following this line of research, our aim was to study how the impact of the behavioral testing on neurobiological mechanisms, such as the Synapsin I and GR expression, could be modulated by the previous experience of EE or standard housing condition in aged Wistar rats. Our study is innovative as it is the first to study the changes in the expression of these proteins after behavioral testing in the dorsal and ventral hippocampi of enriched and standard aged rats, as to date most of the studies have focused on the dorsal hippocampus.

2. Material and methods

2.1. Animals

Thirty-eight 18 month-old male Wistar rats from the vivarium of the University of Seville were used. All the animals had ad libitum access to food and tap water and were maintained at constant room temperature (20–21 °C), with a relative humidity of 65–70% and artificial light–dark cycle of 12 h (8:00–20:00 h light/20:00–8:00 h dark). All the procedures complied with the European Communities Council Directive 2010/63/UE and RD 53/2013, concerning the protection of animals used for experimental and other scientific purposes. All efforts were made to minimize the number of animals used and their suffering. The rats were randomly assigned to four groups: control group (CO: 731.8 ± 16.29 g; $n = 10$), spatial learning group (SPL: 704.4 ± 18.23 g; $n = 10$), environmental enrichment group (EE: 795.1 ± 10.96 g; $n = 9$) and environmental enrichment + spatial learning group (EE + SPL: 689.1 ± 14.11 g; $n = 9$). The weights were not significantly different ($p = 0.21$). The rats were kept in their respective experimental conditions until behavioral experiments were carried out two months later. All the rodents were handled daily for one week (10 min) to accustom them to human contact before the perfusion in the case of the CO and the EE groups, and before the testing in the SPL and EE + SPL groups. Finally, both EE exposure and behavioral tests were carried out during the light-on phase of the rats.

2.2. Housing conditions

Aged rats were housed in large cages of 100 × 95 × 54 cm for a period of two months, 3 h/day (10:00 am/13:00 pm). The stimulating objects were similar in both cages. The cages contained a variety of objects, such as toys, running wheels, ropes, plastic tubes of different diameters, platforms, wooden houses, odorous and sonorous objects and nesting materials. The toys were usually chewable and brightly colored. In each cage, we put a running wheel, allowing the rats to undertake voluntary exercise and it was constant during all the period of environmental enrichment, as well as a yellow platform on which we put some objects. The plastic tubes and the wooden houses allowed the rats to take cover and finally, we introduced odorous materials, such as pieces of orange, chocolate, scented soaps within small balls as well as sonorous objects, such as little bells and rattles. During the rest of the day, the rats were housed in groups (four or five rats per cage) in standard cages (55 × 20 × 34 cm) without stimulating objects. In the EE cage, the rats did not have available food. The configuration of the EE cages was changed once a week and the cages were cleaned twice a week to ensure the welfare of the animals. The CO group was kept in two standard cages, five rats in each cage, without any learning or environmental enrichment experience. The SPL group was kept in the same conditions as the CO group, but with learning experience (Sampedro-Piquero et al., 2014).

2.3. Behavioral procedures

2.3.1. Elevated zero maze (EZM)

The enriched and non-enriched rats were assessed in the EZM in one session of 5 min (10:00 am/12:00 pm). This maze was made of black acrylic in a circular track 10 cm wide, 81 cm in diameter, and elevated 82 cm from the floor (Noldus Information Technology). It was divided into four sections of equal lengths, two open sections and two closed sections with black acrylic walls 35 cm in height. Behavioral measures registered included: (a) closed head dips (the number of times the rat looked over the edge of the maze while a portion of the body was in the closed sections); (b) open head dips (the number of times the rat looked over the edge of the maze while a portion of the body remained in the open sections); (c) duration in open sections; (d) entries into the open sections; (e) latency (the time before the first entry into the open section); (f) fecal boli; and (g) rearing. The movements of the rats were recorded with a camera connected to a computer running the EthoVision 3.1. software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA).

2.3.2. RAWM

The day before the spatial memory task, the rats received a habituation session in which they were given three trials with the platform situated in different starting positions in a small square water tank (47 × 75 × 38 cm). The SPL and EE + SPL groups were trained in a black fiberglass RAWM (10:00 am/13:00 pm) (each arm measuring 80 × 12 cm) that was placed 50 cm above the floor level. The maze had four arms in the shape of a cross. The maze was filled with tap water to a height of 32 cm and a black escape platform was placed 2 cm beneath the surface of the water. The water temperature was kept at 22 ± 1 °C during the task period. The maze was placed in a room with dimmed lights, and there were several extra-maze cues on the walls that the rats could use to navigate. The RAWM testing was performed for four days, six trials/day with 30 s of inter-trial interval. At the beginning of each trial, the rat was immersed in the water, facing the wall, at one of three start positions. The start locations were randomized and the platform was kept in the same arm throughout the entire task (Arm A), and the animals were never released into the water from that arm. Each rat was allowed 60 s to reach the platform and if the rat failed, it was guided to the platform. Once the rat reached the platform, it remained there for 15 s. Between trials, the animal was placed in a small square tank for 30 s. At the end of each day of training, the rats were dried and returned to their home cage. Each trial was recorded and analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands). Latency, distance traveled, reference and working memory errors, as well as the velocity of each day of training were registered.

2.4. Tissue preparations

The day after the RAWM task, the animals were deeply anesthetized (Ketamine 0.4 ml and Xylazine 0.2 ml) and perfused transcardially with 0.9% saline (5 min), followed by 4% paraformaldehyde phosphate-buffer during 30 min (0.1 M; pH 7.4). The brains were postfixed with paraformaldehyde at 4% (0.1 M; pH 7.4) for 3 h, and then transferred into phosphate-buffer (0.1 M; pH 7.4) overnight. The brains were extracted

and introduced in paraffin. Systematic cuts were performed with a microtome (Leica, RM2135, Germany) at 20 μm and the sections were alternated for the two immunohistochemical staining (Synapsin I and GR).

2.5. Synapsin I immunohistochemistry

After deparaffinizing, the sections were permeabilized in Tris buffer saline (TBS) containing 0.1% Triton X-100, followed by a 30 min preincubation in 3% goat serum (Sigma, USA). After this blocking step, we carried out three washes with TBS containing 0.1% Triton X-100. Next, we applied the polyclonal primary antibody, rabbit anti-Synapsin I in 1% bovine serum (Cell Signaling Technology, Inc.) at 1:1600 dilution and the sections were incubated for 24 h at 4 °C. The next day, the sections were washed three times in TBS with Triton X-100, incubated in biotinylated secondary goat anti-rabbit IgG antibody (1:480 dilution; Pierce, USA) in 10% bovine serum for 30 min, and dipped three times in TBS with Triton X-100. The sections were then incubated with an avidin-biotin horseradish-peroxidase complex (Vectastain ABC-Ultrasensitive, EliteKit; Pierce, USA) for 30 min at room temperature, washed twice in TBS with Triton X-100, rinsed in TBS, and then, visualized with DAB (Sigma, USA). Finally, the sections were dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, USA).

2.6. GR immunohistochemistry

Tissue sections were washed three times with TBS (pH 7.4) containing 0.1% Triton X-100 (TBS-T). Endogenous peroxidase was quenched with 3% H_2O_2 in TBS for 30 min. After washing three times with TBS-T, the sections were incubated for 1 h at room temperature in TBS-T containing 10% serum (TBS-T-S, normal goat serum; S-1000, Vector Laboratories). Then, the sections were washed three times with TBS-T and incubated overnight at 4 °C with GR polyclonal rabbit antibody at 1:200 (M-20, Santa Cruz Biotechnology). On the second day, after three washes with TBS-T, the sections were incubated in a secondary antibody for 1 h at room temperature (1:480, biotinylated goat anti-rabbit antibody in TBS, Vector Laboratories). Then, the sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC Ultrasensitive, Elite Kit; Pierce, USA) for 1 h at room temperature, washed three times in TBS and then visualized with diaminobenzidine, as chromogen (DAB, Sigma, USA). Finally, the sections were dehydrated in ethanol, cleared in xylene and coverslipped with Entellan.

In both immunohistochemical techniques, we confirmed the specificity of the immunohistochemical pattern by omitting the primary antibody, resulting in the absence of immunolabeling. In the case of the EE + SPL group, we randomly selected six animals of the total of nine to carry out the immunohistochemical analysis, whereas in the rest of the groups, we used all the animals. The other three brains of the EE + SPL group we used for a different brain analysis that will be part of another experiment.

2.7. Quantitative analysis of Synapsin I and GR expression

The quantification of Synapsin I was done by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) made up of a high precision illuminator, a digital camera and a computer. The mean optical density (OD) for dorsal and ventral Cornu Ammonis 3 (dCA3 and vCA3) was calculated from at least five non-consecutive coronal sections on the right side of the bilateral structure. In each section, five non-overlapping readings were taken, using a square-shaped sampling window that was adjusted for dCA3 and vCA3 size. A total of 25 measurements were taken in dCA3 and vCA3 by a researcher blind to the groups. Background staining was controlled by calculating the average optical density levels from another part of the section without labeling (10 measures) and subtracting these values from the average measure of dCA3 and vCA3.

The quantification of immunopositive cells for GR was carried out in the dorsal (dorsal Cornu Ammonis (dCA1 and dCA3) and dorsal Dentate Gyrus (dDG)) and ventral hippocampus (ventral Cornu Ammonis (vCA1 and vCA3), ventral Dentate Gyrus (vDG) and dorsal and ventral subiculum (dSUB, vSUB)) using the program Leica suite application (Version 2.5.0 R1, Leica Microsystems CMS, Switzerland). Both in the dorsal and ventral hippocampi, we analyzed six non-consecutive sections per animal.

Results show immunopositive cells/area (1000 μm^2). We also calculated the coefficient of error (CE) and the coefficient of variation (CV) of the number of GR immunopositive cells quantified to assess the accuracy of our sampling method. The CE expresses the accuracy of the estimated cell number. Theoretically, the ratio of CE^2/CV^2 should not be higher than 0.5 (Diniz et al., 2010; Gundersen and Jensen, 1992; Slomianka and West, 2005).

The dorsal and ventral hippocampi were anatomically defined according to Paxinos and Watson's atlas (2005), −3.84 mm and −5.80 mm respectively.

2.8. Statistical analysis

Behavioral and brain data were analyzed with SPSS 19.0 (SPSS Inc., Chicago, USA) and were expressed as mean ± SEM. The results were considered statistically significant if $p < 0.05$ and they were represented graphically with SigmaPlot 8.0 (Systat, Richmond, EEUU). On the one hand, to assess the differences between groups in the EZM test, Student's *t*-test for independent samples was carried out. With regard to the RAWM data, we applied an ANOVA of repeated measures (RM ANOVA) (within-factor: days; between-factor: groups). Next, an appropriate post hoc test was used when we found significant differences (pairwise comparisons with Bonferroni adjustment). On the other hand, we used a two-way ANOVA, in which housing condition (standard/enriched) and behavioral testing (no behavioral testing/behavioral testing) were the two factors with two levels each, to analyze possible differences in the Synapsin I density and the GR expression in the dorsal and ventral hippocampi. Appropriate post hoc comparisons were conducted when significant differences were found (Bonferroni test).

3. Results

3.1. Behavioral results

3.1.1. EZM

The *t*-test for independent samples revealed significant differences between groups in the entries into open sections ($T_{17} = 10.25$, $p = 0.003$), the latency to enter into the open section ($T_{17} = 14.12$, $p = 0.001$) and the levels of rearing ($T_{17} = 18.38$, $p = 0.001$). The enriched group showed shorter latency and more entries into the open sections, as well as higher levels of rearing. In contrast, we did not find significant differences between groups in the closed and open head dips ($p = 0.48$ and $p = 0.32$ respectively), in the time spent in the open section ($p = 0.12$) and in the fecal boli

Table 1
Mean values of the activity in the elevated zero-maze for each group.

	Enriched	Standard
Closed head dips	6.22 ± 0.54	5.54 ± 0.84
Open head dips	0.61 ± 0.23	0.27 ± 0.19
Duration in open sections (s)	46.11 ± 5.05	22.81 ± 16.69
Entries into open sections	3.44 ± 0.54*	0.81 ± 0.55
Latency (s)	8 ± 1.63*	226 ± 38.26
Fecal boli	0.67 ± 0.23	0.90 ± 0.64
Rearing	5.89 ± 0.88*	0.91 ± 0.31

Data represent mean ± SEM values.
* $p < 0.05$ significant differences.

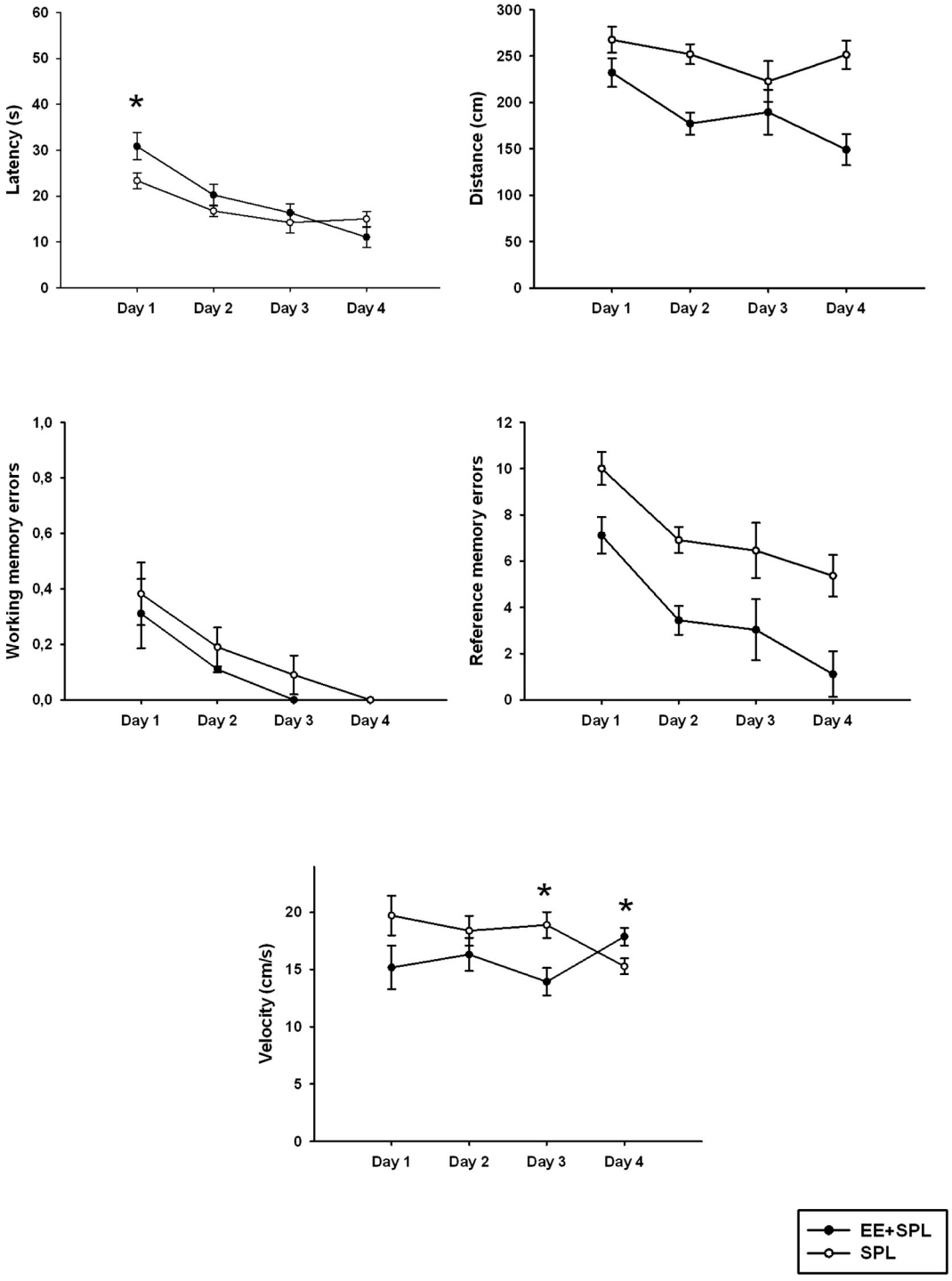


Fig. 1. Behavioral performance in the RAWM (mean ± SEM). The factor groups were significant showing that the EE + SPL group made fewer reference memory errors and traveled shorter distance than the SPL group. In the variables latency and velocity, the interaction groups x days was significant, showing that the EE + SPL had higher latency to find the platform on the first day of training and showed higher velocity on the fourth day. In contrast, the EE + SPL group had lower velocity on the third day of training compared with the SPL group (*$p < 0.05$).

levels ($p = 0.43$). Table 1 shows the average value for each group in these variables.

3.1.2. RAWM

3.1.2.1. Latency. The latency varied as the days progressed ($F_{3,54} = 23.27$, $p = 0.001$). The factor groups was not significant ($F_{1,17} = 1.53$, $p = 0.23$), but the interaction days x groups showed significant differences between groups ($F_{3,54} = 3.44$, $p = 0.023$). We analyzed the interaction with pairwise comparisons with Bonferroni adjustment and we found that the EE + SPL group had higher latency to find the platform compared with the SPL group on the first day of learning ($p = 0.03$) (Fig. 1).

3.1.2.2. Distance traveled. The distance traveled varied as the days went by ($F_{3,54} = 3.62$, $p = 0.02$). The factor groups was significant ($F_{1,17} = 23.52$, $p = 0.001$), showing that the EE + SPL group traveled a shorter distance to find the platform compared to the SPL group. The interaction days x groups was not significant ($F_{3,54} = 2.03$, $p = 0.12$) (Fig. 1).

3.1.2.3. Working memory and reference memory errors. Working memory errors and reference memory errors varied as the days went by ($F_{3,54} = 5.81$, $p = 0.001$, $F_{3,54} = 3.31$, $p = 0.001$ respectively). Regarding the working memory errors, the factor groups and the interaction days x groups were not significant ($F_{1,17} = 1.59$, $p = 0.23$, $F_{1,17} = 1.35$, $p = 0.27$ respectively). Finally, we found that the factor groups were significant in the reference memory errors ($F_{1,17} = 11.88$, $p = 0.003$), showing that the EE + SPL group made fewer reference memory errors. The interaction days x groups was not significant ($F_{1,17} = 1.45$, $p = 0.24$) (Fig. 1).

3.1.2.4. Velocity. The velocity during the task did not vary as the days progressed ($F_{3,54} = 0.31$, $p = 0.82$). The factor groups and the interaction days x groups were significant ($F_{1,17} = 5.70$, $p = 0.03$, $F_{1,17} = 3.34$, $p = 0.03$ respectively). We analyzed the interaction with pairwise comparisons with Bonferroni adjustment and we found that the EE + SPL group had lower velocity on the third day ($p = 0.001$), and higher on the last day of training ($p = 0.02$) (Fig. 1).

3.2. Synapsin I

3.2.1. Dorsal CA3

The two-way ANOVA revealed that the factors housing condition and behavioral testing were significant ($F_{1,33} = 27.70$, $p = 0.001$, $F_{1,33} = 15.56$, $p = 0.001$ respectively), but not the interaction between them ($F_{1,33} = 2.18$, $p = 0.15$) (Fig. 2A).

3.2.2. Ventral CA3

The two-way ANOVA showed that the factor housing condition reached statistical significance ($F_{1,33} = 22.58$, $p = 0.001$), but not the factor behavioral testing ($F_{1,33} = 2.79$, $p = 0.12$) and their interaction ($F_{1,33} = 1.06$, $p = 0.91$) (Fig. 2B).

Fig. 3 shows the effect of the different experimental conditions on the Synapsin I protein expression.

3.3. GR

3.3.1. Dorsal hippocampus

The two-way ANOVA revealed that the factor housing condition was not significant in any of the dorsal hippocampal subfields studied (dCA1, $F_{1,37} = 0.18$, $p = 0.67$; dCA3, $F_{1,37} = 2.15$, $p = 0.18$; dDG, $F_{1,37} = 2.79$, $p = 0.10$). With regard to the behavioral testing factor, it was significant only in the dDG ($F_{1,37} = 71.62$, $p = 0.001$). Finally, their interaction was significant in the dCA1 ($F_{1,37} = 13.98$, $p = 0.001$) and dCA3 ($F_{1,37} = 4.13$, $p = 0.02$) (Fig. 4A).

3.3.2. Ventral hippocampus

The two-way ANOVA showed that the factor housing condition was significant in the vSUB ($F_{1,37} = 118.07$, $p = 0.001$) and vDG ($F_{1,37} = 45.03$, $p = 0.001$). The factor behavioral testing did not reach statistical significance in any subfield studied (vSUB, $F_{1,37} = 1.36$, $p = 0.15$; vDG, $F_{1,37} = 2.21$, $p = 0.17$). Finally, their interaction was significant in the vCA1 ($F_{1,37} = 17.58$, $p = 0.001$), vCA3 ($F_{1,37} = 43.03$, $p = 0.001$) and dSUB ($F_{1,37} = 5.55$, $p = 0.02$), showing that the effect of the behavioral testing on GR expression in these hippocampal subfields depended on the housing condition factor (Fig. 4B).

Fig. 5 shows representative photomicrograph of coronal section showing GR immunopositive cells in the dorsal and ventral hippocampi. Table 2 shows the estimation of the ratio CE^2/CV^2 in the different groups.

4. Discussion

Taking into account our results, we suggest that a previous EE experience, in contrast to a standard housing condition during aging, is a good behavioral intervention to promote better spatial memory performance and a greater control of anxious responses to cope with novel and stressful situations. Thus, we found that enriched rats performed better in the RAWM and showed a reduction of anxiety-related behaviors during the EZM test. This conclusion is supported on the one hand, by the distance traveled and the reference memory errors committed during the RAWM, in which the EE + SPL traveled shorter distance and made fewer reference memory errors than the SPL group and on the other hand, by the short latency of aged enriched rats to enter the open section of the EZM. This last result suggested a reduced emotional reactivity to the conflict approach/avoidance and an improvement in decision taking. Also, the high levels of rearing in this group may have led to better exploration and a faster habituation to the new environment, reducing their anxiety levels when it became familiar. Evidence supports the view that EE attenuates responses to certain anxiety provoking situations and increases exploration time and the search of unfamiliar stimuli (Fox et al., 2006).

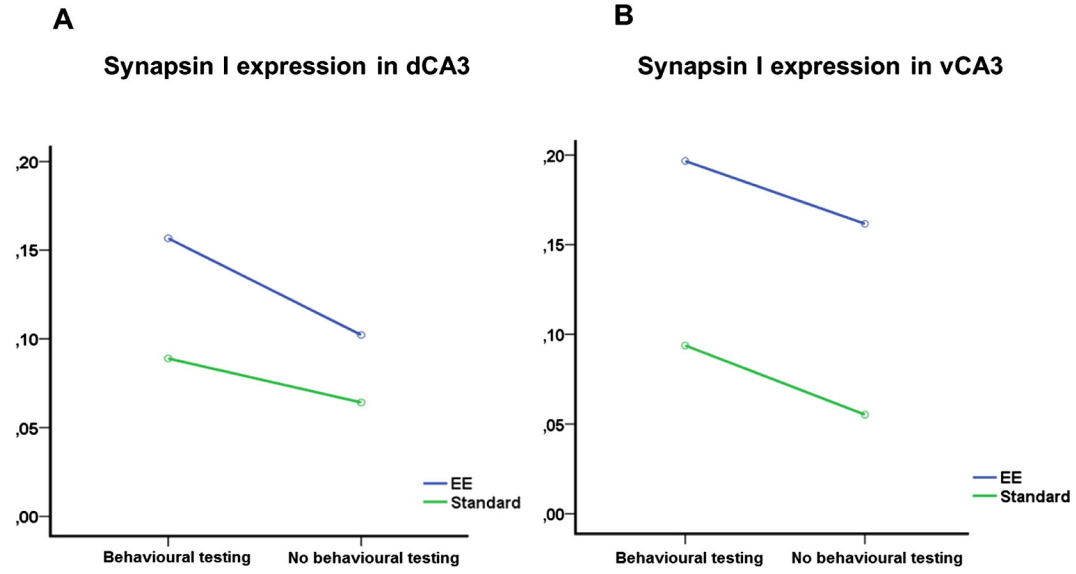
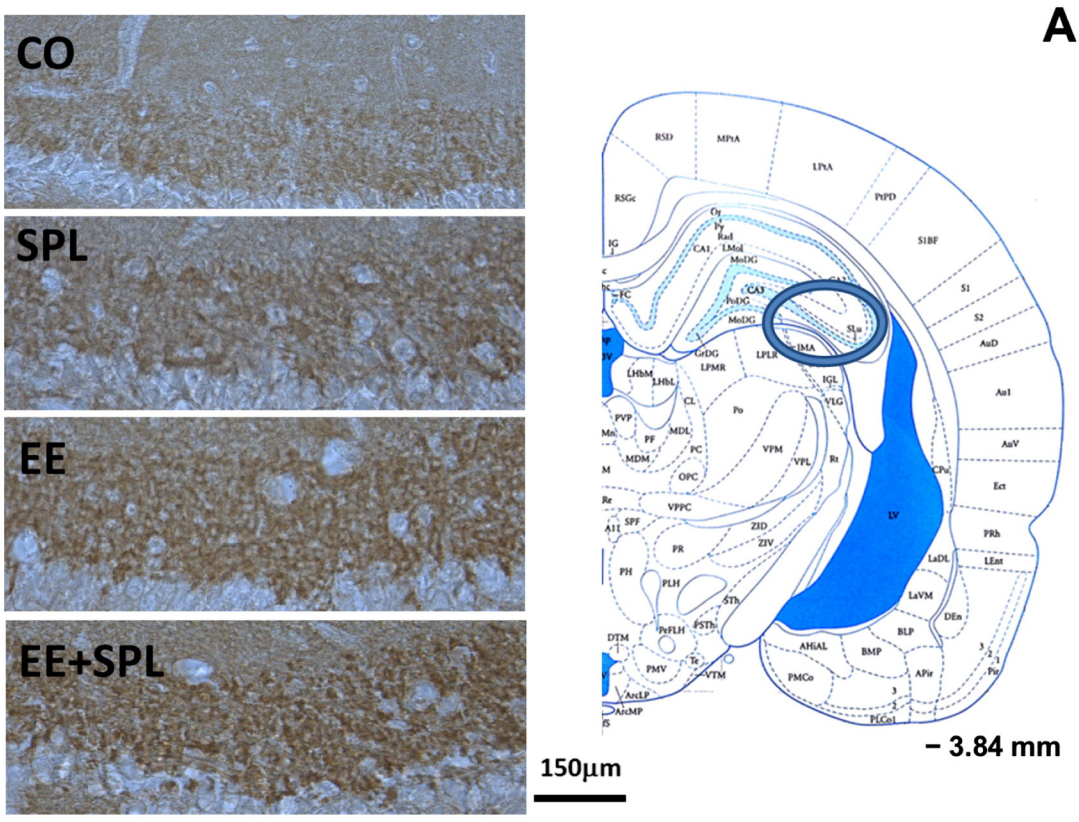


Fig. 2. (A) The Synapsin I density in the dCA3 depended on the housing condition and the behavioral testing factors, ($p = 0.001$), but not the interaction between them ($p = 0.15$). (B) The Synapsin I density in the vCA3 depended on the type of housing condition ($p = 0.001$), but not the behavioral testing factor ($p = 0.12$) and their interaction ($p = 0.91$) (Fig. 2B).



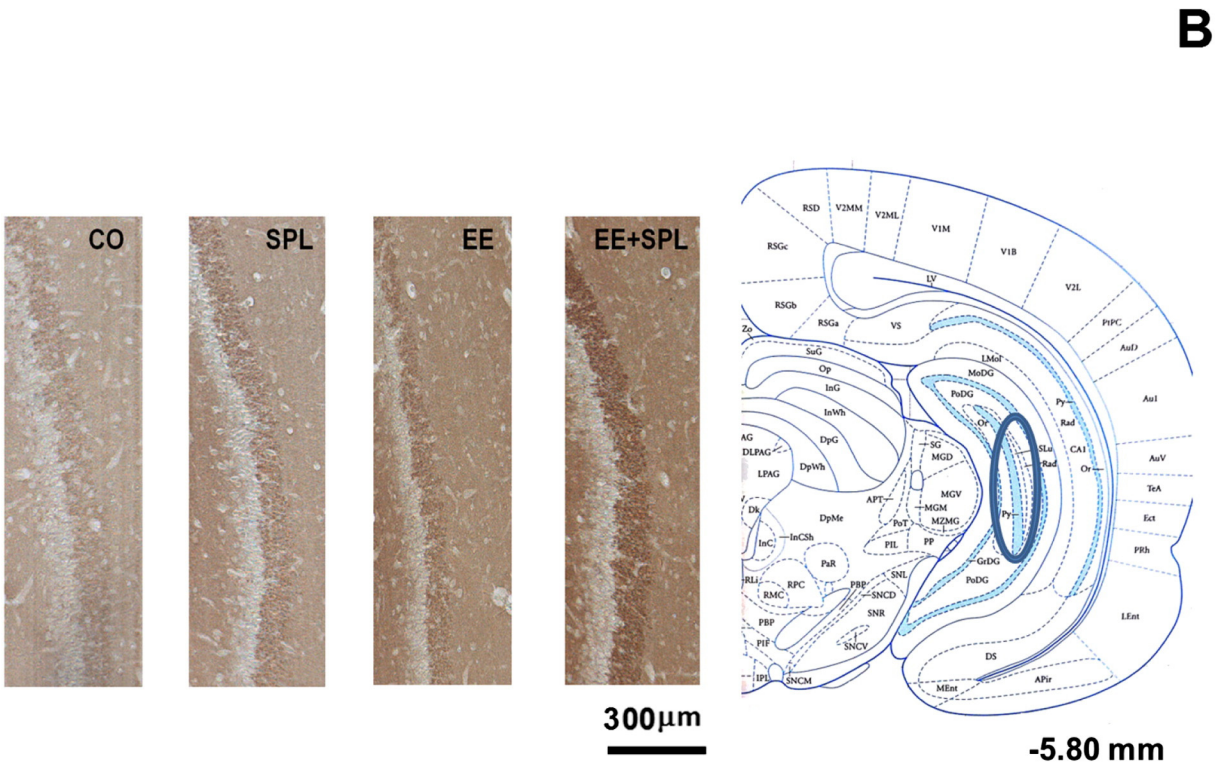


Fig. 3. Representative photomicrographs of Synapsin I density in the dCA3 (A) and the vCA3 (B). The effect of the different experimental conditions on the Synapsin I protein expression is shown. The quantification was done by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England). The photomicrographs of Fig. 4A were made at ×20 objective and in the case of Fig. 4B at ×10 objective.

Our finding that spatial memory is improved by EE in aged rats is consistent with previous studies (Baraldi et al., 2013; Frick and Fernandez, 2003; Sampedro-Piquero et al., 2013). For example, among aged rodents, EE has shown to improve the learning of the Morris water maze (Frick et al., 2003), Hebb-Williams maze (Cummins et al., 1973), incidental learning (Warren et al., 1982) and reverses short-

term memory deficits in a Symbolic Delayed Matching to Sample Task (Soffié et al., 1999). However, we must take into account that the anxiety reduction found in the enriched rats could have been mediating in the better spatial memory performance. In fact, Harris et al. (2009) have concluded that the cognitive benefits of EE occur because enriched rats are less anxious during cognitive testing. Most of the tasks used to assess spatial learning and memory in rodents can be considered stressful, and in the case of the RAWM, although the rats are excellent swimmers, they become stressed when they are submitted to demanding water-spatial memory tasks (Hawley et al., 2012). Interestingly, a recent human research has revealed that when the stressful features of certain testing environments are controlled, making them more appropriate for older people, the cognitive performance in this age group improves (Sindi et al., 2013).

Regarding the neurobiological data, we found that the expression of Synapsin I and GR induced by behavioral testing was differentially modulated in enriched and standard aged rats in some subfields of the dorsal and ventral hippocampi. On the one hand, Synapsin I expression was enhanced in the dCA3, but not in the vCA3, when the rats performed the testing regardless of the previous housing condition. The dorsal hippocampus has a long-established role in certain forms of memory, above all in spatial memory (Bannerman et al., 2004; Potvin et al., 2007), so it was not unusual that the mere performance of the spatial memory task was enough to produce an increase of this presynaptic protein involved in the LTP process. Surprisingly, the EE, regardless if the rats were trained or not, was able to increase the density of Synapsin I in the vCA3, so, it is possible that EE promotes a brain reserve to face subsequent demands, as well as brain lesions. Other studies have also suggested this role of EE in providing a cognitive and brain reserve allowing an efficient use of existing neuronal networks and recruit alternative circuits when required (Nithianantharajah and Hannan, 2006). In fact, enriched rats show a greater ability to adapt and cope with highly demanding situations that have to be solved using complex strategies (Leggio et al., 2005; Petrosini et al., 2009). Human research has revealed that the involvement in activities such as education, aerobic exercise, occupational experiences, leisure activities or mental challenges during aging, also promotes the formation of a brain and cognitive reserve that even could compensate brain disorders, such as dementia (Scarmeas and Stern, 2003).

We focused the Synapsin I analysis in the CA3 because this hippocampal subfield has shown to be important for memory retention (Rolls, 2013), which is probably enhanced by changes, such as neurogenesis, synaptogenesis and LTP. These aspects of the hippocampal functioning have shown to be induced by EE and related with better spatial memory performance (Artola et al., 2006; Duffy et al., 2001; Foster and Dumas, 2001; Foster et al., 1996), so it is likely that the increase of Synapsin I after EE in the vCA3 was a consequence of a higher complexity of CA3 pyramidal neurons promoting higher levels of neurotransmission, and therefore, LTP. Interestingly, recent studies have found that the activation levels of the vCA3 during a spatial memory task were similar to the dorsal part, in contrast to the traditional role of the ventral hippocampus only in emotional functions (Beer et al., 2014).

On the other hand, the behavioral testing increased the GR levels in the dDG of both standard and enriched rats, whereas in the dCA1 and dCA3, the GR expression induced by behavioral testing depended on the previous EE or standard condition experience. Recent studies have shown that GR activation under adaptive levels of stress, may be promoted by EE, leads to strong and long-lasting memories (Finsterwald and Alberini, 2014). For example, GR directly affects hippocampal functions, thus modulating the consolidation of several types of hippocampal-dependent memories, including spatial and contextual

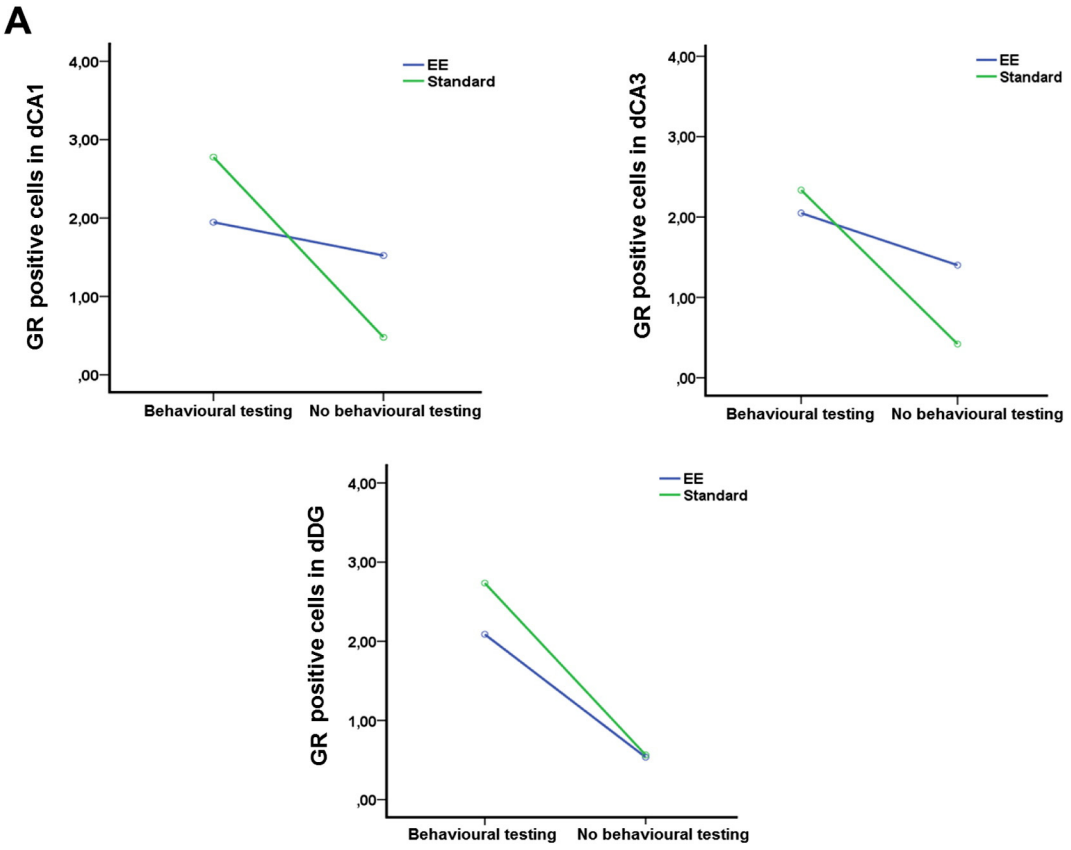


Fig. 4. (A) The GR expression in the dDG increased with the behavioral testing in both enriched and standard rats ($p = 0.001$). In the case of dCA1 and dCA3, the GR expression induced by behavioral testing depended on the previous housing condition ($p = 0.001$, $p = 0.02$ respectively). (B) The GR expression in the vDG and vSUB was enhanced by only the EE experience ($p = 0.001$), whereas in the vCA1, vCA3 and dSUB, the expression of GR induced by behavioral testing factor was modulated by EE factor ($p = 0.001$, $p = 0.001$, $p = 0.02$ respectively).

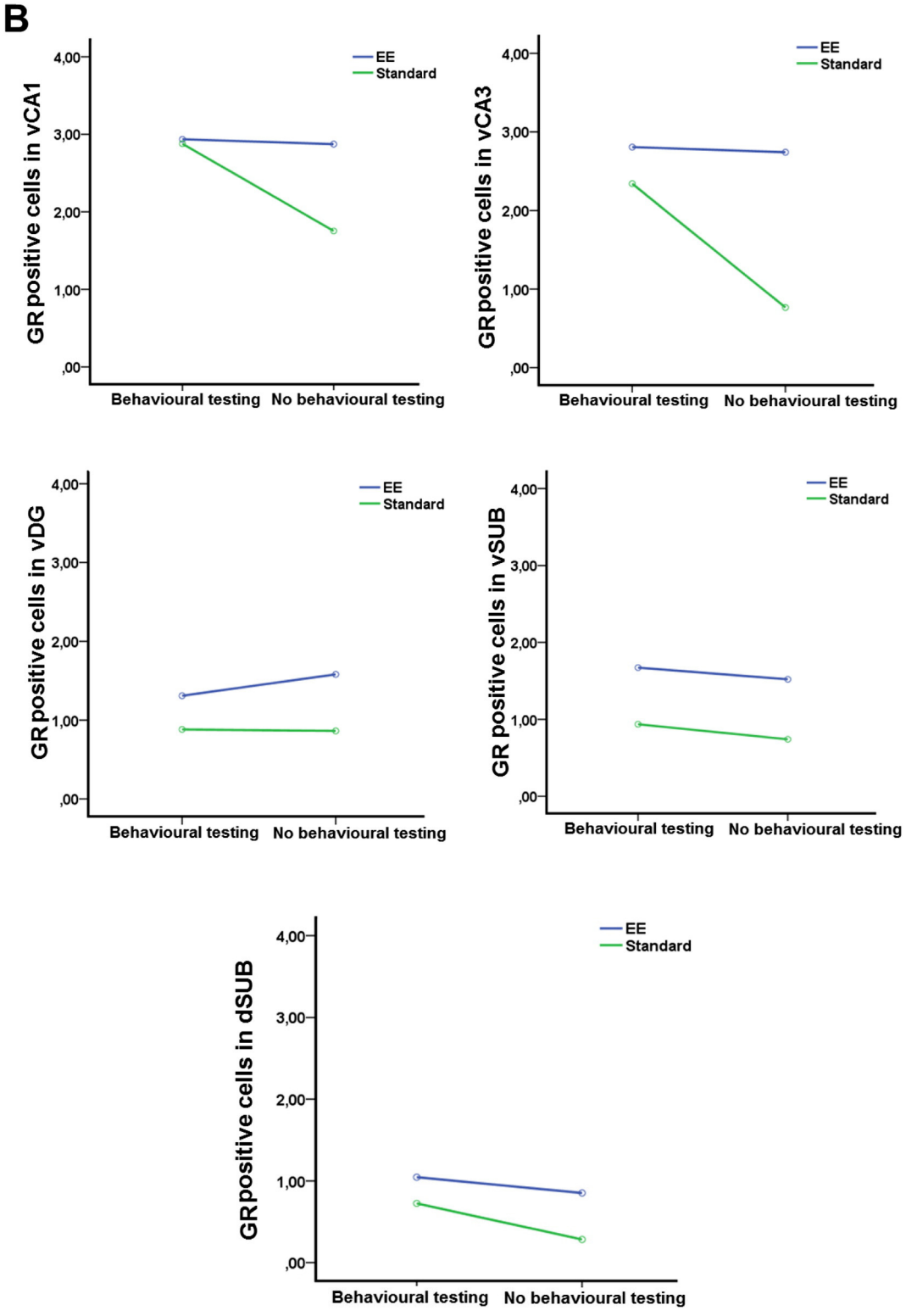


Fig. 4 (*continued*).

memories in rodents and declarative memories in humans (Donley et al., 2005; Eichenbaum, 2000; Gabrieli, 1998; Kim and Diamond, 2002; Roozendaal, 2000; Squire, 2004). Besides, it has been demonstrated that, during learning, GR regulates several intracellular signaling pathways required for memory consolidation and activated by CREB, mitogen-activated protein kinase (MAPK), calcium/calmodulin-dependent protein kinase II (CaMK II), and brain-derived neurotrophic factor (BDNF) (Chen et al., 2012; Finsterwald and Alberini, 2014) which is also known by modulating the Synapsin I phosphorylation (Jovanovic et al., 2000). For example, the MAPK activity induced by GR has shown to enhance the expression of Egr1 which in turn activates Synapsin-I increasing the probability of glutamate release and hypothetically facilitating information processing and memory (Revest et al., 2010; Sandi, 2011).

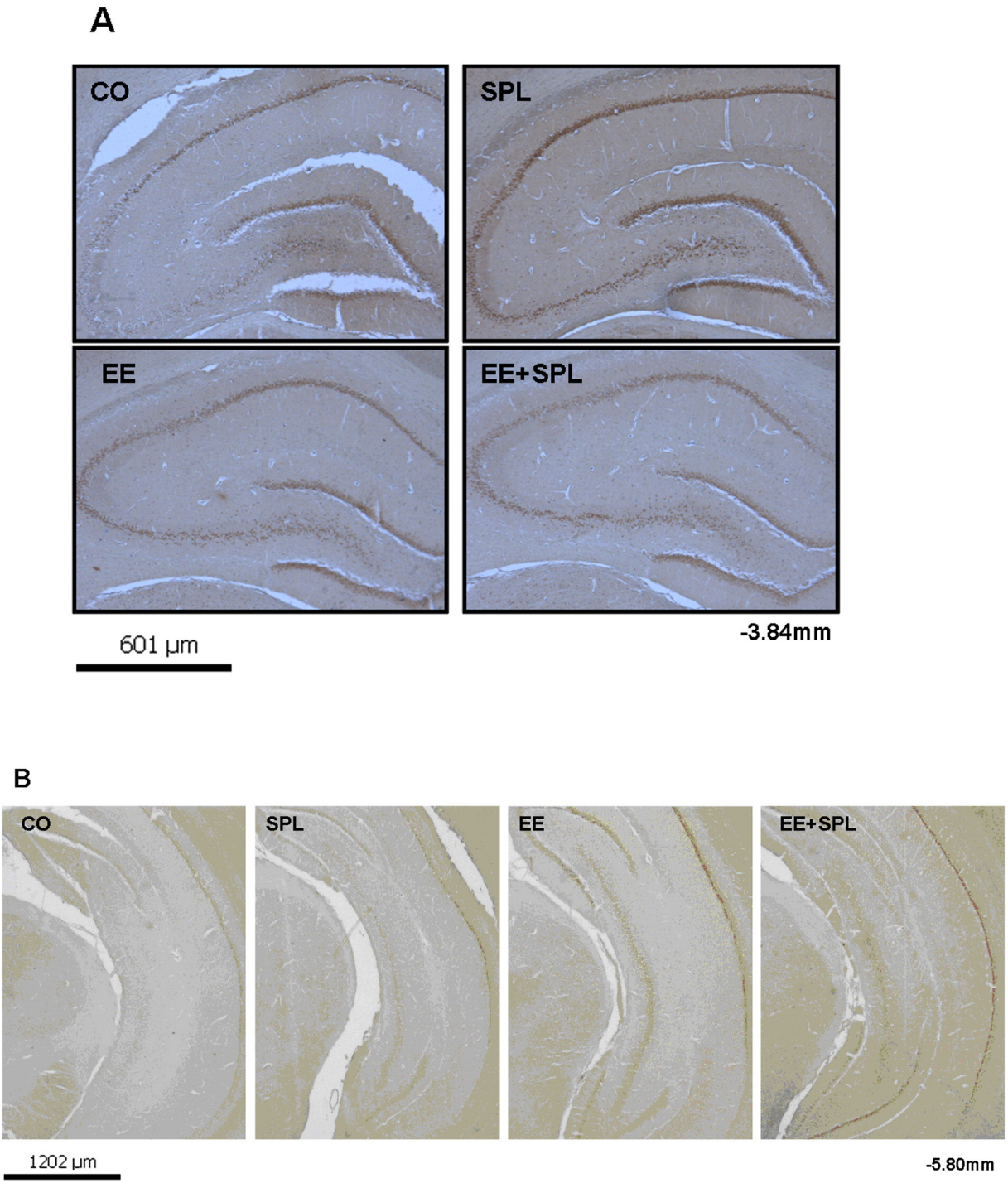


Fig. 5. Representative photomicrographs of coronal sections showing GR immunopositive cells in the dorsal (A) and ventral hippocampi (B) in each experimental condition. The quantification of GR immunopositive cells was made at ×10 objective, but the photomicrographs were made at ×5 objective (A) and ×2.5 objective (B). (Leica suite application, Version 2.5.0 R1, Leica Microsystems CMS, Switzerland).

Noteworthy results were found when we analyzed the GR expression in the ventral region, where the effect of the behavioral testing on this neurobiological mechanism depended on the previous submission to EE in the vCA1, vCA3 and dSUB and even, the EE condition, regardless of the behavioral testing factor, enhanced GR levels in the vDG and vSUB. In this case, it seems to suggest that the ventral hippocampus is more sensitive to the effects of EE, as the Synapsin I results had seemed to suggest. In the case of the vSUB, despite the fact that it has been scarcely studied, exerts a dynamic and inhibitory influence on the HPA axis and the activity of the vDG reduces the innate anxiety-like behavior (Kheirbek et al., 2013; O'Mara et al., 2001). The role of the vCA3 is more unknown, although there is evidence that relates this subfield with the process of memory formation under emotional and stressful situations (Fa et al., 2014).

Nevertheless, we must take into consideration that EE is a multifactorial stimulation program that combines cognitive, social, physical and sensory stimulation, so a lot of variables could have mediated our results and interacted with our observed changes in the Synapsin I and GR expression. For example, EE has shown to enhance the neurogenesis, astrocytic plasticity, as well as neurotrophin levels in the hippocampus

Table 2
Estimation of the ratio CE^2/CV^2 in the different groups.

Dorsal hippocampus					
	dCA1		dCA3		dDG
CO	0.15		0.12		0.06
SPL	0.13		0.02		0.07
EE	0.07		0.05		0.05
EE + SPL	0.04		0.09		0.07
Ventral hippocampus					
	vCA1	vCA3	vDG	vSUB	dSUB
CO	0.06	0.05	0.05	0.10	0.18
SPL	0.06	0.08	0.05	0.07	0.08
EE	0.19	0.20	0.05	0.10	0.29
EE + SPL	0.18	0.10	0.09	0.06	0.05

(Leal-Galicia et al., 2008; Pham et al., 2002; Sampedro-Piquero et al., 2014), although its effects have shown to be not exclusive of this brain region (Harati et al., 2011; Segovia et al., 2008). Besides, the variability between the EE studies (time of onset, duration, objects, type of control group) can provide different results (Simpson and Kelly, 2011).

To sum up, our results seem to suggest that EE is a good intervention to improve the spatial memory performance and reduce the anxiety-related behaviors in aged Wistar rats. In connection with our neurobiological results, we found that in some hippocampal subfields, the GR expression induced by behavioral testing varied depending on the previous EE or standard housing experience, whereas in some ventral subfields EE was enough to increase the GR levels regardless of the rats had been trained or not in the behavioral tasks. On the other hand, Synapsin I expression was enhanced in the dCA3, but not in the vCA3, when the rats performed the behavioral testing both in enriched and standard rats, whereas again the ventral region was more sensitive to the effects of EE. In the future, the knowledge about the brain response to mental, social, sensorial and physical stimulation will permit the designing of therapies for neurocognitive rehabilitation during aging. Moreover, it would be interesting to carry out the analysis of GR and Synapsin I expression, not only at the level of the septal part of the dorsal hippocampus, but also at more posterior levels.

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DISCUSIÓN GENERAL

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Los objetivos generales de esta Tesis doctoral fueron, por un lado, estudiar si la edad a la que se inicia un protocolo de EAM puede ser un factor que influya en sus efectos sobre la conducta y cognición espacial y, por otro lado, analizar el impacto de este programa de estimulación sobre diferentes mecanismos neurobiológicos que subyacen a los procesos de aprendizaje y memoria y que han mostrado estar alterados durante el proceso de envejecimiento.

A continuación, se describirán y discutirán los resultados obtenidos de acuerdo a los objetivos planteados.

Primer objetivo: *Estudiar la conducta de ratas Wistar tras un programa de EAM y de aquellas que vivieron en condiciones estándar de laboratorio.*

a) Estudiar el efecto sobre los niveles basales de ansiedad y de exploración en el laberinto elevado en zero y si este es dependiente de la edad.

b) Estudiar el impacto de este programa de estimulación sobre la memoria de referencia espacial en el laberinto radial acuático y si su efecto es dependiente de la edad.

En cuanto a este primer objetivo, los resultados mostraron que nuestro protocolo de EAM (3h/día, 2 meses) produjo una reducción de respuestas ansiosas cuando los animales, tanto de 5 como de 20, fueron expuestos a un ambiente nuevo, impredecible y estresante en comparación con aquellos otros de su misma edad mantenidos en condiciones estándar. Por otro lado, también observamos que este protocolo de estimulación consiguió mejorar el rendimiento de los animales, independientemente de su edad, en una prueba

de memoria de referencia espacial. Además, nuestros datos parecen indicar que las ratas Wistar que vivieron durante 3h diarias en condiciones de EAM ejecutaron la prueba de memoria espacial empleando una estrategia mucho más precisa que les permitía alcanzar la plataforma oculta cometiendo menos errores.

Primer objetivo (a):

Para la evaluación de los niveles de ansiedad utilizamos el *laberinto elevado en zero* ya que, además de ser una prueba que no requiere un entrenamiento previo, al basarse en la aversión natural de los roedores a los ambientes nuevos (neofobia), ofrece varias ventajas frente al clásico *laberinto elevado en cruz* al eliminar la zona central de este y permitir una exploración ininterrumpida (Braun y cols., 2011; Cook y cols., 2002; Heredia y cols., 2012). El tiempo que los animales permanecen en el área abierta es la variable más empleada como reflejo de los niveles de ansiedad, considerándose que un tiempo mayor es indicativo de niveles reducidos. Del mismo modo, otras variables como el tiempo que tarda el animal en salir por primera vez al área abierta (latencia), el número de veces que asoma su cabeza a este área (*head dip*), o la cantidad de bolas fecales como respuesta a la activación del sistema nervioso autónomo, son empleadas también como índices de ansiedad (a menos latencia menos ansiedad, a más *head dips* menos ansiedad, a más bolas fecales más ansiedad). No obstante, uno de los principales inconvenientes de este tipo de pruebas es que no permiten discriminar entre los niveles de exploración y los de ansiedad de forma independiente y, aunque algunos autores sugieren que un alto grado de exploración es indicativo de bajos niveles de ansiedad (Larsson y cols., 2002), nosotros optamos por

considerar estos parámetros independientes. De esta forma, y como complementarias a las variables anteriores, se decidió incluir otras medidas específicas de exploración como el número de entradas en cada una de las áreas (abiertas y cerradas), la frecuencia de *rearing* o la distancia recorrida. En el caso de las ratas de 5 meses, debido a sus mayores niveles de actividad en comparación con los roedores viejos, calculamos el tiempo de permanencia en el área abierta por cada una de las entradas realizadas (variable: *tiempo por entrada*), con el fin de ajustar la influencia de los altos niveles de exploración sobre el tiempo de permanencia en el área abierta (Heredia y cols., 2012).

Los resultados mostraron que nuestro protocolo de EAM redujo las conductas ansiosas y aumentó los niveles de exploración en ambos grupos de edad. Así, los animales enriquecidos de 5 meses mostraron una menor latencia para entrar por primera vez al área abierta, mayor tiempo de permanencia en ella así como, más *head dips* y menor defecación durante la prueba. La variable *tiempo por entrada*, la cual nos ofrece, como ya se comentó previamente, información sobre el nivel de ansiedad del animal corregido el efecto de la exploración, también apuntó a un efecto ansiolítico del EAM. En cuanto a los niveles de exploración, las ratas enriquecidas recorrieron una distancia mayor, entrando más veces en las áreas abiertas y realizando más conductas de *rearing.* Respecto a las ratas de 20 meses expuestas a EAM, estas también mostraron una menor latencia para entrar por primera vez al área abierta y, en relación a los niveles de exploración, presentaron una mayor frecuencia de *rearing* así como, más entradas a las zonas abiertas. En este grupo no calculamos la variable *tiempo por entrada* al no haber encontrado diferencias significativas en el tiempo total de permanencia en el área abierta.

De acuerdo a nuestros resultados, varios estudios han mostrado que el EAM produce un efecto ansiolítico cuando los animales son expuestos a estresores psicogénicos, como es el caso de un ambiente nuevo (Fox y cols., 2006). Este efecto es fácilmente comprensible si tenemos en cuenta que la exposición a la novedad es una de las características inherentes del propio protocolo de EAM (Van de Weerd y cols., 2002). El cambio constante de los estímulos es considerado un mecanismo de inoculación de estrés, puesto que la repetida introducción de objetos nuevos es comparable a la exposición frecuente a estresores de carácter leve (Larsson y cols., 2002). Sin embargo, otros autores sugieren que el EAM es capaz de reducir los niveles de ansiedad al aumentar el sentido de control que los animales tienen sobre el ambiente (Larsson y cols., 2002; Roy y cols., 2001). También, el hecho de que los animales sean emocionalmente menos reactivos en situaciones nuevas les podría permitir explorar el ambiente más eficientemente y, de este modo, conseguir una habituación más rápida y una reducción de los niveles de ansiedad. En nuestro caso, también hemos observado que conductas relacionadas con la exploración del laberinto aumentaron en los grupos enriquecidos de ambas edades. Incluso, aunque la exposición a EAM sea restringida a períodos cortos diarios, como es en nuestro caso, también se ha observado que es capaz de aumentar la frecuencia y duración de las conductas exploratorias (Widman y cols., 1992; Widman y Rosellini, 1990).

Es interesante destacar también que una excesiva estimulación ambiental puede producir un aumento de ansiedad en ciertas cepas de roedores, sugiriéndose que los efectos del EAM sobre la respuesta de ansiedad puede que sigan la forma de *U* invertida, siendo necesario controlar

variables como el grado de exposición o la complejidad del ambiente (Huff y cols., 2003; Van de Weerd y cols., 1994). En relación a esto, se ha observado que pocos elementos son necesarios en el EAM para obtener resultados positivos sobre la respuesta de ansiedad (Brillaud y cols., 2005).

Primer objetivo (b):

Para la evaluación de la memoria espacial empleamos el laberinto radial acuático. Una de las ventajas que nos ofrece, frente a otras pruebas, es que nos permite evaluar de forma simultánea la memoria de trabajo (recuerdo del brazo incorrecto visitado previamente) y de referencia (recuerdo de los brazos que no contienen la plataforma). Otra ventaja es que no necesitamos privar de alimento al animal, siendo ésta una variable que afecta al funcionamiento cerebral así como, a los niveles de ansiedad de los roedores. Con este laberinto evitamos también la conducta de tigmotaxis, típica de los primeros ensayos en el laberinto de Morris, y que puede generar fatiga en los animales.

Con respecto a los resultados obtenidos observamos que las ratas que fueron expuestas a EAM, independientemente de su edad, tuvieron un mejor rendimiento que los grupos estabulados en condiciones estándar al mostrar una menor latencia para alcanzar la plataforma y al recorrer una menor distancia en el primer ensayo de cada sesión de aprendizaje. Esta última variable, junto con la latencia del primer ensayo de cada día de entrenamiento, en la que no encontramos diferencias significativas entre grupos, han sido incluidas con el objetivo de valorar la consolidación de la memoria espacial, altamente sensible al proceso del envejecimiento (Kumar y cols., 2012).

Además, nuestros resultados mostraron que el que los animales enriquecidos alcanzasen antes la plataforma no fue resultado de una mayor

velocidad durante la prueba y, por tanto, de una posible mejora en sus capacidades físicas debido al mayor espacio disponible en la jaula de EAM, ya que tanto las ratas de 5 como de 20 meses que recibieron EAM mostraron una menor velocidad comparadas con sus respectivos grupos control. De este modo, en el caso de las ratas de 5 meses que fueron expuestas a EAM, la menor latencia para alcanzar la plataforma podría haber sido debida a la corta distancia recorrida durante todas las sesiones de entrenamiento en comparación con aquellas de su misma edad estabuladas en condiciones estándar. En nuestro primer experimento, no observamos que los roedores viejos recorriesen menos distancia que su grupo control, sin embargo, un análisis más exhaustivo de los datos conductuales, realizado en trabajos posteriores y en el que ya se tuvo en cuenta el número de errores así como, el tiempo en el centro del laberinto, nos permitió observar que las ratas de 20 meses que recibieron EAM mostraron un número menor de entradas en los brazos que no contenían la plataforma (errores de memoria de referencia) así como, una mayor permanencia en el centro del laberinto. Estos resultados parecen indicar que los animales viejos expuestos a EAM realizaron la prueba siguiendo una estrategia más precisa y eficiente, en la que nadaban hasta el centro del laberinto y en base a las pistas alocéntricas que lo rodeaban decidían a qué brazo entrar cometiendo así menos errores. Por el contrario, el grupo de 20 meses que no recibió EAM es probable que buscase la plataforma aleatoriamente cometiendo significativamente más errores de referencia.

Estudios previos que siguen la línea de nuestros resultados han encontrado con frecuencia que las ratas viejas tienen especial dificultad en aquellas pruebas espaciales que requieren una estrategia alocéntrica para su

ejecución (Begega y cols., 2012; Klencklen y cols., 2012; Moffat y cols., 2006). De este modo, una cuestión importante sería determinar si el peor rendimiento observado en los roedores viejos no enriquecidos es debido a un declive en las funciones de memoria espacial o a un fallo en las operaciones necesarias para el procesamiento y uso de la información espacial. El hecho de que las ratas viejas expuestas diariamente a EAM mostrasen un buen rendimiento, comparable incluso al de ratas de 5 meses, no significa que este protocolo de estimulación sea una condición necesaria para que los animales viejos aprendan una tarea de memoria espacial, pero sí pudo aumentar la velocidad de adquisición (Schrijver y cols., 2002) o permitir un uso más preciso de las pistas distales disponibles (Speisman y cols., 2013).

No obstante, también existen estudios en los que no se ha encontrado una mejora de la memoria espacial tras un protocolo de EAM. Por ejemplo, en el trabajo de Harburger y cols. (2007) su protocolo de EAM de 24 horas diarias mejoró el rendimiento de ratones viejos en el MWM, pero no el de los jóvenes y adultos. La falta de efecto del EAM en los roedores de estas edades es sorprendente, pero pudo deberse a factores como una corta duración de este protocolo de estimulación (4 semanas) o a que el rendimiento de los ratones de estas edades era ya asintótico y, por tanto, no susceptible de mejora por el EAM. También hay que tener en cuenta que las capacidades cognitivas de una rata son muy superiores a las del ratón y, por tanto, puede que se beneficien más de las experiencias de EAM (Wishaw, 1995).

Evidentemente, creemos que todos estos cambios conductuales y cognitivos son reflejo del efecto que ha tenido nuestro protocolo de EAM sobre la plasticidad cerebral. Por tal motivo, el segundo objetivo de esta Tesis

doctoral se centró en el estudio de algunos de los cambios neurobiológicos que pueden subyacer a estos resultados conductuales.

Segundo objetivo: *Analizar el impacto de las condiciones experimentales CO; EAM; AP; EAM+AP sobre diferentes mecanismos subyacentes a los procesos de aprendizaje y memoria:*

a) La actividad metabólica neuronal (actividad citocromo c oxidasa) y la red de activación asociada con cada condición experimental.

b) La plasticidad astrocítica (número y morfología de células positivas a la proteína ácida fibrilar glial, GFAP).

c) Los niveles de Sinapsina I en el hipocampo dorsal y ventral.

d) La expresión de receptores de glucocorticoides en el hipocampo dorsal y ventral.

Segundo objetivo (a):

Con el objetivo de conocer el patrón de activación de diferentes regiones cerebrales utilizamos el análisis cuantitativo de la actividad de la Citocromo c oxidasa (COx). La COx es la enzima terminal o complejo IV del canal de transporte de electrones de la mitocondria y su actividad representa un índice de las demandas energéticas de las neuronas tras una estimulación prolongada (Méndez-López y cols., 2009, 2012; Rubio y cols., 2012; Villarreal y cols., 2002). Así, decidimos emplear este índice de actividad metabólica, y no otros, como por ejemplo 2-desoxiglucosa, ya que ha mostrado ser mucho más preciso a la hora de reflejar la actividad neuronal que ocurre tras periodos largos de tiempo, desde horas a incluso semanas y, por tanto, muestra la historia de actividad metabólica de una región cerebral específica (González-Lima y Cada, 1994).

En el análisis de la actividad COx utilizamos ratas Wistar tanto de 5 como de 20 meses bajo las condiciones experimentales descritas en la Figura 7. Los resultados mostraron, al igual que los trabajos de otros investigadores, que la actividad COx generalmente no sufre cambios durante el proceso de envejecimiento (Gorini y cols., 1989; Vertechy y cols., 1993; Villarreal y cols., 2002). Así, no encontramos diferencias significativas entre los grupos control (grupos 5/CO y 20/CO) de 5 y 20 meses en la actividad COx de las regiones estudiadas, con la excepción del septum lateral, en donde el grupo de ratas de 20 meses mostró una mayor actividad metabólica. El septum lateral está implicado en funciones afectivas y motivacionales regulando las respuestas conductuales a las demandas del ambiente (Sheenan y cols., 2004). De este modo, la mayor actividad en esta región puede que sea una respuesta compensatoria a la hiperactividad del eje HPA descrita en roedores viejos debido a su rol en reducir la respuesta de miedo y ansiedad. Cuando las ratas jóvenes y viejas fueron expuestas a EAM (grupos 5/EAM y 20/EAM) observamos diferencias entre grupos en regiones cerebrales con un rol tanto en la activación (amígdala central) como en la inhibición (cortezas cingulada y prelímbica) del núcleo paraventricular hipotalámico.

Por otro lado, es posible que la actividad COx refleje principalmente aquellos cambios que ocurren en el consumo energético neuronal tras demandas importantes, como por ejemplo, durante una prueba de memoria. Conforme a esta hipótesis, observamos que los roedores viejos entrenados en una tarea de memoria de referencia en el laberinto radial acuático tuvieron una mayor actividad COx en las diferentes subdivisiones que componen la corteza prefrontal medial (cortezas cingulada, prelímbica, infralímbica), en el núcleo del

lecho de la estría terminal y en la amígdala central, en comparación con las ratas de 5 meses (grupos 5/AP y 20/AP). La corteza prefrontal medial tiene un rol complejo en la regulación de la respuesta de estrés, con sus diferentes subdivisiones implicadas en distintas funciones (Blanco y cols., 2009; Cerqueira y cols., 2008). Las cortezas cingulada y prelímbica quizás suprimen la actividad de la corteza infralímbica, implicada en la activación del núcleo paraventricular hipotalámico y por tanto del eje HPA, a través de sus acciones inhibitorias sobre esta corteza (Cerqueira y cols., 2008). Igualmente, la mayor actividad metabólica del núcleo del lecho de la estría terminal y de la amígdala central parece apoyar la interpretación de una mayor activación del eje neuroendocrino en los roedores viejos que realizaron la prueba de memoria espacial. En relación a esto, se ha observado que la mayoría de las pruebas empleadas para estudiar los procesos de aprendizaje y memoria en roedores pueden ser consideradas como tareas estresantes y, como consecuencia de su naturaleza aversiva, elicitan la activación de los sistemas de estrés (Aguilar-Vallés y cols., 2005), que normalmente ya se encuentran hiperactivados en los roedores viejos. Por último, una mayor activación metabólica de la corteza prefrontal medial también se ha relacionado con un esfuerzo superior a la hora de recuperar la información espacial y con el grado de dificultad de la tarea (Leger y cols., 2012).

La condición de EAM hizo que el grupo de 5 meses que fue posteriormente evaluado en la prueba de memoria espacial (grupos 5/EAM + AP y 20/EAM+AP) mostrase mayor actividad metabólica en todas las regiones cerebrales estudiadas respecto al grupo de 20 meses, con la excepción de la corteza parietal, posiblemente más implicada en estrategias espaciales de tipo

egocéntrico (Schindler y Bartels, 2013; Weniger y cols., 2009). Un incremento de la actividad metabólica ha sido relacionado con un mejor rendimiento conductual (Leger y cols., 2012), e incluso puede que el EAM facilitase esa mejor ejecución al aumentar las conexiones sinápticas entre neuronas, o incluso su proliferación y supervivencia, lo cual podría generar una mayor demanda metabólica.

También quisimos analizar si existían diferencias en la actividad COx entre las distintas condiciones experimentales que componen un mismo grupo de edad. Respecto a esto, observamos que apenas existieron cambios significativos entre los grupos control y los que recibieron EAM (grupos 5/CO y 5/EAM; grupos 20/CO y 20/EAM) ni a los 5 ni a los 20 meses de edad. Sin embargo, cuando comparamos la actividad COx de aquellos grupos que realizaron la prueba de memoria espacial con los que además de esto, fueron expuestos previamente a EAM, observamos que en el caso de las ratas de 5 meses (grupos 5/AP y 5/EAM + AP), el EAM aumentó la actividad metabólica neuronal de la corteza prefrontal medial, implicada también en procesos atencionales, toma de decisiones, flexibilidad conductual, integración de información espacial así como, en el control de la ansiedad, previamente comentado, lo cual parece explicar, al menos en parte, el mejor rendimiento de este grupo (Cerqueira y cols., 2008; Robinson y cols., 2011; Vertes 2006). Por otro lado, y en contraste al resultado observado en el grupo de animales de 5 meses, en las ratas viejas expuestas a EAM anteriormente a la prueba de memoria espacial (grupos 20/AP y 20/EAM + AP), la actividad COx estuvo significativamente reducida en comparación al grupo que no recibió estimulación. A pesar de que en el grupo de ratas jóvenes la mayor actividad

COx supuso un mejor rendimiento, en este caso, la reducida actividad metabólica no fue correlato de una mala ejecución. De este modo, es posible que el EAM haya provocado una respuesta mucho más eficiente, al favorecer un buen rendimiento conductual, pero con una reducida demanda energética o lo que es lo mismo, al facilitar una mejor adquisición de la tarea reduciéndose las necesidades metabólicas de las regiones implicadas en el proceso de memoria espacial.

Otro de nuestros objetivos fue el estudio de redes cerebrales. Para ello, se empleó el Análisis de Componentes Principales (ACP) (Begega y cols., 2012; Castilla-Ortega y cols., 2010; Cracchiolo y cols., 2007; Salmon y cols., 2009) que nos permitió comprender y diferenciar la red cerebral que subyacía a la actividad metabólica de cada grupo de edad a través de las correlaciones entre las distintas regiones estudiadas. El ACP reveló la misma red funcional en ambos grupos de edad, pero las regiones cerebrales que la componían contribuían de forma ligeramente diferente dependiendo de la edad de los animales. Por ejemplo, en el grupo de 5 meses, independientemente de la condición experimental, regiones corticales e hipocampales tuvieron una mayor relevancia dentro de la red, mientras que en el grupo de 20 meses, independientemente también de la condición experimental, regiones más implicadas en respuestas de ansiedad, tanto en el inicio de estas como en su regulación, tuvieron una ligera mayor contribución (núcleo del lecho de la estría terminal o amígdala, entre otras). Este análisis estadístico nos permitió, mediante el cálculo de las puntuaciones factoriales, analizar las posibles diferencias que existían en la red obtenida entre las condiciones experimentales que componían cada grupo de edad. De este modo,

observamos que tanto en el caso del grupo de 5 como de 20 meses, no existían diferencias significativas en la red entre el grupo control y el que fue expuesto a EAM (grupos CO y EAM). Por otro lado, en el caso de las ratas de 5 meses, el grupo que recibió EAM previo a la prueba de memoria (grupo EAM + AP) fue el que mostró una mayor actividad en la red, sobre todo en regiones corticales e hipocampales, lo cual podría explicar parte de su mejor rendimiento. El hipocampo ha sido relacionado con la formación de asociaciones entre estímulos y con la elaboración de un *mapa cognitivo* necesario para el correcto rendimiento de nuestra prueba de memoria espacial (Aggleton y cols., 2000; Redish, 2001). En el caso del grupo de 20 meses, fue interesante encontrar que los grupos que recibieron EAM (grupos EAM y EAM + AP) mostraron una reducida activación en esta red en la que, en su caso, predominó la contribución de regiones implicadas en la respuesta de ansiedad. Este resultado parece seguir la misma línea de lo observado a nivel conductual, en donde nuestro protocolo de EAM provocó una reducción de conductas ansiosas, así como un buen rendimiento en la prueba de memoria.

Estudios recientes han mostrado que toda condición experimental que provoca cambios en las demandas energéticas de las neuronas puede afectar a la función sináptica (Liu y cols., 2002; Wu y cols., 2004). La plasticidad neuronal se basa en el consumo de energía y, por tanto, se ve influida por todas aquellas experiencias que afectan al estado de energía de la neurona, como el ejercicio físico (Lee y cols., 2000, 2002; Mattson y cols., 2004; Sampedro-Piquero y cols., 2013), el aprendizaje o la estimulación ambiental. Así, se piensa que el efecto que tienen todas estas experiencias sobre la plasticidad neuronal quizás se basa en su capacidad para acceder a la

maquinaria mitocondrial, crítica en el mantenimiento de las demandas de energía celular y en el metabolismo, como vimos con nuestro análisis de la actividad COx (Vaynman y cols., 2006). De este modo, nos planteamos estudiar los cambios que aparecían como consecuencia de las diferentes condiciones experimentales en elementos que forman parte de la función sináptica, como son los astrocitos y la proteína presináptica Sinapsina I.

Segundo objetivo (b):

En este estudio sólo utilizamos ratas Wistar de 20 meses de edad asignadas aleatoriamente a las condiciones experimentales descritas en la Figura 7.

Como ya ha sido descrito en el marco teórico, los astrocitos forman parte activa de la transmisión sináptica, regulan el metabolismo neuronal y tienen funciones neuroprotectoras (Auld y Robitaille, 2003; Romero y cols., 2014; Slezak y Pfrieger, 2003). La elección de la proteína GFAP, y no de otros marcadores como S100β, vimentina o la glutamina sintetasa para analizar las propiedades de los astrocitos, fue debido a que estos otros marcadores presentan serios problemas y desventajas cuando se les compara con la proteína GFAP. Por ejemplo, la S100β y la glutamina sintetasa son proteínas capaces de teñir bien los núcleos, pero no las ramificaciones astrocíticas. Por otro lado, la proteína vimentina, al igual que la GFAP, es un buen marcador, pero se expresa principalmente en células gliales inmaduras (Saur y cols., 2014). Además de todo lo anterior, la proteína GFAP es requerida para la formación de procesos astrocíticos estables.

A pesar del rol tan importante que tienen los astrocitos en el mantenimiento neuronal, hay muchas cuestiones acerca de cómo los cambios

que ocurren en ellos, relacionados con el envejecimiento y el EAM, afectan a las funciones cognitivas. Nuestros resultados mostraron que el aprendizaje de una prueba de memoria espacial (grupo AP) y, sobre todo, la exposición de los roedores viejos a EAM (grupo EAM), aumentó el número de astrocitos inmunopositivos a la proteína GFAP y su complejidad morfológica en ciertas subregiones hipocampales, en comparación al grupo de roedores de 20 meses que fueron estabulados en condiciones estándar (grupo CO).

Es interesante destacar que las ratas que recibieron EAM muestran un aumento de estas células en el giro dentado dorsal (GD), ya sea en condiciones basales o tras realizar la tarea de memoria espacial (grupos EAM y EAM + AP). Sin embargo, mientras que en CA1 dorsal no observamos diferencias significativas entre grupos, al igual que Viola y cols. (2009) describieron previamente, en CA3 dorsal, todas las condiciones experimentales (AP; EAM; EAM + AP) provocaron un aumento del número de astrocitos en comparación con los animales del grupo control (grupo CO). Sin embargo, entre estas tres condiciones experimentales (AP; EAM; EAM + AP) no observamos diferencias significativas.

El incremento del número de astrocitos en el GD dorsal podría estar relacionado con los efectos específicos que tiene el EAM sobre la neurogénesis y la formación de nuevas sinapsis, junto con el hecho de que el GD parece contar con un microambiente específico que favorece la proliferación celular (Bekiari y cols., 2014; Kempermann, 2011; Morrens y cols., 2012). Incluso, se ha observado que los astrocitos situados en la capa granular del GD favorecen los procesos de neurogénesis desde la etapa prenatal a postnatal (Sun y cols., 2014). Del mismo modo, el aprendizaje de una prueba de memoria espacial

(grupo AP), con independencia de que los animales hayan sido expuestos o no a EAM, también produjo un aumento del número de astrocitos en el GD dorsal. En apoyo a este resultado, Jahanshahi y cols. (2007) ya observaron que el número de astrocitos en esta subregión hipocampal estaba relacionado con la duración del protocolo de aprendizaje. Así, sus datos mostraron que el aprendizaje de una prueba de memoria de referencia espacial, incluso con menos sesiones que el protocolo utilizado en nuestros trabajos, consiguió aumentar el número de astrocitos en el GD dorsal.

La transmisión sináptica parece estar modulada por cambios dinámicos en los procesos astrocíticos. Los astrocitos muestran cambios morfológicos en materia de minutos, tanto espontáneos como inducidos por actividad, y expresan moléculas conocidas como gliotransmisores que influyen en la sinapsis (Rodnight y Gottfried, 2013; Volterra y Meldolesi, 2005). Por ejemplo, el gliotransmisor D-serina es fundamental para la inducción de la PLP al actuar como agonista del receptor NMDA (Mothet y cols., 2006; Slezak y cols., 2006), o incluso, la secreción de colesterol por parte de los astrocitos es clave en la biogénesis de vesículas sinápticas a través de su acción sobre la proteína Sinapsina I (Göritz y cols., 2002; Pfrieger, 2003). De este modo, otro de nuestros objetivos fue estudiar los cambios morfológicos que surgieron en estas células vinculados a las distintas condiciones experimentales (CO; EAM; AP; EAM + AP). Nuestros resultados mostraron que la longitud de las ramificaciones astrocíticas así como, el número de intersecciones y nodos, estudiado todo ello a través del método de Sholl (Sholl, 1953), aumentaron como consecuencia del EAM, dando lugar a astrocitos con una morfología más desarrollada en el hipocampo dorsal. Los largos y más ramificados procesos

astrocíticos tendrían la capacidad de modular más fácilmente los elementos pre y postsinápticos (Ben Achour y Pascual, 2010; Theodosis y cols., 2008; Wang y Bordey, 2008). Además, la comunicación entre neuronas y astrocitos es de carácter bidireccional, por lo que factores tróficos secretados por las neuronas y aumentados por el EAM, como el factor de crecimiento nervioso (BDNF), podría estar participando en estimular un mayor desarrollo del astrocito (Kuzumaki y cols., 2011; Parpura y Verkhratsky, 2012). En nuestro caso, al ser animales de 20 meses, puede que también esa mayor complejidad observada tras el EAM sea una respuesta compensatoria a las alteraciones que aparecen con la edad en la comunicación sináptica (Billard, 2006; Pfrieger, 2010). En relación a esto último, durante el envejecimiento se han descrito una serie de cambios sinápticos que requieren un remodelamiento de las redes neuronales, lo cual precisa de estabilidad homeostática así como, de neuroprotección, proporcionada principalmente por los astrocitos (Rodríguez y cols., 2014; Schipke y Kettenmann, 2004; Tasker y cols., 2012).

Segundo objetivo (c):

En este estudio sólo utilizamos ratas Wistar de 20 meses de edad divididas en las condiciones experimentales descritas en la Figura 7.

Otro elemento que también ha sido estudiado en esta Tesis, y que ha mostrado ser un marcador de la actividad sináptica, es la expresión de la proteína presináptica Sinapsina I. Esta proteína se encuentra asociada a la superficie de las vesículas sinápticas, junto con proteínas del citoesqueleto como la actina. La Sinapsina I es fosforilada por varias proteínas kinasas, las cuales modulan la unión que mantiene con las vesículas sinápticas, permitiendo la movilización de estas últimas y la liberación del neurotransmisor

al espacio sináptico (Cesca y cols., 2010; Cheetham y cols., 2003). Hemos elegido estudiar el patrón de expresión de esta proteína en el hipocampo dorsal y ventral ya que uno de los procesos implicados en la PLP es el aumento de la liberación del neurotransmisor, lo cual es mediado en parte por la actividad de Sinapsina I (Hilfiker y cols., 1999). En el caso de la región CA3 dorsal, la expresión de Sinapsina I aumentó tras la prueba de memoria espacial, independientemente de si los animales habían sido expuestos o no a EAM, mientras que en la región CA3 ventral, la mera exposición a EAM fue suficiente para incrementar los niveles de esta proteína. El hipocampo dorsal tiene un rol muy establecido en ciertos tipos de memoria, fundamentalmente espacial, así, no fue extraño observar que el aprendizaje de una prueba de memoria de referencia fuese suficiente para inducir un incremento de esta proteína implicada en la PLP (Bannerman y cols., 2004; Potvin y cols., 2007). De acuerdo a nuestro resultado, el estudio previo de Gómez-Pinilla y cols. (2001) también encontró un aumento de Sinapsina I en el hipocampo dorsal tras una prueba de memoria de referencia en el laberinto de Morris. Respecto al aumento promovido por el EAM en CA3 ventral, estudios inmunohistoquímicos, como el nuestro, revelaron que la expresión de esta proteína está íntimamente relacionada con procesos de crecimiento neuronal y de sinaptogénesis, sugiriéndose una relación entre el inicio de su expresión y la maduración de la red neuronal (De Camilli y cols., 1983). De este modo, debido a que el EAM promueve la aparición de nuevas sinapsis (Birch y cols., 2013), y estas llegan a ser maduras y funcionales tras solamente un mes desde su formación, puede que necesiten de una mayor expresión de Sinapsina I para el mantenimiento de su estabilidad. Este aumento de Sinapsina I podría entenderse como una

reserva cerebral que protege a las nuevas sinapsis, e incluso, serviría para hacer frente a posteriores demandas. En relación a esto, y como ya se ha comentado en el apartado introductorio, el EAM tiene la capacidad de promover la formación de una *reserva cerebral* que permite un uso eficiente de las redes neuronales existentes así como, la integración de las nuevas (Nithianantharajah y Hannan, 2006; Petrosini y cols., 2009).

Segundo objetivo (d):

En este estudio sólo utilizamos ratas Wistar de 20 meses de edad asignadas aleatoriamente a las condiciones experimentales descritas en la Figura 7.

Finalmente, y como factor modulador de la actividad sináptica, otro de nuestros objetivos fue estudiar la expresión de RGs en el hipocampo dorsal y ventral. Estos receptores son los encargados de mediar la retroalimentación negativa sobre el eje HPA, por lo que el análisis de su expresión puede explicar, en parte, la reducción de conductas de tipo ansioso en animales expuestos a EAM. Al mismo tiempo, al actuar sobre las sinapsis glutamatérgicas son capaces de modular la transmisión sináptica, la plasticidad, el aprendizaje y la memoria (Barnes, 2011; Myers y cols., 2014; Popoli y cols., 2011; Salehi y cols., 2010; Sandi, 2011; ter Horst y cols., 2012). Además, los RGs han mostrado actuar no sólo sobre el eje HPA, sino también sobre otras vías de señalización, como la B-cell CLL/lymphoma 2 (Bcl-2) implicada en proporcionar neuroprotección (Hunsberger y cols., 2009).

Nuestros resultados revelaron que en el hipocampo dorsal, el EAM previo a la prueba de memoria (grupo EAM + AP) redujo los niveles de los RGs en CA1 y CA3, mientras que en el GD es el aprendizaje espacial, y no el EAM,

el que aumentó su expresión. En contraste a los resultados observados en el hipocampo dorsal, la expresión de estos receptores en el hipocampo ventral fue mucho más susceptible a los efectos del EAM. Así, el impacto de la prueba de memoria espacial sobre la expresión de estos receptores dependió de la exposición previa a EAM (grupo EAM + AP), e incluso el EAM (grupo EAM) por sí solo aumentó los niveles de estos receptores en el GD y el subiculum ventral.

A la hora de comprender este patrón diferencial de expresión, estudios previos ya observaron que el EAM induce la expresión de estos receptores en subcampos específicos del hipocampo y no de forma homogénea (Olsson y cols., 1994; Vivinetto y cols., 2013; Weiss y cols., 2004). En el caso del hipocampo ventral son varios los estudios que lo han relacionado con funciones reguladoras de la respuesta de ansiedad (Bannerman y cols., 2004; Bertoglio y cols., 2006; Trent y Menard, 2010). Así, el aumento de RGs observado tras el EAM podría entenderse como una respuesta compensatoria y adaptativa al aumento de secreción de GCs que provoca esta intervención. Aunque este dato pueda parecer contradictorio, al considerarse tradicionalmente que elevados niveles de GCs tienen un efecto negativo sobre la salud, varios estudios mostraron que intervenciones positivas como el EAM, o el ejercicio aeróbico, provocan un exceso de secreción de esta hormona (de Kloet y cols., 2009). Esto es debido a que los niveles de GCs en sangre no son reflejo de la valencia emocional de un estímulo, sino una muestra de la actividad conductual y, por tanto, de los requerimientos metabólicos de los tejidos activos (Buwalda y cols., 2012). De este modo, en el caso del EAM la actividad locomotora requerida para manipular todos los estímulos adecuadamente y la exposición

frecuente a la novedad provocan la activación del eje HPA. Sin embargo, la diferencia entre esta intervención y estresores negativos parece encontrarse en los mecanismos de retroalimentación negativa, mediados por los RGs, los cuales, si su expresión está aumentada, promueven resiliencia, y si está reducida, enfermedad (de Kloet y cols., 2009). Además, también parece que el estado de bienestar del sujeto en el momento de liberación de los GCs es un elemento clave a la hora entender los efectos de esta hormona sobre el cerebro y la conducta (Buwalda y cols., 2012).

El hipocampo dorsal, por el contrario, parece estar mucho más implicado en funciones cognitivas y, especialmente en la memoria espacial (Bannerman y cols., 2004; Fanselow y Dong, 2010; Miyoshi y cols., 2012). Los RGs modulan la consolidación de este tipo de memoria al afectar sobre distintas vías de señalización que regulan la fosforilación de Sinapsina I (Finsterwald y Alberini, 2014; Jovanovic y cols., 2000). La modulación que ejercen estos receptores sobre la memoria espacial depende de su grado de activación: una activación dentro de unos límites normales favorece un buen rendimiento, mientras que niveles bajos o por encima de lo normal tienen el efecto opuesto (Jöels y cols., 2006; Roozendaal, 2002). De este modo, el EAM podría favorecer que la expresión y activación de los RGs se mantengan en unos niveles normales, permitiendo un buen rendimiento en la prueba de memoria espacial, mientras que la ejecución comprometida del grupo que no fue expuesto a EAM (grupo AP) pudo deberse, en parte, a una activación en exceso de estos receptores como consecuencia del carácter estresante de la prueba y de la secreción abundante de GCs.

En conjunto, estos resultados sugieren que el EAM es un protocolo de estimulación multifactorial capaz de producir efectos positivos sobre la conducta y cognición, independientemente de la edad de los animales. En el caso de los roedores viejos parece, además, que estos beneficios tienen que ver con una serie de cambios en mecanismos neurobiológicos que participan en la actividad y comunicación neuronal así como, en factores que modulan estas funciones. Todos estos cambios podrían formar parte de la conocida como *reserva cerebral*, descrita en el apartado introductorio y que es posible potenciar incluso en edades avanzadas.

Como es obvio, los trabajos que componen esta Tesis doctoral tienen varias limitaciones que pueden ser mejoradas en posteriores líneas futuras. Por ejemplo, sería interesante incluir más grupos de diferentes edades para valorar los beneficios del EAM a lo largo del ciclo vital, al igual que comprobar si variaciones en el tiempo de exposición a nuestro protocolo de EAM puede cambiar los efectos observados. Por otro lado, nuestros estudios a nivel cerebral fueron limitados a los grupos de ratas de 20 meses, con la excepción de la actividad COx, por lo que hubiera resultado interesante haber analizado la expresión de los mecanismos neurobiológicos discutidos en esta Tesis doctoral en el grupo de ratas jóvenes, y ver si el patrón de expresión es similar o no al de las ratas viejas. Como línea futura también consideramos que sería necesario el estudio de la expresión de Sinapsina I, GFAP y de los RGs en diferentes regiones cerebrales y no sólo en el hipocampo, como en estos primeros trabajos. La razón de centrar nuestros estudios en esta región cerebral fue el hecho de que el hipocampo, debido a su rol bien establecido tanto en los procesos de memoria como en el control de la ansiedad, es una de

las regiones más susceptibles al proceso de envejecimiento y que podría, por tanto, responder con mayor intensidad al EAM. Finalmente, como línea futura sería interesante estudiar si los beneficios observados con nuestro protocolo de EAM se mantienen a lo largo de la vida o finalizan tras un tiempo sin recibir estimulación, pudiendo incluso ser la edad una variable que module la duración de sus efectos positivos.

CONCLUSIONES

CONCLUSIONES

1. Nuestro protocolo de EAM redujo conductas de tipo ansioso, aumentó los niveles de exploración y mejoró el rendimiento en una prueba de memoria de referencia espacial independientemente de la edad de los animales.

2. El ACP reveló que la misma red de actividad metabólica (actividad COx) subyacía a los grupos de 5 y 20 meses de edad, pero la contribución de las distintas regiones cerebrales a esta red fue ligeramente diferente dependiendo de la edad de los animales.

3. Nuestro protocolo de EAM aumentó el número de astrocitos inmunopositivos a la proteína GFAP en el GD dorsal, así como su complejidad morfológica en diferentes subregiones del hipocampo dorsal en ratas Wistar de 20 meses de edad.

4. El aumento de Sinapsina I en CA3 dorsal dependió tanto de la condición de estabulación previa como de la experiencia de aprendizaje, pero no de la interacción entre ambos factores. En el caso de CA3 ventral, el aumento de expresión de esta proteína dependió solamente de la exposición a EAM.

5. El aprendizaje de una prueba de memoria espacial aumentó los niveles de los RGs en el GD dorsal, mientras que en CA1 y CA3 dorsal, el grupo de animales estabulado en condiciones estándar y sometido a la prueba de memoria mostró un aumento de los niveles de este receptor en comparación con los estabulados en EAM. Por otro lado, en el hipocampo ventral, el EAM previo a la prueba de memoria, e incluso por sí solo, aumentó los niveles de este receptor.

CONCLUSIONS

1. Our EE protocol reduced anxious behavior, increased the exploration levels and improved the behavioral performance in a reference memory task, regardless of the age of the animals.

2. The PCA showed that the same network of metabolic activity (COx activity) underlay the groups aged 5 and 20 months, but the contribution of the brain regions that made up this network was slightly different depending on the age of the animals.

3. Our EE protocol increased the number of GFAP immunopositive astrocytes in the dorsal DG, as well as their morphological complexity in different subregions of the dorsal hippocampus in 20 month-old Wistar rats.

4. The increase of Synapsin I in the dorsal CA3 depended on both the previous housing condition and the learning experience, but not the interaction between these factors. In the case of the ventral CA3, the increase of Synapsin I was only dependent on the EE.

5. The learning of a spatial memory task enhanced the GR expression in the dorsal DG, whereas in the dorsal CA1 and CA3, the group of rats housed in standard conditions and submitted to the memory task showed an increase in the expression of this receptor compared with the enriched group. On the other hand, in the ventral hippocampus, EE prior to the memory task, and even its mere effect, increased the levels of this receptor.

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