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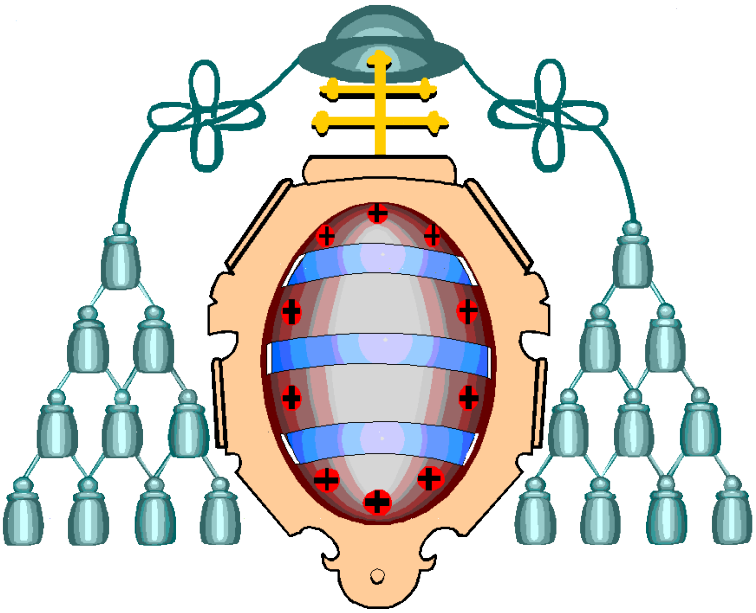

PROGRAMA DE DOCTORADO: PSICOLOGÍA
DEPARTAMENTO DE PSICOLOGÍA

Bases neurales del recuerdo y la extinción de la memoria espacial

Neural basis underlying spatial memory retrieval and extinction

TESIS DOCTORAL

Marta Méndez López

Oviedo, 2015

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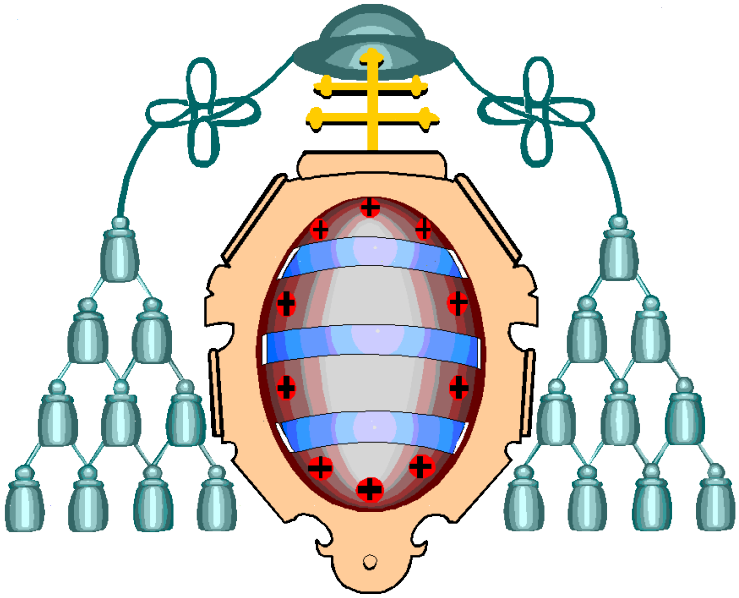

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TESIS DOCTORAL

DOCTORANDO: MARTA MÉNDEZ LÓPEZ

DIRECTORA: NÉLIDA Mª CONEJO JIMÉNEZ

Oviedo, 2015

La Tesis doctoral presentada por Marta Méndez López para obtener el grado de Doctor por la Universidad de Oviedo, es el resultado de diferentes investigaciones que han dado lugar a 8 estudios (cuatro artículos publicados y cuatro manuscritos en elaboración). Los trabajos se han llevado a cabo durante un período de 4 años en el laboratorio de Neurociencias, del Área de Psicobiología (Departamento de Psicología) de la Universidad de Oviedo, y en el *Laboratory of Pharmacology and Experimental Therapeutics,* IBILI, Universidad de Coimbra (Portugal). Previamente, en el año 2011 obtuvo la suficiencia investigadora mediante la superación del Master de Investigación en Neurociencias, obteniendo la máxima calificación. Los artículos que se presentan en esta Tesis han sido publicados en revistas internacionales con un factor de impacto global de 13.144 (de acuerdo a ISI Journal Citation Reports, 2013).

- Méndez-Couz M., Conejo N.M, Vallejo G, Arias JL. Brain functional network changes following prelimbic area inactivation in a spatial memory extinction task. Behavioural Brain Research. 287:247-255. 2015. DOI:10.1016/j.bbr.2015.03.033 .Factor de Impacto 3.391.

- Méndez-Couz M., Conejo N.M., González-Pardo H., Arias J.L. Functional interactions between dentate gyrus, striatum and anterior thalamic nuclei on spatial memory retrieval. Brain Research. 1605:59-69. 2015. DOI: 10.1016/j.brainres.2015.02.005. Factor de Impacto 2.828.

- Méndez-Couz M., Conejo N.M., Vallejo G., Arias J.L. “Spatial memory extinction: a c-Fos protein mapping study". Behavioural Brain Research 260(214)101-110. 2013. DOI: 10.1016/j.bbr.2013.11.032. Factor de Impacto 3.391.

- Conejo N.M., Cimadevilla J., González-Pardo H., Méndez-Couz M., Arias JL. "Hippocampal inactivation with TTX impairs long-term spatial memory retrieval and modifies brain metabolic activity". PloS ONE. 28;8(5):e64749. 2013. DOI: 10.1371/journal.pone.0064749. Factor de Impacto 3.534.

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To Whom It May Concern,

I am the principle investigator of my laboratory which is part of the UCSF Center for Integrative Neuroscience at the University of California, San Francisco. This is to certify that the graduate student Ms. Marta Méndez López collaborated in a research project aimed to elucidate the neural the mechanisms underlying spatial reward learning in my laboratory at UCSF, between May 1st and July 30th, 2014.

Specifically, she worked with the graduate student Ms. Marielena Sosa in a preliminary phase of the project. During three months, the student participated in the behavioral training of the experimental animals (Long Evans male rats) in a rewarded spatial linear track memory task. At the same time, the student learned the necessary methods and techniques to build a microdrive array carrying 21 independent removable tetrodes using previously described methods by the host lab (Frank et al. 2004, *J Neurosci* 24: 7681-7689) and targeting brain areas of interest, particularly the dorsal and ventral hippocampus. Following pretraining, rats were implanted with the aforementioned microdrive array. Over the next 20 days, tetrodes were lowered to its corresponding target in the dorsal or ventral CA1 or CA3 hippocampal subfields. Once the tetrodes were implanted, the animal was trained in a W-track rewarded spatial task and electrophysiological data were collected during the training process, EEG waveforms were recorded by each tetrode during the entire procedure. In order to confirm the correct tetrode placement, electrolytic lesions were performed at the end of each training session and later confirmed by brain extraction, tissue processing and histological study.

In order to comply with the USCF security regulations, prior to perform any research task, the student took every necessary course of safe laboratory procedures. Those courses included instruction on personal protective equipment, safety handling and disposal of chemicals, LARC Barrier Orientation, laboratory animal handling and euthanasia, among other general lab security courses given by UCSF.

In addition, Ms Marta Méndez had the opportunity to assist the Doctorate program in Neurosciences Journal Club and follow the research outcomes during weekly lab meetings, where graduate students and postdoctoral colleges discussed their current and future work. At the same time, she attended several seminars and conferences given by invited national and international specialists visiting UCSF.

San Francisco (CA), August 1st, 2014.

School of Psychology
Head of School Professor Ed Wilding BSc MSc PhD

Ysgol Seicoleg
Pennaeth yr Ysgol Yr Athro Ed Wilding BSc MSc PhD

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Cardiff, May 8, 20015

I, Patricia Gasalla Canto, PhD in Psychology (Learning Sciences), Research Associate at the School of Psychology (College of Biomedical & Life Sciences), Cardiff University (UK), have the following appraisal concerning the work of Ms. Marta Méndez López towards her PhD:

The candidate present eight papers, four of them were already published in distinct journals that are indexed in ISI with an impact factor higher than 2.8 and the rest are submitted to analogous scientific journals.

Taken together, the papers account for a very coherent line of research, which focus on the study of the neural bases of spatial learning , adding novel experimental manipulations in animal models and observing its effects on behavioral and biological (histochemistry, immunohistochemistry and Western blot) measures as dependent variables, as it is expected in psychobiological research.

Well outlined state of the art, high-quality references, very clear research questions, fine organization, and good readability are noticeable features in every of the applicant's manuscripts.

Also, data seem to be driven from very sound experimental methods and techniques, after which proper statistics are applied to achieve robust results, allowing the candidate to draw convincing conclusions.

For the above mentioned reasons and to the best of my knowledge, the papers show a very consistent use of all skills that are required to produce original scientific contributions, with valuable impact, to the field of psychobiology.

Patricia Gasalla Canto

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UNIVERSITA' DEGLI STUDI DI CAGLIARI

Dipartimento di Scienze Biomediche

I, Camino Alvarez Fidalgo, PhD in Biomedical Sciences (Neurosciences), Post Doc Research Fellow at Department of Biomedical Sciences, Università degli studi di Cagliari (Sardinia, Italy) have the following appraisal concerning the work of Marta Méndez López (MS):

REPORT

The work of the candidate Marta Méndez López to the Doctorate Degree in the University of Oviedo is composed by eight papers, four of them were already published in distinct journals with a global impact factor of 13,14 (ISI, Journal citation Reports ,2013) and the rest are submitted to analogous scientific journals.

In general, the presented work explored the contribution of different cerebral brain areas that account for learning and memory, and specifically for the retrieval and extinction phases of a spatial memory task carried out in the Morris Water Maze.

- Papers one to three focus on the neural basis underlying the retrieval of a spatial memory task both under physiological conditions and experimental manipulations, as hippocampus *Cornnus Ammonis 1* or the prelimbic area of prefrontal cortex reversible lesion animal models, using the GABA-A agonist Muscimol as a temporal inactivating drug. Then metabolic brain activity of selected brain structures was measured in order to shed light on neural networks supporting this process.
- Papers four to seven aim to elucidate the brain networks and structures that account for the extinction of a previously acquired reference spatial memory task, using as well as the afore mentioned ones, different experimental manipulations and cellular-molecular biology techniques to measure its effects at a behavioral and physiological levels.
- Paper eight seeks the determination of the NPY system involvement in spatial learning and memory, for that purpose an evaluation of the effects of intracerebral administration NPY Y_2 receptor antagonist BIIE0246 on the dorsal CA1 hippocampus in rodents was performed, to evaluate its effects on anxiety, spontaneous horizontal activity and spatial memory tasks.

The referred papers showed extremely relevant work, bibliographically well based with well stablished methodologies and an upper level of explanation and integration of the results. Therefore, it seems clear than the candidate fulfils the conditions to present its work before a Doctorate Jury.

Cagliari, 8th May, 2015.

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Sito: dipartimenti.unica.it/scienzebiomediche

Contenido

RESUMEN

El aprendizaje y la memoria espacial son habilidades vitales en los animales para orientarse y recordar la posición de lugares de interés en su ambiente, así como para mantener el sentido de la dirección y la localización mientras nos movemos en él, y para adaptarnos a nuevos entornos. La memoria espacial representa una de las funciones cognitivas más básicas y esenciales, además de ser particularmente compleja, ya que el sistema nervioso integra información multisensorial de nuestro entorno a lo largo del tiempo y en el espacio.

Por el momento, se conoce que la memoria espacial se compone de varias fases, que a su vez implican una participación diferencial y temporalmente dependiente de distintas estructuras y redes cerebrales, mayoritariamente del sistema límbico. En la actualidad, numerosos estudios apoyan la teoría prevalente, en la que se propone una implicación del circuito corteza prefrontal-hipocampo en la adquisición de esta tarea. Sin embargo, las bases anatómicas, así como los sistemas de neurotransmisión que sustentan las fases posteriores de esta memoria, tales como el de recuerdo a posteriori, o la extinción de la misma, aún son motivo de controversia.

Por todo lo anterior, en este trabajo nos planteamos estudiar las bases neurales que sustentan las fases de recuerdo y de extinción de la memoria espacial, así como la implicación del sistema del neuropéptido Y, tradicionalmente ligado a conductas de tipo ansioso, en la memoria espacial.

Los resultados de nuestro trabajo muestran, que tras haber adquirido una tarea de memoria de referencia espacial en el laberinto acuático de Morris, los animales son capaces de ejecutar con éxito la tarea de recuerdo una o varias semanas más tarde, y que la conducta se extingue tras cuatro sesiones de extinción, sin recuperación espontánea a las 24 horas.

Cuando se estudian las bases moleculares que subyacen a cada una de estas fases se observó que, tras una tarea de recuerdo de memoria de referencia espacial, se producen cambios a nivel de la corteza prefrontal, el cuerpo estriado, el núcleo anterodorsal del tálamo y el hipocampo, tanto en su porción dorsal como ventral. Por otro lado, la extinción de esta tarea se relaciona con cambios en áreas del sistema límbico y áreas asociadas, tales como las

cortezas prefrontal, la parietal y la retroesplenial, el hipocampo, núcleos amigdalinos, y los cuerpos mamilares. Además, el recuerdo de la memoria espacial depende de la integridad de la corteza prelímbica y del sistema hipocampal, y la inactivación temporal de estas áreas se asocia con cambios funcionales en las redes neurales del circuito hipocampo-prefrontal que lo sustentan. En cuanto a la extinción, la inactivación temporal del área prelímbica cambia las redes neurales implicadas en la misma, aunque su integridad no parece esencial para la ejecución de la tarea. Asimismo, la inactivación hipocampal altera la tarea de extinción de la memoria.

Por último, los cambios regionales de expresión del neuropéptido Y, así como de sus receptores Y1R e Y2R a nivel de la corteza prefrontal y del hipocampo dorsal, se asocian con la mejora de la ejecución de una tarea de memoria espacial, observándose cambios asociados en la actividad metabólica en áreas que previamente habían sido relacionadas con esta función, tales como la corteza prefrontal, el tálamo, el hipocampo y los cuerpos mamilares.

En conjunto, nuestros resultados apoyan que redes distribuidas del circuito prefrontal-hipocampo y estructuras funcionalmente asociadas, como los núcleos del talámicos anteriores, el cuerpo estriado o los cuerpos mamilares subyacen al recuerdo y la extinción de la memoria espacial, teniendo un papel determinante en este tipo de memoria el sistema del neuropéptido Y.

ABSTRACT

Learning and memory are essential abilities for animal life and survival, in order to be oriented in the environment and to remember specific places, to maintain their body orientation while navigating and to adapt themselves in a new environment. Spatial memory represents one of the essential cognitive functions, and its particularly complex as the nervous system integrates information provided by multiple sensory entries through time and space.

Currently, it is known that spatial memory has several phases involving different brain structures and networks, mostly limbic structures, which show differential and time-dependent involvement during spatial memory acquisition. However, although much is known about the acquisition of spatial memory, the anatomical basis and the neurotransmitter systems underlying the late phase of this memory process, as well as during the retrieval or extinction of the previously acquired memory are still a controversial issue.

Taken the aforementioned into account, in the present work we aim to elucidate the brain structures and related brain functional networks underlying spatial memory retrieval and extinction, using the Morris water maze paradigm for this purpose. Additionally, the influence of the neuropeptide Y neurotransmitter system in spatial memory process was assessed.

Results show that animals trained in a hidden platform-reference spatial memory task in the Morris water maze are able to successfully complete a retrieval task one week after acquiring the task. Similarly, the previously learned task is successfully extinguished after four extinction sessions and it does not present spontaneous recovery 24 h later.

When the molecular mechanism underlying these processes was analyzed, our results revealed that extinction of the spatial memory is related with neural networks that included functional coupling between the prefrontal, parietal and retrosplenial cortices, as well as the hippocampus, the amygdala and the mammillary bodies. Similarly, retrieval of the previously learned task was associated with changes in the metabolic activity of the prefrontal cortex, the striatum, the anterodorsal thalamic nucleus and both dorsal and ventral hippocampal portions.

Additionally, results show that retrieval of the spatial reference memory task was impaired both by temporal prelimbic area temporal inactivation, and by unilateral or bilateral inactivation of the dorsal hippocampus. Retrieval of spatial memory depends on the integrity of the hipocampal system even several weeks after the initial training. Accordingly, brain networks including the prefrontal-hippocampus circuitry vary according to the degree of the hippocampal blockade. Similarly, temporal inactivation of the prelimbic area also altered the brain functional networks involved in the spatial memory extinction task, although its integrity seems not to be essential for the task completion. Moreover, inactivation of the dorsal hippocampus impaired spatial memory extinction.

Lastly, expression levels of neuropeptide Y and its Y_2R-Y_1R receptors in the prefrontal cortex and the hippocampus were associated with performance in the spatial memory task. Similarly, the metabolic activity changed in brain areas typically related to spatial memory like the prefrontal cortex, the thalamic nuclei, the hippocampus and the mammillary bodies.

In summary, our results support widespread brain circuits underlying the retrieval and extinction of spatial memory involving a network between the prefrontal cortex and the hippocampus and additional networks comprising functional related structures like the striatum, thalamic nuclei or the mammillary bodies, with a leading role the system neuropeptide Y in this type of memory.

Introducción

INTRODUCCIÓN

1.1.CLASIFICACIÓN DE LA MEMORIA

El aprendizaje y memoria, junto a la selección natural, permite que los seres vivos puedan adaptarse a las condiciones cambiantes del medio externo. Se entiende como **aprendizaje** el hecho de que la experiencia produzca cambios en el sistema nervioso que puedan ser duraderos y se manifiesten en el comportamiento de los individuos (Morgado, 2005). Por otro lado, la **memoria** se define como un proceso neurocognitivo que permite registrar, codificar, consolidar, almacenar, acceder y recuperar la información aprendida (Estevez-Gonzalez, Garcia-Sanchez, & Barraquer-Bordas, 1997), dando a nuestra vida un sentido de continuidad (Morgado-Bernal, 2011; Morgado, 2005). El aprendizaje y la memoria son capacidades tan importantes para nosotros que representan, para muchos autores, el "pegamento que sostiene nuestra vida mental", puesto que sin su poder de unificación todos los aspectos conscientes e inconscientes de nuestra vida estarían rotos en tantos fragmentos como segundos tiene un día, nuestra vida estaría así vacía y sin sentido (Kandel, Dudai, & Mayford, 2014).

No es pues de extrañar que desde tiempos remotos el ser humano se haya sentido atraído por la idea de entender los mecanismos fisiológicos del aprendizaje, pero no ha sido hasta la segunda mitad del siglo XX cuando los avances científicos y técnicos han permitido empezar a desentrañar los mecanismos que subyacen a los procesos de aprendizaje y memoria (Lopez-Rojas, Almaguer-Melian, & Bergado-Rosado, 2007).

1.1.1. CLASIFICACIÓN Y TIPOS DE MEMORIA

Existen distintos tipos de memoria de acuerdo con diversas clasificaciones propuestas (Maine de Biran, 1929; Ryle, 1949; Bruner, 1969; Winograd, 1975;(O'Keefe & Nadel, 1978) Cohen y Squire, 1980; Graf y Schacter, 1985; Tulvin, 1985; Squire y Zola-Morgan, 1988). Atendiendo a una clasificación temporal (Estevez-Gonzalez et al., 1997; R. F. Thompson & Kim, 1996), la memoria se puede dividir en: *memoria sensorial*, *memoria a corto plazo* y *memoria a largo plazo*. A su vez la *memoria a largo plazo* se subdivide en: *memoria no declarativa o implícita* y *memoria declarativa o explícita* (Fig. 1).

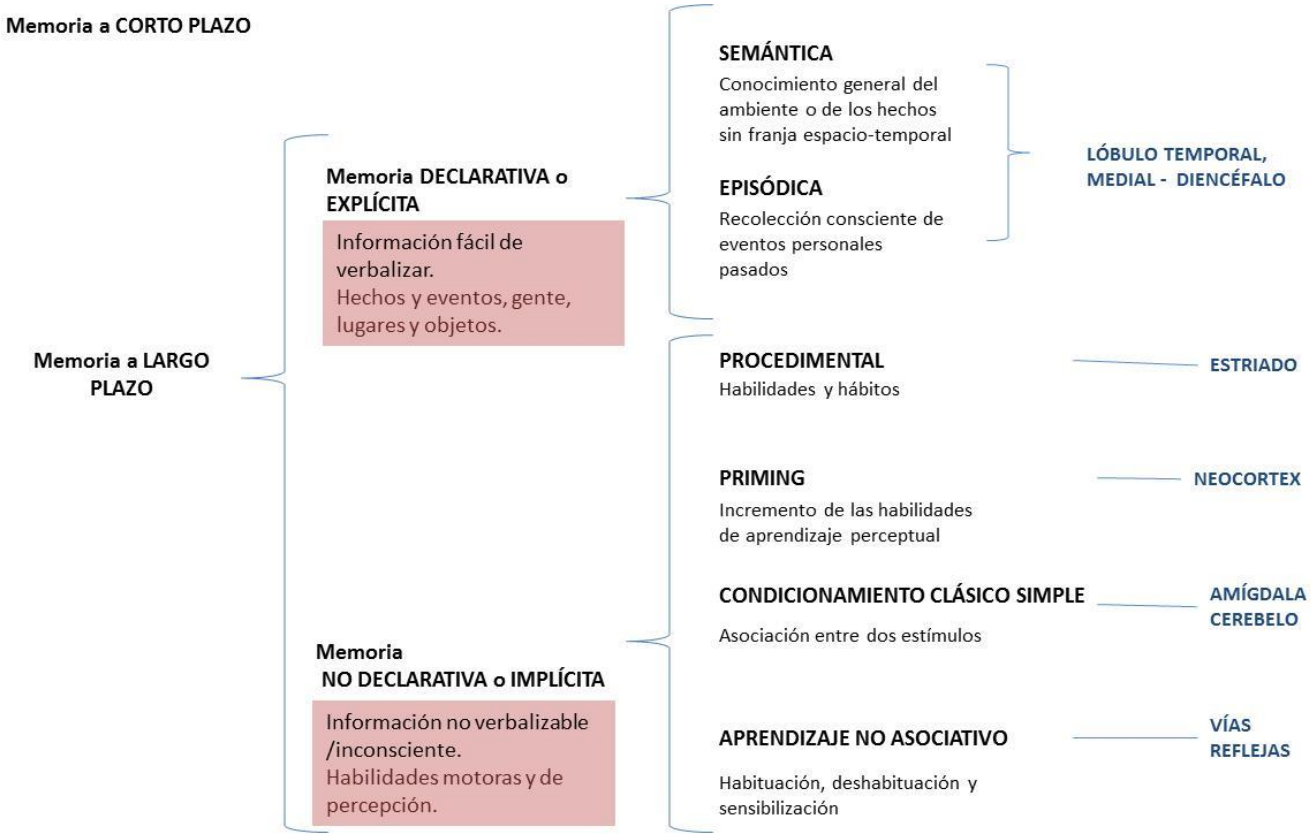

Figura 1: Tipos de memoria existentes atendiendo a una posible clasificación temporal de la memoria y a las estructuras cerebrales que tienen un papel más relevante en cada una de ellas. Adaptado de Thompson y Kim (1996).

De acuerdo con esta clasificación, la *memoria no declarativa (o no declarativa)* consiste en habilidades y cambios en el rendimiento y en la conducta que registramos por la influencia de experiencias pasadas. Se adquiere de forma gradual (Morgado, 2005) y no suele requerir intención ni consciencia (Estevez-Gonzalez et al., 1997), se relaciona principalmente con estructuras cerebrales como el cuerpo estriado y la amígdala, el cerebelo, y en animales invertebrados simples vías reflejas (Davis et al., 1994; Kandel, 2001; Quirk, Repa, & LeDoux, 1995; R. F. Thompson & Kim, 1996). Sin embargo, la *memoria declarativa o explícita* es la capacidad para adquirir información de hechos (*memoria semántica*) y eventos (*memoria episódica*). Puede adquirirse en pocos ensayos y se considera como una memoria de expresión cambiante y flexible. En humanos esta memoria puede expresarse de forma verbal e implica

un conocimiento consciente. Su adquisición se relaciona con el sistema hipocampal y otras estructuras del lóbulo temporal medial, pasando en fases finales del mismo a ser más dependiente de la corteza cerebral (Kandel et al., 2014; Kandel & Pittenger, 1999; Morgado, 2005).

En humanos, este tipo de memoria se puede evaluar mediante imágenes de tomografía por emisión de positrones (PET), en las que, por ejemplo, se ve una clara activación del hipocampo derecho cuando un sujeto recorre calles de un laberinto virtual (Maguire et al., 1998). Aunque por su naturaleza consciente la memoria declarativa parece más susceptible de estudiarse en humanos, su presencia en animales también ha sido demostrada (Eichenbaum, 2000; Ergorul & Eichenbaum, 2004; Tang et al., 1999). Una forma de evaluar la *memoria declarativa* en roedores es mediante pruebas que evalúen la capacidad de orientación espacial, considerado concretamente un subtipo de memoria episódica (Nadel & Hardt, 2011; Nadel & Moscovitch, 2001; O'Keefe & Nadel, 1978), puesto que implica el almacenamiento de información dentro de un marco espacio-temporal.

En la naturaleza, los roedores deben emplear alguna estrategia que les permita recordar donde se ubica el alimento, y esta capacidad puede estudiarse en el laboratorio gracias al empleo de laberintos cuya solución depende del uso de información espacial (Santin, Rubio, Begega, Miranda, & Arias, 2000). Una de las pruebas más ampliamente utilizadas para estudiar la memoria espacial en roedores es el laberinto acuático de Morris (R. Morris, 1981, 1984). Consiste en un tanque de agua circular de reducidas dimensiones en la que los animales deben encontrar una plataforma oculta (sumergida por debajo de la superficie del agua) guiándose por información visual disponible en el entorno espacial. Por su simplicidad, rapidez de aprendizaje y similitud con el entorno natural de los roedores, este laberinto es uno de los más empleados en el estudio de la memoria espacial en estos animales (Vicens, Redolat, & Carrasco, 2003).

1.1.2. DINÁMICA TEMPORAL DE LA MEMORIA.

Cajal (1852-1934), cuya intuición llevó a inferir la función del sistema nervioso a partir de su morfología, fue el primero en proponer la plasticidad en el número y fuerza de las conexiones neuronales como la base física del aprendizaje y el soporte de la memoria (Morgado-Bernal, 2011).

Mucho más tarde, en 1973 Lomo y Bliss descubrieron que una estimulación de frecuencia moderadamente alta en determinadas células del hipocampo producía incrementos estables y duraderos de la respuesta postsináptica, lo que se denominó potenciación sináptica a largo plazo (PLP). Estudios posteriores in vitro confirmaron que se trataba de un fenómeno duradero y de inducción rápida, específica para cada estímulo y con capacidad asociativa a los mismos, lo que convertía a la PLP en un buen candidato a mecanismo celular del aprendizaje y la memoria (Bliss & Collingridge, 1993; Lomo, 2003).

Por otra parte, la memoria a corto plazo o retención consciente de una información durante un tiempo breve se basa en cambios efímeros, eléctricos o moleculares, en las redes neurales implicadas. Pero si, como consecuencia de la repetición de la experiencia estos cambios persisten, pueden activar el metabolismo celular para dar lugar a la síntesis de nuevas proteínas y cambios estructurales. Se da así una implicación simultánea de genes y sinapsis, en un proceso conocido como consolidación de la memoria (Alvarez & Squire, 1994; Dudai, 2004; Kandel, 2001; Kandel et al., 2014; McGaugh, 2002). Su resultado es el establecimiento de una memoria a largo plazo basada en cambios estructurales persistentes, como la formación de nuevas espinas dendríticas (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004).

Tras esta mencionada fase, puede existir un recuerdo, que se basa no solo en la información originalmente adquirida, sino también en los nuevos conocimientos, motivaciones, sentimientos y experiencias de toda índole que el sujeto recuerda. Es por tanto, un proceso activo cuyo resultado puede no ser idéntico a la experiencia original (Buckner & Wheeler, 2001; Maviel, Durkin, Menzaghi, & Bontempi, 2004). Los estímulos que activan el recuerdo podrían tener efectos diferentes según su duración. Los breves tienden a iniciar un proceso conocido como reconsolidación de la memoria, que consiste en que cuando una memoria ya consolidada se reactiva, se vuelve nuevamente lábil y es capaz de alterarse o recomponerse si en ese momento se introduce nueva información y, por tanto, en la memoria espacial los circuitos cerebrales implicados en la consolidación se reactivan en cada sesión de entrenamiento (Nader, 2003; Nader, Hardt, & Lanius, 2013; Nader, Schafe, & LeDoux, 2000; Sara, 2000). Además, las áreas implicadas pueden ser diferentes durante la consolidación temprana o durante la reconsolidación posterior a la reactivación de la memoria (Nader, 2003; Nader et al., 2000; Sara, 2000).

Del mismo modo que les conviene a los animales aprender y recordar respuestas para determinados estímulos, es adaptativo que la conducta se modifique una vez que las circunstancias ya no precisan de ella. El proceso por el que las respuestas condicionadas a los estímulos van decreciendo se denomina extinción. Para muchos autores, los mecanismos moleculares en la adquisición y o la consolidación de la memoria para la extinción serían similares a los de la adquisición y/o consolidación del miedo condicionado original (Bonini et al., 2011; Bruchey, Shumake, & Gonzalez-Lima, 2007; Myskiw, Fiorenza, Izquierdo, & Izquierdo, 2010; Szapiro, Vianna, McGaugh, Medina, & Izquierdo, 2003; Vianna, Igaz, Coitinho, Medina, & Izquierdo, 2003). Es decir, estaríamos hablando de un nuevo aprendizaje y la formación de una nueva memoria que, sin borrar la traza original, inhibe su expresión.

1.2.MEMORIA ESPACIAL

Tanto los humanos como el resto de los animales necesitamos disponer de un sistema de orientación espacial para organizar nuestras conductas en relación al entorno que nos rodea en cada momento. Muchas de estas conductas, como la búsqueda de comida o refugio, los comportamientos reproductivos y parentales o la huida de un potencial depredador y regreso al nido, requieren que seamos capaces de orientarnos en el espacio (Santin et al., 2000).

Se podría definir a la navegación espacial como una conducta compleja orientada a una meta, que requiere conocer tanto el lugar en el que el animal está como aquel al que quiere dirigirse. Este conocimiento requiere la codificación de información multimodal, que concierne la posición del cuerpo en relación con el entorno (R. Wang & Spelke, 2002). Esta capacidad de emplear distintas estrategias de navegación para encontrar un lugar en un entorno visitado con anterioridad se denomina Aprendizaje Espacial (Kessels, de Haan, Kappelle, & Postma, 2001). Por otra parte, la memoria espacial consistiría en múltiples mecanismos especializados en codificar, almacenar y recuperar información aprendida acerca de rutas, configuraciones y localizaciones espaciales (Kessels et al., 2001; Vicens et al., 2003).

Como hemos comentado anteriormente, la memoria espacial se considera principalmente declarativa y de tipo episódica. Desde el punto de vista temporal, al igual que en la memoria general, existen formas a corto y a largo plazo.

Existen numerosos estudios de aprendizaje y consolidación de la memoria en el que se utilizan distintas tareas y paradigmas en roedores. En el caso de la memoria implícita, como el Condicionamiento Pavloviano clásico, se puede estudiar con la tendencia natural de los roedores utilizados en experimentación, a escapar de un área altamente iluminada hacia una con condiciones de baja iluminación (step-*through method*), o la misma tendencia a evitar una descarga eléctrica que se señala o se deja de señalar mediante un estímulo condicionado, como un determinado tono (Evitación inhibitoria, *two way avoidance*) entre otros. Por otra parte, la memoria explicita (hipocampo-dependiente) (Eichenbaum, 1997a) tal como la memoria espacial, se ha estudiado tradicionalmente en laberintos radiales de brazos o laberintos de agua. Estos laberintos se utilizan con frecuencia para determinar si diferentes tratamientos o condiciones experimentales afectan a la memoria y el aprendizaje. Su resolución depende del uso de información espacial (Morgado-Bernal, 2011).

El primer intento de explicar la ejecución de tareas espaciales en animales se atribuye a E.C Tolman (1948). Este autor se basó en los resultados de estudios de discriminación espacial empleando el laberinto en T, para sugerir que los animales aprendían asociaciones entre lugares concretos y una recompensa. De este modo se formaba lo que denominó "Mapa cognitivo" de la estancia en la que se encontraban, permitiéndoles acceder al reforzador. De esta manera se forma una representación interna de la meta y su relación con los elementos de la estancia serían los que les llevarían a asociar la presencia o ausencia de recompensas con lugares (Delamater & Lattal, 2014; Tolman, 1948).

Estas afirmaciones se contraponían a las asociacionistas prevalentes en la época, de autores como Hull (1943) que defendían una respuesta de giro a derecha o izquierda desde el punto de elección, es decir, un aprendizaje de respuesta sin formación de representaciones a modo de mapa espacial.

Específicamente, Tolman distingue entre mapa cognitivo y la adquisición de hábitos según los contenidos de las representaciones y la flexibilidad con que se expresan las memorias. Siguiendo esta línea, Eichenbaum (Eichenbaum, 1997a, 1997b) defendió que *los animales aprenden a anticiparse a estímulos y a conectar los elementos del mapa, pudiendo crear inferencias en la navegación que les permite llegar a soluciones nuevas o rutas más cortas*, lo que denominó memoria relacional.

Ya en 1978, Okefee y Nadel publicaron su famoso libro "*The hipocampus as a cognitive map"* en el que proponen al hipocampo como la estructura cerebral que interviene en la confección de un mapa cognitivo, dándole a este concepto una entidad neurofisiológica, además de psicológica.

Estos autores consideraron dos sistemas distintos de navegación, estrategias táxicas y cartográficas, tal como se observa en la Tabla 1. Las táxicas serían aquellas en las que se emplean estrategias de orientación propioceptivas, existirían dos subtipos: De guía o De orientación; mientras que en las cartográficas se forman y emplean mapas espaciales del entorno.

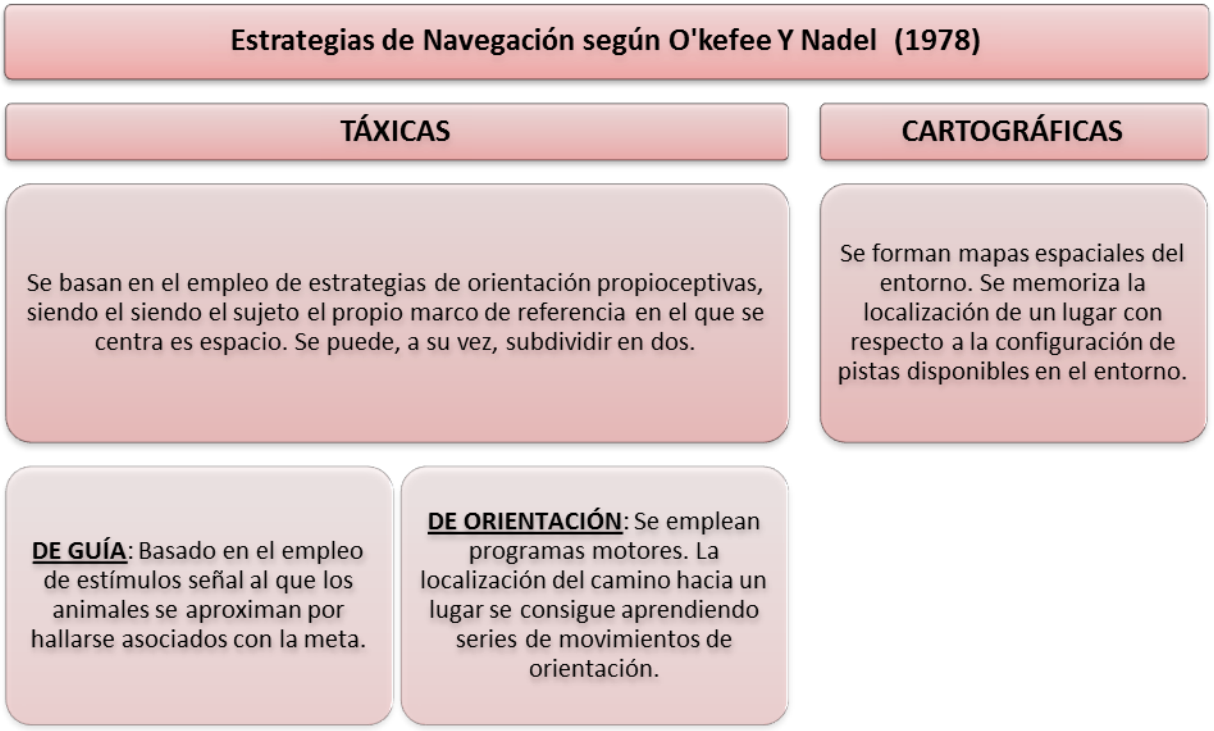

Tabla 1: Clasificación de las estrategias de Aprendizaje según O'kefee y Nadel en su libro *The hippocampus as a cognitive map* (1978).

1.2.1. EVALUACIÓN DEL APRENDIZAJE DE ORIENTACIÓN EN ANIMALES DE EXPERIMENTACIÓN, EL LABERINTO ACUÁTICO DE MORRIS.

Pocos años después de la publicación del libro de O'keefe y Nadel, R. Morris (1981) propuso que la localización espacial no dependía exclusivamente de la presencia de pistas locales, presentando uno de los paradigmas de mayor uso para el estudio de la orientación espacial en roedores, el laberinto acuático de Morris, conocido por sus siglas en inglés MWM (*Morris Water Maze*), que dio a conocer en el mismo año y con el que se publicaron experimentos pioneros en años posteriores (R. Morris, 1981, 1984), incluyendo los experimentos del grupo canadiense dirigido por Whishaw, Kolb y Sutherland que contribuyeron notablemente a su popularidad (Kolb, Mackintosh, Whishaw, & Sutherland, 1984; Kolb, Pittman, Sutherland, & Whishaw, 1982; Kolb, Sutherland, & Whishaw, 1983).

Aunque existen múltiples laberintos para el estudio de estos procesos en roedores, tal como el laberinto radial de ocho brazos (Amin, Pearce, Brown, & Aggleton, 2006) o el simplificado laberinto en T (Fidalgo, Conejo, Gonzalez-Pardo, & Arias, 2014b), el MWM sigue siendo una de las pruebas más utilizadas en Neurociencia de la Conducta, y particularmente en el caso que nos ocupa, para estudiar procesos de aprendizaje y memoria espacial. Su sensibilidad a diferentes manipulaciones experimentales ha hecho que las tareas que se puedan estudiar en él se incluyan como pruebas conductuales para evaluar el impacto de diferentes alteraciones del sistema nervioso, tales como daño cerebral (Conejo, Cimadevilla, Gonzalez-Pardo, Mendez-Couz, & Arias, 2013; R. G. Morris, Garrud, Rawlins, & O'Keefe, 1982), envejecimiento (Sampedro-Piquero, Zancada-Menendez, Begega, Mendez, & Arias, 2013; Villarreal, Gonzalez-Lima, Berndt, & Barea-Rodriguez, 2002) o distintos procesos neurodegenerativos (Arias, Fidalgo, Felipo, & Arias, 2014).

En el caso que nos ocupa, este paradigma ha sido de enorme utilidad en el estudio de las distintos tipos y fases que se dan en la memoria de orientación espacial, tanto en memoria a corto plazo, en la memoria de trabajo, y en el caso de la memoria de referencia espacial (Mendez-Lopez, Mendez, Lopez, & Arias, 2009b; Santin et al., 2003; Xavier, Oliveira-Filho, & Santos, 1999). Dentro de esta última, el laberinto acuático de Morris se ha utilizado para el estudio de las bases neurales que están implicadas tanto en los momentos iniciales de adquisición de la memoria de referencia espacial (Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2007; Okada & Okaichi, 2009) como en fases de consolidación (Leon, Bruno, Allard, Nader, & Cuello, 2010; Riedel et al., 1999) e incluso el recuerdo (Leon et al., 2010; Loureiro, Cholvin, et

al., 2012; Nanry, Mundy, & Tilson, 1989) y más recientemente la extinción de la tarea previamente adquirida en el laberinto acuático de Morris (Lattal, Mullen, & Abel, 2003; Mendez-Couz, Conejo, Vallejo, & Arias, 2014b).

1.2.2. BASES NEURALES DE LA MEMORIA ESPACIAL

Gracias a los estudios experimentales en los que se emplean paradigmas tales como el comentado Laberinto acuático de Morris, entre otros, en los últimos años los conocimientos sobre las bases neuroanatómicas del aprendizaje espacial en roedores se han incrementado progresivamente. A pesar de que la mayoría de los trabajos proponen a la formación hipocampal como mediadora en distintos sistemas de memoria implicados en la conducta (Alvarez & Squire, 1994; Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Muller & Stead, 1996; Muller, Stead, & Pach, 1996; Olton & Papas, 1979; Squire & Zola-Morgan, 1991), estudios más recientes enfatizan el papel de otras estructuras cerebrales interconectadas que median en ella, tales como los núcleos talámicos anteriores (Kolb et al., 1982; Mendez-Couz, Conejo, Gonzalez-Pardo, & Arias, 2015; van Groen, Kadish, & Michael Wyss, 2002), el núcleo accumbens (Seamans & Phillips, 1994) la corteza parietal, (Buckner & Wheeler, 2001; Kesner, DiMattia, & Crutcher, 1987; Kolb et al., 1983; Maguire et al., 1998), los cuerpos mamilares (Vann, 2010, 2011; Vann & Aggleton, 2004) o la corteza perirrinal (Bussey, Dias, Amin, Muir, & Aggleton, 2001; Jenkins, Amin, Brown, & Aggleton, 2006).

El modelo prevalente de consolidación de la memoria apoya una reorganización temporal de los circuitos neurales que sustentan la memoria a largo plazo (Bontempi et al., 1999; Frankland & Bontempi, 2005), específicamente, esta hipótesis se sustenta en las interacciones entre el hipocampo dorsal y el cortex prefrontal medial (mPFC). De acuerdo con este modelo, las memorias inicialmente hipocampo-dependientes serían almacenadas en un momento posterior en redes hipocampo corticales, para finalmente pasar a ser dependientes del neocortex (Frankland & Bontempi, 2005; Leon et al., 2010; McClelland, McNaughton, & O'Reilly, 1995; Smith & Squire, 2009; Squire & Alvarez, 1995). Existen numerosas evidencias que indican que existen diferentes circuitos neuronales que son activados durante las etapas tempranas o tardías de la adquisición de la memoria de referencia espacial (Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2004; Fidalgo et al., 2014b) o durante la consolidación de dicha memoria (Deiana, Platt, & Riedel, 2011). Sin embargo, los circuitos que subyacen a las fases posteriores como el recuerdo a largo plazo, o la extinción de esa memoria cuando la conducta no resulta adaptativa son aún un tema que provoca cierta controversia científica.

Centrándonos en la corteza prefrontal, numerosos estudios apuntan a su implicación directa en los procesos de memoria espacial, bien en la posición en el espacio en el que se encuentra un objeto (Kesner & Holbrook, 1987), así como en tareas de memoria espacial, tanto a corto como a largo plazo y memoria de trabajo (Blum, Hebert, & Dash, 2006; Churchwell & Kesner, 2011; Ragozzino, Adams, & Kesner, 1998) o en memoria de referencia espacial evaluada en laberinto de Morris (Conejo et al., 2013; Churchwell, Morris, Musso, & Kesner, 2010; Leon et al., 2010; G. W. Wang & Cai, 2008) entre otros.

El papel de la corteza prefrontal medial en la organización de la conducta de orientación espacial ha sido demostrado tanto en seres humanos como en otros primates no homínidos (Squire & Zola-Morgan, 1991). A pesar de la antigua polémica acerca de la existencia de la corteza prefrontal en mamíferos no primates, se ha confirmado que las ratas poseen corteza prefrontal medial que es comparable a la corteza prefrontal en humanos (Heidbreder & Groenewegen, 2003). Funcionalmente esta área ha sido implicada en procesos atencionales, el control de respuestas autonómicas y la emoción, así como en el procesamiento de la información espacial y la flexibilidad conductual (Heidbreder & Groenewegen, 2003; Ragozzino, 2007; Ragozzino, Detrick, & Kesner, 1999; Rich & Shapiro, 2007). La corteza prefrontal medial de la rata es una estructura funcionalmente heterogénea que puede dividirse en al menos tres áreas citoarquitectónicamente distinas: la corteza cingulada anterior, la corteza prelímbica (PL) y la corteza infralímbica (IL).

Dentro de la corteza prefrontal medial, en el área prelímbica, ha sido relacionada con respuestas vegetativas asociadas a la flexibilidad conductual en tareas de orientación espacial (Delatour & Gisquet-Verrier, 1999, 2001) así como con aspectos emocionales de la conducta. Estructuralmente, se puede subdividir en una zona dorsal y una zona ventral. La primera de ellas se conecta con zonas sensorimotoras y áreas corticales de asociación (Heidbreder & Groenewegen, 2003), en cambio, la zona ventral del área prelímbica recibe conexiones de algunas estructuras del circuito límbico, como el área CA1 ventral del hipocampo o el subículo (Degenetais, Thierry, Glowinski, & Gioanni, 2003) así como del complejo amigdalino (A. J. McDonald, 1998; R. J. McDonald & White, 1993). Pero son las conexiones entre el área ventral del área prelímbica y el hipocampo dorsal las que han hecho pensar en un papel significativo de ambas estructuras en la memoria espacial (Heidbreder & Groenewegen, 2003). Además, trabajos en los que se miden los cambios en el metabolismo oxidativo inducidos por el entrenamiento en el laberinto acuático de Morris han demostrado la implicación del área

prelímbica, junto con la región CA1 del hipocampo y los núcleos talámicos en la adquisición de la memoria de referencia espacial (Conejo, Gonzalez-Pardo, Gonzalez-Lima, & Arias, 2010; Conejo, Gonzalez-Pardo, et al., 2007). En este sentido, en estudios previos de nuestro grupo, basados en el análisis de cambios en el metabolismo oxidativo cerebral a lo largo de una tarea de adquisición de aprendizaje espacial, llevado a cabo en el laberinto acuático de Morris, se observa una implicación creciente de dicha área en las fases más tardías del aprendizaje, mientras que su participación es más discreta al inicio del mismo (Conejo et al., 2010). Esta activación tardía de la corteza prelímbica a lo largo del aprendizaje podría hacernos pensar en una función ligada a fases de consolidación o recuerdo de memoria espacial, más que a la propia adquisición, ya que, como se ha comentado previamente, ésta se da desde los primeros momentos del aprendizaje.

1.2.2.1. Circuitos neurales en la fase de recuerdo

Como previamente hemos comentado, existe controversia acerca de las áreas y circuitos implicados en el recuerdo de la memoria espacial. En esta línea, los estudios de lesión del córtex prefrontal muestran déficits tanto en la adquisición como en el recuerdo a corto plazo de la memoria espacial (Lacroix, White, & Feldon, 2002). Así, los trabajos que defienden una reorganización temporal de los circuitos neurales que subyacen el almacenamiento de la memoria a largo plazo (Bontempi et al., 1999; Frankland & Bontempi, 2005, 2006; Winocur, Moscovitch, & Bontempi, 2010) estarían de acuerdo con el incremento de la implicación de la corteza prefrontal observado por Conejo et al. (2010) a lo largo de un estudio de orientación espacial. Una de las hipótesis propuesta es que los recuerdos son almacenados en las redes hipocampo-corticales y posteriormente en la corteza, pero durante el recuerdo la mPFC puede actuar como un comparador "de desajuste", inhibiendo la actividad hipocampal para prevenir la re-codificación de las memorias ya existentes (Frankland & Bontempi, 2005). Estos trabajos parecen poner de manifiesto la importancia de las redes corticales-hipocampo y en particular la región prelímbica para la consolidación y el recuerdo de la memoria espacial, pero aún no se conoce con exactitud la contribución temporal-dependiente de esta estructura ni sus interacciones a lo largo de las fases de la memoria remota espacial.

Por añadidura al papel del hipocampo y del córtex prefrontal, otras regiones cerebrales han sido propuestas como soporte del recuerdo de la memoria espacial. En particular, se han señalado estructuras como los núcleos tálamicos (Loureiro, Cholvin, et al.,

2012), el estriado (Iaria, Petrides, Dagher, Pike, & Bohbot, 2003) o la menos mencionada porción ventral del hipocampo (Loureiro, Lecourtier, et al., 2012).

Análogamente, Aggleton and Brown (1999) postularon un "sistema hipocampal extendido" en el que el tálamo conjuntamente con el hipocampo se requerirían para la ejecución con éxito de tareas de memoria espacial. Es más, en este sentido, se ha apuntado a algunas subdivisiones del estriado como regiones clave en los sistemas de memoria procedimental, implícito o de hábito (Fidalgo, Conejo, Gonzalez-Pardo, & Arias, 2012; Packard, Hirsh, & White, 1989), y de forma paralela, han sido asociados con flexibilidad de comportamiento (Palencia & Ragozzino, 2005; Ragozzino, 2007). Por otra parte, a la porción ventral del hipocampo se le considera usualmente asociada con la modulación del estrés, las emociones y los afectos (Bannerman et al., 2004; Moser & Moser, 1998), asociando casi de manera exclusiva a la memoria espacial con la porción dorsal de esta estructura. Sin embargo, en los últimos años nuevos estudios han propuesto una continuidad funcional del hipocampo que se requeriría para comportamientos basados en aprendizajes rápidos de lugar (Bast, Wilson, Witter, & Morris, 2009), lo que también ha sido visto en tareas de recuerdo reciente de orientación espacial, mostrando en roedores una doble activación del hipocampo dorsal y ventral durante este tipo de tareas (Bontempi et al., 1999; Maviel et al., 2004).

1.2.2.2. Circuitos neurales implicados en Extinción

Como hemos explicado con anterioridad, si una conducta deja de sernos útil, no sería adaptativo seguir expresándola sin cambios. Esa conducta, por lo tanto, podría entrar en un proceso de extinción. Aunque la literatura sobre las estructuras y redes neurales de esta fase de la memoria espacial son muy escasos, de acuerdo a algunos autores (Huston, Schulz, & Topic, 2009) los procesos que ocurren en el laberinto de Morris seguirían las normas clásicas que subyacen al aprendizaje instrumental.

Siguiendo la mencionada teoría, se ha propuesto que los mecanismos moleculares que sustentan la adquisición y la consolidación de los procesos de extinción son similares a los que ocurren durante la adquisición o la consolidación del proceso original, específicamente descrito en aprendizajes de condicionamiento de miedo al contexto (Lattal et al., 2003; Szapiro et al., 2003). De este modo, la extinción podría entenderse como un nuevo

aprendizaje, lo que implica nueva formación de memoria, aunque preservando la memoria original. Este proceso estaría asociado, por tanto, con un descenso en la respuesta a las tareas de memoria (Bouton, Westbrook, Corcoran, & Maren, 2006).

Sin embargo, aunque existen multitud de estudios que tratan de explicar los circuitos neurales implicados en esta fase en aprendizajes de condicionamiento (Bouton et al., 2006; Bruchey et al., 2007; Cammarota, Bevilaqua, Vianna, Medina, & Izquierdo, 2007; Lattal et al., 2003; Szapiro et al., 2003; Telch et al., 2014; Vianna et al., 2003), los procesos neurales que subyacen a la extinción en el caso de un aprendizaje de orientación espacial no han sido esclarecidos.

Tomando como referencia los circuitos que subyacen a la consolidación de esa orientación espacial, se ha visto que existen múltiples conexiones entre estructuras corticales y subcorticales, como ya hemos descrito anteriormente (Bontempi et al., 1999; Conejo, Gonzalez-Pardo, et al., 2007; Fidalgo, Conejo, Gonzalez-Pardo, & Arias, 2014a; Maviel et al., 2004; Mendez-Lopez et al., 2009b). Los escasos estudios sobre extinción de una memoria de referencia espacial se han centrado principalmente en sus efectos a nivel de comportamiento (Huston, Silva, Komorowski, Schulz, & Topic, 2013; Prados, Manteiga, & Sansa, 2003; Prados, Sansa, & Artigas, 2008; Schulz, Huston, Buddenberg, & Topic, 2007; Topic et al., 2005; Vargas-Lopez, Lamprea, & Munera, 2011).

En los últimos años, algunos estudios han tratado de revelar cuáles serían las bases neurales de la extinción espacial, de una manera regional, aunque en la mayoría de los casos se han centrado en las consecuencias de esa extinción de la memoria espacial, como la indefensión o desesperación inducida por estos procesos de extinción. (Huston et al., 2009; Huston et al., 2013; Huston, van den Brink, Komorowski, Huq, & Topic, 2012; Topic, Oitzl, Meijer, Huston, & de Souza Silva, 2008). Este modelo animal de indefensión se utiliza actualmente para estudiar los procesos de depresión, ya que este es una de las características típicas del trastorno(Huston et al., 2013).

Más recientemente, unos pocos autores, como Porte et al. (2011) han tratado de establecer un mapa cerebral de este proceso, incluyendo estructuras como el hipocampo o la amígdala. Por otra parte, estructuras tales como cortex prefrontal medial o los cuerpos mamilares han sido relacionadas de manera paralela e independiente tanto en estudios de otras fases de la memoria espacial (Conejo et al., 2010; Loureiro, Lecourtier, et al., 2012;

Mendez-Lopez et al., 2009b; Vann, 2010, 2011) o en procesos de extinción de un aprendizaje previamente adquirido, lo cual ha sido ampliamente discutido en revisiones como las de Delamater (2004) o las más recientes del mismo autor (Delamater & Westbrook, 2014) o de Bouton et al. (2006).

1.3.TÉCNICAS Y MÉTODOS DE ESTUDIO PARA EL ESCLARECIMIENTO LAS ESTRUCTURAS IMPLICADAS EN MEMORIA ESPACIAL

1.3.1. INACTIVACIÓN CEREBRAL

Basándonos en la literatura, podemos pensar que la complejidad y variedad de procesos implicados en el aprendizaje y la memoria requieren una compleja aproximación para avanzar en la comprensión de los mecanismos neurobiológicos que los sustentan (Vazdarjanova, Cahill, & McGaugh, 2001). Sin embargo, aunque se ha avanzado mucho a nivel celular y molecular, se cree que solo se conseguirá una idea completa de los mecanismos que subyacen a los procesos de memoria avanzando en el conocimiento de la organización funcional de los circuitos cerebrales implicados en ella a nivel de sistema, aunque ésta sea la aproximación la más complicada de todas, según los trabajos de Kandel and Pittenger (1999).

El desarrollo de una gran variedad de técnicas de inactivación reversible ha dado lugar a un amplio rango de herramientas que nos permiten investigar los diferentes niveles de aprendizaje y organización de los procesos de memoria, desde que se introdujeron en los años 60 del siglo pasado (Breen & Mc, 1961; J. Bures & Buresova, 1960a, 1960b). Estas técnicas presentan numerosas ventajas frente a las más antiguas técnicas de inactivación o lesión permanentes. Por ejemplo, si un área se ve implicada en las diferentes etapas de la memoria, como adquisición, consolidación, recuerdo o extinción, la inactivación permanente de dicha área escondería su función en los procesos subsecuentes, sin embargo, esto no ocurre cuando se inactiva en una ventana temporal específica (E. J. Bures & Buresova, 1990). Además, si la misma área se requiere para varios procesos, mediante estas técnicas pueden conocerse sus papeles independientes en las distintas etapas, lo que puede ser muy útil cuando se pretende estudiar circuitos cerebrales que actúan en procesos independientes pero que se solapan temporalmente (E. J. Bures & Buresova, 1990; J. Bures, 1995; Rashidy-Pour, Motaghed-Larijani, & Bures, 1995). Otra de las grandes ventajas es el tamaño del área

lesionada, que es mucho menor y más precisa en este tipo de lesiones (Ambrogi Lorenzini, Baldi, Bucherelli, Sacchetti, & Tassoni, 1997; J. Bures, 1995; Riedel et al., 1999; Sun & Laviolette, 2012). Para una revisión completa del tema se puede consultar la revisión de Gallo (2007).

Para inactivar reversiblemente un área cerebral se pueden inyectar determinados fármacos en las áreas de interés. Éstos pueden ser de dos tipos: bloqueantes de sodio como la *Tetradotoxina* (TTX) o anestésicos locales que impiden la iniciación y la transmisión de potenciales de acción tanto en cuerpos celulares como axones, o bien agonistas y antagonistas de neurotransmisores que interfieren con la actividad neuronal al nivel de sinápsis.

Ambos requieren inyectar el fármaco a través de una microinyección (Martin & Ghez, 1999). Esta técnica es muy ventajosa para los estudios de comportamiento, ya que la cánula de inyección puede ser insertada en cánulas guías implantadas de forma crónica, además, en áreas cerebrales profundas, el procedimiento de microinyección permite la desactivación de regiones cerebrales más pequeñas que las técnicas de inyección usadas con anterioridad, por tanto, el daño al tejido circundante se minimiza debido al menor diámetro de la cánula (Lomber, 1999).

Según este mismo autor, el uso de antagonistas de receptores de neurotransmisores se convierte en una herramienta muy útil para inactivar sistemas y regiones específicas porque tiene ventaja de inactivar temporalmente las neuronas de un área evitando las fibras de paso (Lomber, 1999). Para regiones específicas tales como el hipocampo, los antagonistas de los principales receptores transmisores excitatorios, como el glutamato, pueden bloquear la actividad neuronal en el área.

En concreto, los agonistas de los receptores de $GABA_A$ que hiperpolarizan neuronas impidiendo la generación de un potencial de acción, se adecuan a la mayoría de las regiones cerebrales, porque los receptores $GABA_A$ tienen una amplia distribución en el sistema nervioso central. Entre estos fármacos, el agonista de $GABA_A$ *Muscimol* es el más común en los estudios de inactivación reversible. Al contrario que el bloqueo de corta duración provocado por GABA, que tiene una duración similar a los anestésicos locales, los efectos del muscimol persisten de 12 a 24 h. (Martin & Ghez, 1999; Riedel et al., 1999) lo que proporciona mayores garantías al asegurar que las pruebas realizadas tras la inyección del fármaco se realizan bajo su efecto. Sus ventajas en el campo de la memoria han hecho que sea utilizado en numerosos

estudios, por ejemplo para evaluar su implicación en la extinción del miedo condicionado, mediante la inactivación del área prelímbica (PL), infralímbica (IL), amígdala basolateral e hipocampo ventral (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011). Este fármaco también se ha empleado en estudios de adquisición (G. W. Wang & Cai, 2008), consolidación o recuerdo reciente de memoria espacial (Blum et al., 2006; Cholvin et al., 2014; Hobin, Ji, & Maren, 2006; Wartman, Gabel, & Holahan, 2014).

1.3.2. ANÁLISIS DEL METABOLISMO OXIDATIVO CEREBRAL EN EL ESTUDIO DE LA MEMORIA ESPACIAL

Una de las técnicas más utilizadas para el estudio del metabolismo oxidativo es *la tinción histoquímica de la citocromo c oxidasa,* que consiste en el marcaje del complejo IV de la cadena de transporte de electrones, también conocido *como citocromo c-oxidasa* (CO), enzima presente en la membrana mitocondrial interna, que pertenece a la clase óxido-reductasa. Esta enzima cataliza el último paso en la cadena de transporte electrónico acoplado al proceso de fosforilación oxidativa, permitiendo la producción de energía en forma de ATP. La enzima CO es muy antigua desde un punto de vista evolutivo, ya que está presente en la membrana de algunas células procariotas y se encuentra presente en todas las células eucarióticas (Wong-Riley, 1989).

La actividad CO es considerada como un marcador fiable de actividad neuronal. Su papel crítico en el metabolismo energético y la dependencia del cerebro del metabolismo aeróbico apoya la teoría de que el nivel de actividad de la CO dentro de las neuronas se correlaciona positivamente con su nivel de actividad funcional (Wong-Riley, 1989). Además, la actividad de esta enzima representa un índice de capacidad metabólica mitocondrial (Bertoni-Freddari et al., 2001), que se asocia con las demandas energéticas de las neuronas tras una estimulación prolongada (Gonzalez-Lima & Jones, 1994; Wong-Riley, 1989, 2012). La actividad neuronal comprende la síntesis de neurotransmisores y otras moléculas, el transporte axoplasmático y el transporte activo de iones, siendo éste último el mayor consumidor de energía. Al aumentar la actividad neuronal la neurona puede obtener energía mediante dos fuentes diferentes: para restablecer el potencial de membrana utilizan la Na^+-K^+ ATPasa, en los demás casos se necesita un aumento del flujo sanguíneo y del consumo de glucosa que producirá un aumento de la respiración celular con el consiguiente aumento de actividad de la CO y síntesis de ATP (Kennedy, des Rosiers, Reivich, & Sokoloff, 1974)

El estudio histológico de la actividad de esta enzima ya ha sido usado previamente para elaborar mapas de los cambios regionales en el metabolismo cerebral que se subyacen la ejecución de una gran variedad de comportamientos en diferentes especies animales (Agin, Chicher, & Chichery, 2001; Puga, Barrett, Bastida, & Gonzalez-Lima, 2007). Es más, algunos autores han dado a conocer cambios en la actividad CO relacionados con tareas de memoria y aprendizaje que se realizaban en el laberinto acuático de agua (Conejo et al., 2013; Mendez-Couz et al., 2015; Mendez-Couz et al., 2014b; Riha, Rojas, & Gonzalez-Lima, 2011; Villarreal et al., 2002). Este método ha demostrado ser de utilidad para detectar tanto las diferencias en la capacidad metabólica de determinadas regiones cerebrales de manera específica, como para investigar su conectividad funcionalidad (Sakata, Coomber, Gonzalez-Lima, & Crews, 2000). Siguiendo esta línea, las regiones cerebrales que están funcionalmente acopladas y los consiguientes cambios en estas asociaciones pueden expresarse como los cambios en la fuerza de las correlaciones directas de actividad CO entre regiones cerebrales (Puga et al., 2007; Sakata et al., 2000).

1.3.3. ANÁLISIS MEDIANTE TÉCNICAS INMUNOHISTOQUÍMICAS

1.3.3.1. EXPRESIÓN DE GENES DE EXPRESIÓN TEMPRANA

Actualmente se sabe que la formación de memorias implica reajustes estructurales de las sinapsis y que estos procesos requieren la síntesis de nuevas proteínas (Tischmeyer & Grimm, 1999). Uno de los primeros procesos que ocurren tras la aparición de un nuevo estímulo es la expresión de factores de transcripción. Estos factores son complejos proteicos cuya función es la coordinación y la regulación de la transcripción génica. Estos complejos interaccionan con regiones específicas del ADN tras haber sido activadas. AP-1 (*Activating Protein* 1) es una familia de factores de transcripción dentro de la cual se incluyen las familias de fos y jun, proteínas que se fusionan formando dímeros o heterodímeros que se unen al ADN y regulan de esta manera numerosos procesos celulares. Estas proteínas son sintetizadas a partir de unos genes denominados genes de expresión temprana (IEGs), que se activan en respuesta a cascadas de señalización intracelular. En los últimos años se han hecho esfuerzos por identificar y caracterizar genes de activación temprana (IEG) inducidos por actividad neuronal como herramienta para explorar las bases celulares y moleculares de la actividad

cerebral (Amin et al., 2006; Jenkins et al., 2006; Pothuizen, Davies, Albasser, Aggleton, & Vann, 2009; Tischmeyer & Grimm, 1999; Ugajin, Kunieda, & Kubo, 2013).

Uno de los genes de expresión temprana más utilizados como marcador de la actividad neuronal es c-fos. El interés por utilizar la proteína c-Fos como marcador surgió en los años 80 cuando se observó que esta proteína controlaba varios fenómenos incluyendo el aprendizaje y la memoria (Kaczmarek, Lapinska-Dzwonek, & Szymczak, 2002), lo que desencadenó su utilización en numerosos estudios relacionados con estos fenómenos conductuales. Concretamente, la expresión de c-fos se sigue utilizando para estudiar procesos como condicionamiento del miedo (Conejo, Gonzalez Pardo, Lopez, Cantora, & Arias, 2007) (B. M. Thompson et al., 2010), aversión al sabor (Dossat, Lilly, Kay, & Williams, 2011), aprendizaje espacial (Mendez-Couz, Conejo, Vallejo, & Arias, 2014a; Mendez-Lopez, Mendez, Lopez, & Arias, 2009a; Mendez et al., 2008; Santin et al., 2003; Vann, Brown, & Aggleton, 2000) entre otros.

El gen c-fos pertenece a la familia de los genes de expresión temprana (IEGs) porque su inducción es una de las primeras respuestas celulares que sucede tras la aplicación de una gran variedad de estímulos, como estímulos osmóticos, lumínicos, visuales, sensoriales, estrés o conductuales (Sharp, Sagar, & Swanson, 1993). Además, su inducción es rápida y transitoria, por lo que después de una estimulación celular, sus niveles retornan al nivel basal en varias horas (Sharp et al., 1993). La proteína que este gen codifica, proteína c-Fos, causa la despolarización de la membrana y el la apertura de canales de calcio dependientes de voltaje, lo que da lugar a cambios en la actividad neuronal (Morgan & Curran, 1989). La inducción de la expresión de c-fos se ha relacionado con la actividad neuronal que sucede debido a la estimulación por aprendizaje. (Radulovic, Kammermeier, & Spiess, 1998; Tischmeyer & Grimm, 1999). Además, existen ejemplos en la literatura que han demostrado que esta técnica resulta de utilidad para estudiar la plasticidad neuronal que se requiere para los estudios de memoria espacial (Mendez-Couz et al., 2014b; Mendez-Lopez et al., 2009a; Pothuizen et al., 2009; Tischmeyer & Grimm, 1999; Vanelzakker et al., 2011).

1.3.3.2. ANÁLISIS DE LA EXPRESIÓN DEL NEUROPÉPTIDO Y.

Desde hace décadas se conoce que el neuropéptido Y tiene múltiples funciones en el sistema nervioso central (SNC) y periférico de los mamíferos, jugando un papel importante en funciones como la ansiedad, la conducta digestiva, la regulación de la presión arterial, en los

ritmos circadianos biológicos o la conducta (Thorsell & Heilig, 2002). El papel del neuropéptido Y (NPY) como modulador de los procesos de memoria ha sido menos estudiado, aunque se sabe que su administración en el SNC de roedores mejora la retención de tareas de memoria implícita, como la evitación activa, y que parece tener un efecto temporalmente dependiente (Flood, Hernandez, & Morley, 1987).

En este sentido, es sabido que influye sobre la memoria a corto y largo plazo de manera dosis-dependiente en forma de U invertida, con bajas dosis mejorando la memoria a bajas dosis y deteriorándola a altas (Flood et al., 1987; Thomas & Ahlers, 1991). Además, sus efectos sobre la memoria parecen ser dependientes de la región cerebral donde actúe. De esta forma, la administración del NPY intracerebral a nivel de la porción rostral del hipocampo o del septum mejora la retención de la memoria en un test de evitación activa de un shock eléctrico en un laberinto en T mientras que su administración a nivel del hipocampo caudal o la amígdala deteriora la memoria, posiblemente por su efecto sobre la ansiedad o el estrés difícil de discriminar en este tipo de test (Flood et al., 1987; Heilig, 2004).

Hasta el momento se han descrito al menos 6 tipos de receptores para el NPY (Goncalves, Martins, Baptista, Ambrosio, & Silva, 2015). Los más estudiados en cuanto a su efecto sobre la memoria son el receptor NPY Y_1 (Y_1R), Y_2 ($Y2R_)$ y NPY Y_5 (Y_5R), (Xapelli, Agasse, Ferreira, Silva, & Malva, 2006) ya que se sabe que en roedores el sistema NPY a nivel cerebral ejerce sus efectos a través de ellos. Como se podría pensar, se encuentran, entre otras, en regiones estrechamente relacionadas con la memoria espacial. Concretamente, el RNA mensajero para Y1 y los receptores proteicos codificados se ha encontrado en la corteza cerebral, el giro dentado del hipocampo, la amígdala, y distintos núcleos talámicos e hipotalámicos y en niveles moderados en la corteza frontoparietal y diversas capas piramidales del Cuerno de Ammon (Jacques, Tong, Dumont, Shen, & Quirion, 1996; Larsen, Mikkelsen, Jessop, Lightman, & Chowdrey, 1993). Por otro lado, el receptor Y_2 es mayoritario en el SNC y se detecta sobre todo en la formación hipocampal, principalmente en la región CA3, pero también en el núcleo del lecho de la estría terminal (BNST), septum lateral, amígdala e hipotálamo, la corteza piriforme, la sustancia negra, entre otros. (Dumont, St-Pierre, & Quirion, 1996; Gustafson et al., 1997; Parker & Herzog, 1999).

Actualmente existen pocos trabajos acerca del efecto del sistema NPY en memoria espacial, aunque la escasa literatura existente apunta a una implicación de este sistema en la misma (Thorsell et al., 2000). No existe, sin embargo, un acuerdo común acerca de la mayor o

menor implicación de receptores Y_1 o Y_2. En este sentido, se ha visto un efecto potenciador de la neurogénesis en el giro dentado del hipocampo mediado por el receptor Y_1 (Howell et al., 2005) que podría relacionarse con un efecto neuroprotector o bien facilitador de la memoria espacial. Por otra parte, también se han demostrado efectos neuroprotectores del receptor Y_2 contra cambios producidos por drogas como la neuroanfetamina a nivel hipocampal (Goncalves et al., 2012) y otras drogas de abuso (Goncalves et al., 2015) así como contra los efectos del estrés oxidativo en el comportamiento depresivo y las alteraciones de memoria espacial en modelos murinos de alzhéimer (dos Santos et al., 2013).

1.3.4. ANÁLISIS POR WESTERN BLOT.

La técnica de *western blot* es a día de hoy una de las más utilizadas en biología molecular, y no ha tardado demasiado en ser incluida entre uno de los métodos habituales de estudio de los cambios moleculares debidos a la conducta.

Esta técnica nació en los años 70 para evaluar de manera específica una proteína concreta. Se desarrolló de manera paralela por dos laboratorios distintos usando distintos geles, poliacrilamida-urea en el caso de Towbin y SDS-poliacrilamida en el de Burnette, como describen en sus recientes revisiones Towbin (2009) y Burnette (2009) respectivamente. Este último método, S*odium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis* o SDS-PAGE, es el más empleado actualmente. Se ha convertido en una herramienta de estudio muy útil en el campo que nos ocupa gracias a su utilidad para estudiar la presencia, abundancia relativa, masa molecular relativa o modificaciones postraduccionales de proteínas. En esta línea, existen numerosos estudios que muestran su aplicabilidad en el análisis de la memoria espacial. Como ejemplo de esta última utilidad, podríamos citar el estudio de Porte et al. (2011), quienes mostraron cambios en el nivel de fosforilación del factor de transcripción de la proteína de unión a elementos de respuesta de AMP cíclico (conocido por sus siglas en inglés CREB), de manera regional en el CA1 del hipocampo tras una tarea de extinción de memoria espacial. En la misma línea se encuentran estudios de expresión de la subunidad I de la proteína citocromo C oxidasa en relación a una tarea de orientación espacial, en este caso para el estudio de las estructuras implicadas en la ejecución de un aprendizaje de respuesta (Fidalgo, Conejo, Gonzalez-Pardo, Lazo, & Arias, 2012), aunque en este caso no se encontraron diferencias regionales en la expresión de dicha subunidad asociadas con el aprendizaje de discriminación visual.

Objetivos

OBJETIVOS

El aprendizaje y la memoria espacial son vitales en los animales para orientarse y aprender la posición de lugares de interés en su ambiente, para mantener el sentido de la dirección y la localización mientras nos movemos en él, y para adaptarnos a nuevos entornos. Esta habilidad, conocida como orientación espacial, o memoria espacial, representa una de las funciones cognitivas más básicas y esenciales, y tiene la característica de ser particularmente compleja, ya que el sistema nervioso ha de ser capaz de integrar la información multisensorial de nuestro entorno a lo largo del tiempo y en el espacio.

Hasta el momento, se conoce que esta memoria espacial se compone de varias fases, que a su vez implican a distintas estructuras cerebrales mayoritariamente del sistema límbico. Además, se sabe que estas estructuras presentan una participación diferencial y temporalmente dependiente, tanto de forma discreta como en su relación funcional con otras estructuras, para formar redes neurales de distinta manera a lo largo del proceso de adquisición de un aprendizaje de orientación espacial. (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Frankland & Bontempi, 2005; Maviel, Durkin, Menzaghi, & Bontempi, 2004). Así pues, aunque la hipótesis prevalente, que ha sido apoyada por numerosos trabajos recientes entre los que se encuentran muchos de nuestro grupo de investigación, es que la memoria espacial depende del estrecho acoplamiento entre estructuras límbicas como la corteza prefrontal medial y el hipocampo, en las diferentes fases de estos procesos; (Aggleton & Pearce, 2001; Conejo, Gonzalez-Pardo, Gonzalez-Lima, & Arias, 2010; Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2007; Fidalgo, Conejo, Gonzalez-Pardo, & Arias, 2014; Mendez-Lopez, Mendez, Lopez, Cimadevilla, & Arias, 2009; Okada & Okaichi, 2009; Remondes & Schuman, 2004; Santin, Rubio, Begega, Miranda, & Arias, 2000) todavía no se han esclarecido las redes cerebrales que sustentan otras fases posteriores de la memoria espacial, tales como el de recuerdo posterior, o la extinción de la misma cuando dicha conducta ya no resulta adaptativa.

El discernimiento de las bases neurales de estos procesos es indispensable para conocer y poder intervenir terapéuticamente y de forma óptima en trastornos psicológicos que impliquen mecanismos de aprendizaje y memoria, como trastornos de ansiedad, o en

aquellos procesos en los que este tipo de memoria, que además resulta un buen indicador de deterioro cognitivo, se ve alterada, tales como demencias, lesiones o traumatismos cerebrales u otras alteraciones neuropsiquiátricas que cursan con alteraciones nmésicas, tales como la encefalopatía hepática o la esquizofrenia.

Por todo lo anterior, en el presente trabajo estudiamos las estructuras y redes neurales implicadas en los procesos de recuerdo y extinción de una tarea de memoria espacial en el laberinto acuático de Morris. Con este fin nos planteamos los siguientes objetivos:

Primer Objetivo: Evaluar conductualmente a ratas machos adultas en una tarea de aprendizaje de orientación espacial que implica componentes de memoria a largo plazo. Analizar la capacidad de recuerdo de la tarea a la semana de finalizar el aprendizaje en un grupo de animales, y en otro grupo la capacidad de extinción de la conducta aprendida. (Artículos 1,4 y 5).

Segundo Objetivo: Estudiar los cambios inmediatamente evocados en la actividad cerebral tras la tarea de aprendizaje espacial, mediante inmunohistoquímica para la proteína de expresión temprana c-Fos (Artículo 5).

Tercer Objetivo: Analizar, en los mismos animales, los cambios en la actividad metabólica cerebral a nivel regional, haciendo uso de la histoquímica de la citocromo oxidasa (CO) (Artículos 1 y 4).

Cuarto Objetivo: Realizar análisis de correlación aplicados a los datos de la actividad CO para determinar la relación entre diferentes estructuras cerebrales, de tal manera que podamos conocer y comprender las posibles vías o redes neurales, así como los mecanismos plásticos cerebrales que sustentan los procesos de recuerdo y extinción de la memoria espacial. (Artículos 1 y 4).

Quinto Objetivo: Evaluar los efectos de la inactivación cerebral de aquellas regiones cerebrales del sistema límbico previamente analizadas, en las fases de recuerdo y extinción en la orientación espacial en roedores. Así como estudiar los efectos de dichas inactivaciones en la participación diferencial y en las redes funcionales formadas por las estructuras relacionadas, en la ejecución de la tarea de recuerdo o extinción de la memoria espacial. (Artículos 2, 3, 6 y 7).

Sexto Objetivo: Determinar los cambios en la expresión de proteínas de señalización intracelular implicadas en los cambios en plasticidad sináptica, que se creen asociados con los procesos de memoria, tales como el neuropéptido Y (NPY) en aquellas estructuras cerebrales que muestran una relación directa con la conducta evaluada y provocadas por la inactivación cerebral (Artículo 8).

Material y Métodos

MATERIAL Y METODOS

3.1.ANIMALES

Para la realización de esta tesis doctoral se emplearon ratas macho adultas (*Rattus norvegicus)* de la cepa Wistar (260-360g) procedentes del bioterio de la Universidad de Oviedo y del bioterio de la Universidad de Sevilla. Los animales de experimentación fueron seleccionados aleatoriamente de diferentes camadas y se alojaron en grupos de 5 animales por jaula, manteniéndose durante el experimento un ciclo de luz/oscuridad de 12 horas (periodo de luz: 08:00-20:00 h), temperatura ambiental de 23±2ºC, humedad absoluta de 65±5%, con acceso libre a comida y bebida. Todos los procedimientos experimentales fueron aprobados por el Comité Veterinario del Bioterio de la Universidad Oviedo y su manipulación posterior se realizó, en todo momento, de acuerdo con la Directiva del Consejo de las Comunidades Europeas del 24 de noviembre de 1986 (86/609/EEC) y legislado en nuestro país mediante el Real Decreto 53/2013.

De acuerdo a los requerimientos propios de cada experimento, los animales se dividieron en distintos grupos experimentales sometidos diversas manipulaciones y protocolos conductuales. La división específica en grupos y los procedimientos a los que éstos fueron sometidos en cada experimento se muestran en detalle en cada uno de los Artículos.

3.2.APARATOS

3.2.1. LABERINTO ACUÁTICO DE AGUA

Para evaluar de aprendizaje de orientación espacial en roedores (Artículos 1-8) se utilizó el laberinto acuático de Morris (Morris *Water Maze* MWM), que consiste en una piscina circular negra (150cm de diámetro y 40 cm. de altura) fabricada en fibra de vidrio y sustentada sobre una plataforma de 35 cm. de altura (Morris, 1984). La piscina estaba situada en el centro de una habitación de 16m^2, rodeada de numerosas pistas visuales como figuras geométricas de colores o globos fijados a paneles negros que se colocaron en paneles negros alrededor del laberinto. Estas pistas visuales son utilizadas por el animal para guiarse, siguiendo una estrategia alocéntrica. El nivel del agua era de 30 cm. y su temperatura de 21±2ºC. La piscina

se dividió en cuatro cuadrantes imaginarios iguales (A, B, C y D), en uno de los cuales se sumergió una plataforma circular (plataforma de escape) con un diámetro de 10 cm. y una altura de 28 cm. quedando oculta 2 cm. por debajo del nivel del agua. Cada ensayo se grabó mediante un sistema de grabación de ruta basada en imágenes (EthoVision XT, Noldus, Wageningen, Paises Bajos) mediante el que se registró tanto el tiempo que los animales pasaban en cada cuadrante virtual o en el lugar de la plataforma, como la trayectoria seguida por los animales o el tiempo que tardaban en alcanzar el objetivo (denominado "latencias de escape") (Figura 2).

En todos los casos, los ensayos de los experimentos realizados en este paradigma tuvieron lugar entre las 09:00-14:00h.

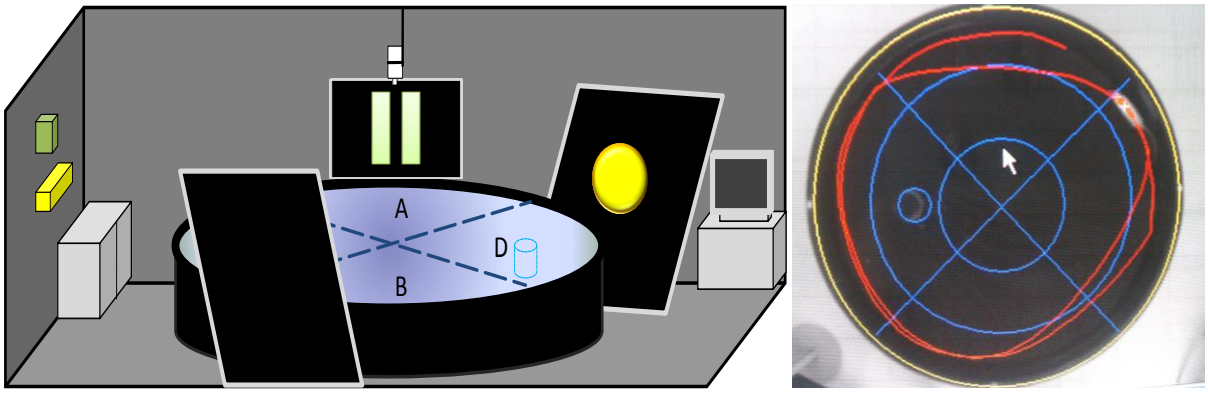

Figura 2: Izquierda: Representación esquemática de la conformación de la sala en la que se llevó a cabo el entrenamiento y evaluación de la memoria espacial. Derecha: Imagen generada por el software Ethovision en el que se aprecia la trayectoria típica de un animal en el primer día de entrenamiento.

3.2.2. ACTÍMETROS

La evaluación de la actividad horizontal espontánea (Artículo 8) se evaluó mediante el uso de cajas de actímetros. Cada una de ellas está formada por una caja transparente de material acrílico que incorpora una cámara de video a modo de registro en la parte superior (Noldus PhenoTyper, Países Bajos) (FIGURA3). A lo largo de cada sesión se obtuvieron grabaciones automáticas de la distancia recorrida por los animales usando al igual que se

explicó en el apartado anterior, un software de análisis de seguimiento en vídeo (EthoVision XT, Noldus, Wageningen, Países Bajos).

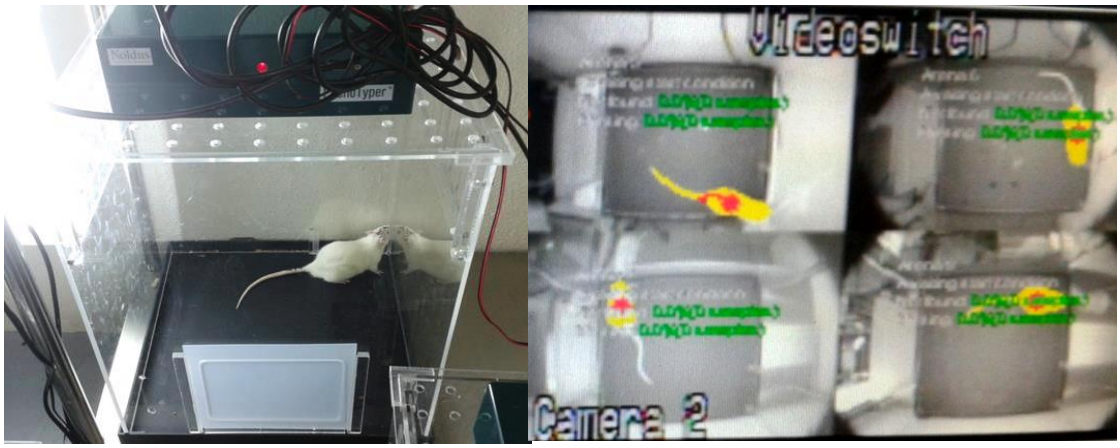

Figura 3: En la imagen izquierda se muestra un momento de habituación de un protocolo de medición de la actividad horizontal espontánea en el cual se puede observar al animal explorando libremente el interior de la caja de actímetro. Derecha: Ejemplo de registro automático de la actividad mediante el software integrado.

3.2.3. LABERINTO EN CERO ELEVADO

El laberinto en Cero Elevado (EZM) se utilizó para medir conductas de ansiedad (Artículo 8). El aparato consta de una plataforma circular plana de material acrílico de 10 cm de ancho y 81 cm de diámetro que se encuentra elevada 81 cm del suelo (Noldus Information Technology). La superficie del laberinto circular está dividida funcionalmente en cuatro secciones de igual longitud, dos áreas "abiertas" y dos "cerradas". Estas últimas se encuentran flanqueadas por paredes del mismo material que el suelo, de 35 cm de altura. Las variables de estudio registradas incluyeron la distancia total caminada por los animales y el tiempo que éstos estuvieron en los brazos abiertos del laberinto. Los movimientos del animal se grabaron utilizando el mismo software mencionado para el caso anterior (FIGURA 4).

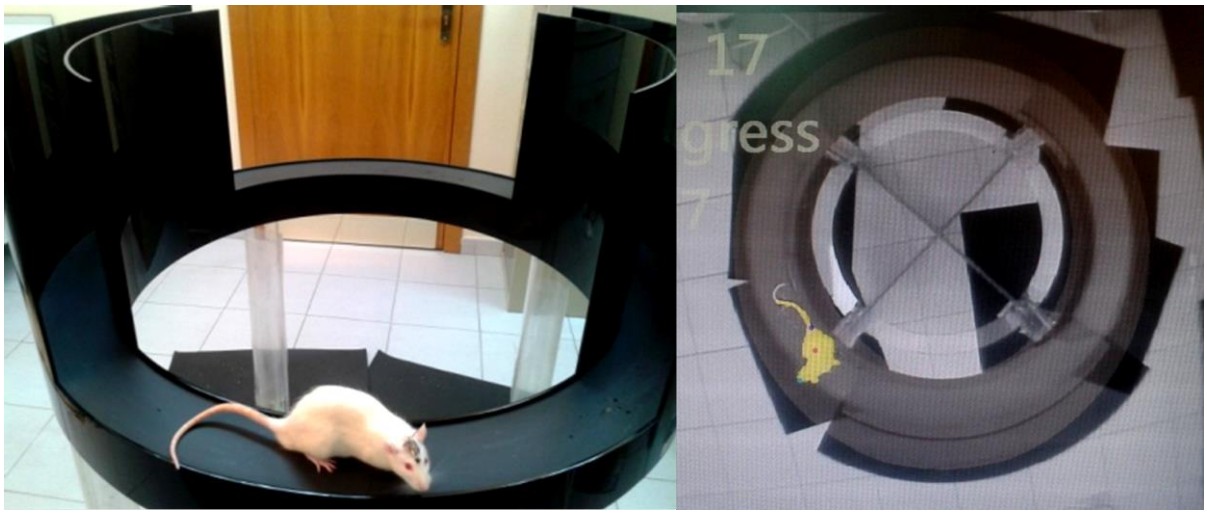

Figura 4: Imágenes de un momento de la prueba de ansiedad en el EZM (izquierda), y una imagen registrada por EthoVision Pro Noldus durante esta prueba (derecha)

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3.2.4. PROTOCOLO EXPERIMENTAL CONDUCTUAL

Pruebas neurológicas

Una semana antes de comenzar con las fases conductuales de todos los experimentos, los animales fueron manipulados diariamente durante un periodo de 5-10 minutos, reduciendo de este modo los comportamientos de ansiedad asociado con el manejo experimental. Durante este periodo se les pasó una batería de pruebas neurológicas para descartar posibles alteraciones. Concretamente la evaluación neurológica consistió en evaluar la abducción de las patas traseras, el asimiento, los reflejos de extensión y flexión, la respuesta vestibular/auditiva, la sacudida de cabeza, la respuesta geotáctica negativa y el reflejo de enderezamiento (Bures, Buresova, & Huston, 1976).

En el caso de los animales sometidos a cirugía estereotáxica (Art. 2, 3, 6-8), tras el periodo de recuperación postoperatoria, fueron nuevamente evaluados mediante la misma batería de pruebas neurológicas anteriormente descrita, con el fin de descartar posibles alteraciones motoras o sensoriales causadas por el procedimiento de cirugía.

Protocolos de memoria de referencia espacial en el laberinto acuático de Morris.

Para evaluar la capacidad de memoria de referencia se instruyó a los animales mediante un protocolo que consistía en liberar a la rata en la superficie del agua del laberinto acuático, siempre orientada hacia la pared mismo y usando como punto de inicio los cuatro puntos cardinales diferentes en los cuales la piscina fue virtualmente dividida. Dichos puntos fueron seleccionados en un orden pseudoaleatorio. La rata debía llegar desde estos puntos hasta la plataforma y obtener así la recompensa de dejar de nadar. Cada prueba tuvo una duración máxima de 60 segundos, tras los cuales el animal se guió hacia la plataforma si aún no la ha encontrado, y se le dejó permanecer en la misma durante 15 segundos, mientras que el intervalo entre pruebas fue de 30 segundos.

Habituación

La primera parte de este protocolo, cuyas fases están representadas en la FIGURA 5, consistió en un día de habituación, cuya finalidad era la de reducir el estrés que puede producir el contacto con el agua así como el procedimiento utilizado para el entrenamiento en la piscina de Morris. Los animales se trasladaron a la habitación en su caja de estabulación y se dejaron allí durante cinco minutos antes de cualquier manipulación. Posteriormente se les sometió a dos sesiones de cuatro ensayos de entrenamiento en cada una, durante los que la plataforma se encontraba visible 2 cm. por encima del agua. Para ello, los animales fueron liberados pseudoaleatoriamente desde cada uno de los puntos en los que divide la piscina.

Adquisición

Los cinco días siguientes corresponden a la fase de aprendizaje de la tarea de memoria de referencia. Durante esta etapa los animales siguieron un protocolo de entrenamiento con una sesión de cuatro ensayos diaria. A diferencia de la fase de habituación, la plataforma se situó sumergida 2 cm. por debajo de la superficie del agua y por tanto no visible para los animales.

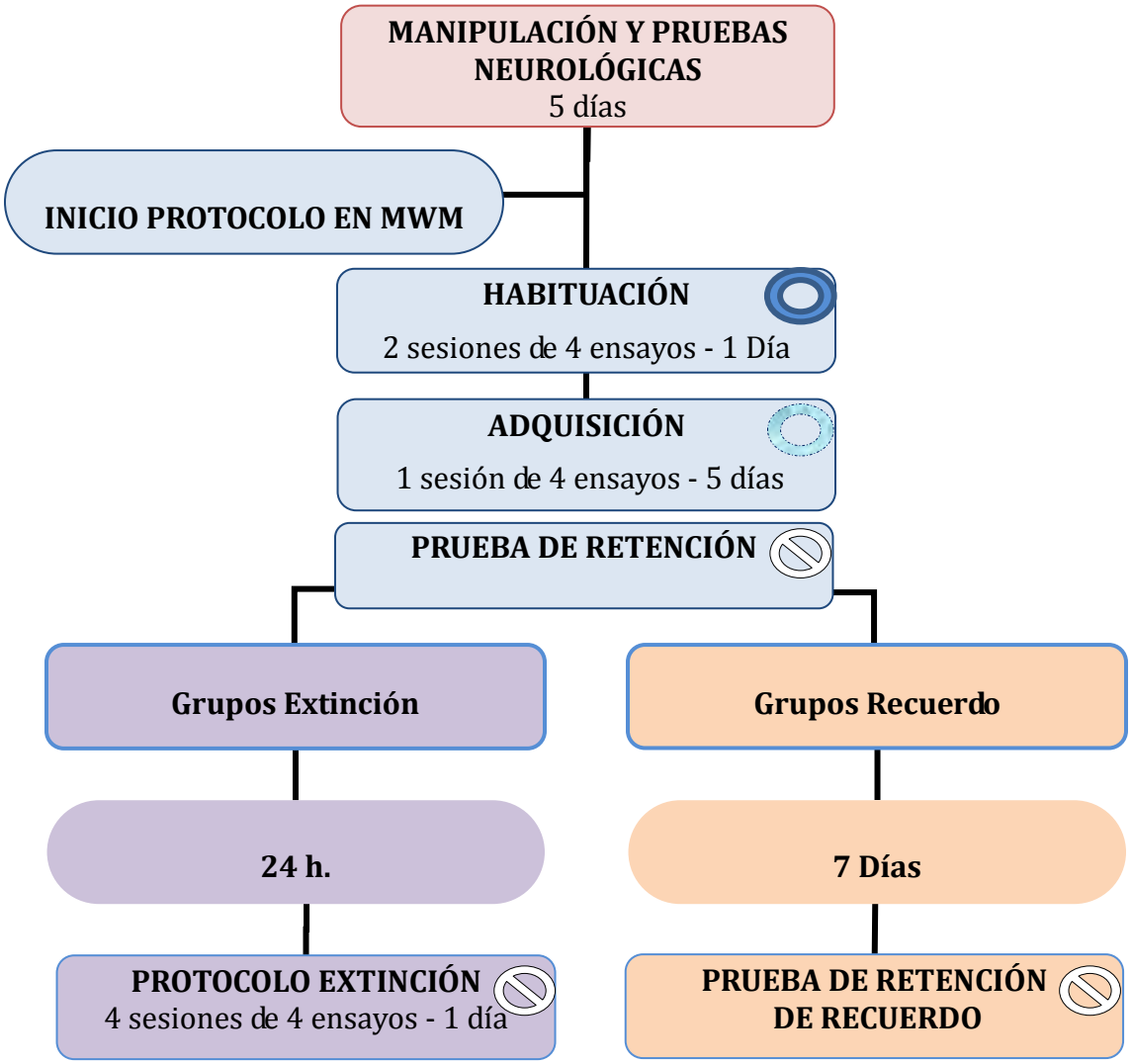


Figura 5: Línea temporal de los experimentos de memoria de referencia llevados a cabo en el laberinto acuático de Morris (MWM). El círculo con línea continua representa a la plataforma visible en el laberinto, línea discontinua: plataforma invisible y círculo con línea diagonal: ausencia de plataforma.

El quinto día, una vez finalizado el aprendizaje, se quitó la plataforma, para realizar la prueba de retención o ensayo de prueba (transfer). Esta prueba consiste en retirar la plataforma del laberinto y posteriormente liberar al animal desde el cuadrante contralateral al que estaba localizada previamente la plataforma, dejándole nadar durante 60 segundos. Posteriormente se evalúa tanto el tiempo medio que el animal emplea para llegar a la plataforma, denominado "latencias de escape", así como cuánto tiempo se encuentra la rata en el cuadrante de escape (D). Para evitar la extinción temprana de la conducta tras la prueba de

retención se añade un ensayo igual que los explicados en la fase de adquisición, en el que la plataforma se encuentra nuevamente presente en el laberinto. En este caso los animales son liberados desde el cuadrante B.

Recuerdo

En los experimentos en que se evaluó la capacidad de recuerdo a largo plazo los animales fueron devueltos al animalario y se estabularon en condiciones normales durante 7 (Art. 1, 2) o 30 días (Art. 2). Pasado este periodo se les sometió a una nueva prueba de retención en condiciones idénticas a la anteriormente explicada (FIGURA 5).

Extinción

Para evaluar la capacidad de extinción de la conducta previamente adquirida (Art. 4-7) se siguió el protocolo de extinción propuesto por Rossato, Bevilaqua, Medina, Izquierdo, and Cammarota (2006). De acuerdo con este procedimiento, se retiró la plataforma del laberinto y los animales se sometieron a cuatro sesiones que incluían cuatro ensayos de un minuto de duración cada uno, 16 en total, en el que nadaron libremente en el laberinto. El intervalo entre ensayos fue de 30 segundos y el tiempo entre sesiones fue de 30 min. (FIGURA 5).

Protocolo de estudio de la influencia del sistema NPY en la memoria espacial

Por otro lado, se valoraron los efectos de la administración del antagonista del receptor NPY Y_2R en conductas de hiperactividad, posibles efectos anxigénicos o ansiolíticos y sus efectos en memoria espacial (explicado detalladamente en el Artículo 8). Para ello se usaron distintos paradigmas conductuales que se explican a continuación.

En la FIGURA 5 se observa la línea temporal de realización de los experimentos a los que se sometieron los dos grupos de animales que se incluyeron en este Artículo. Como se puede observar, la infusión de la droga cuyos efectos pretendíamos analizar sucedió siempre 30 minutos antes de cualquier prueba conductual (actividad horizontal espontánea, conductas de tipo ansioso y test de aprendizaje en el laberinto acuático de Morris).

Actividad horizontal espontánea

La primera prueba realizada a los animales del Artículo 8 consistió en el análisis de la actividad horizontal espontánea de los animales. Con este fin, las ratas fueron trasladadas por separado a la habitación en la que se encontraban los actímetros. Una vez allí se colocó a cada animal en un actímetro, y se les permitió explorar el interior de la caja a modo de habituación durante 5 min. Pasado este tiempo los animales exploraron libremente el laberinto durante 30 minutos, en los que se midió la actividad horizontal.

Los resultados obtenidos fueron analizados en periodos de 5 en 5 minutos.

Entre cada sesión de experimentación las cajas se limpiaron con etanol para eliminar posibles rastros olfativos dejados por los animales.

Ansiedad

Dos días después de la primera prueba (Ver Figura 6) los animales se sometieron a una prueba para evaluar la conducta de tipo ansioso. Para ello, media hora después de la infusión del fármaco o su correspondiente control vehículo suero salino, las ratas se trasladaron desde el animalario en el que se encontraban estabuladas y se llevaron a la habitación experimental en el que se dispuso el laberinto en cero elevado (EZM), en jaulas individuales.

Una vez allí se les dejó reposar durante 5 min. a modo de habituación al nuevo ambiente. Después de este periodo los animales se colocaron en el centro del brazo abierto dejándolas explorar el laberinto libremente.

En este caso se analizó el tiempo que los animales emplearon explorando el brazo abierto, así como la distancia total recorrida.

Después de finalizar cada sesión el laberinto se limpió con etanol al 70% para eliminar cualquier pista olfativa dejada por el animal al explorar el laberinto.

Memoria espacial. Protocolo de aprendizaje Masivo en MWM.

Tal y como se muestra en la FIGURA 6, dos días después de finalizar el protocolo en el EZM los animales iniciaron el protocolo de memoria de referencia espacial en el laberinto

acuático de Morris para evaluar el efecto de la infusión del antagonista de Y_2R a nivel de hipocampo dorsal en la ejecución de tareas de memoria espacial. Las condiciones de la habitación y aparato fueron las mismas que para los experimentos anteriormente descritos. Las fases de las que constó la prueba quedan descritas en la figura 6).

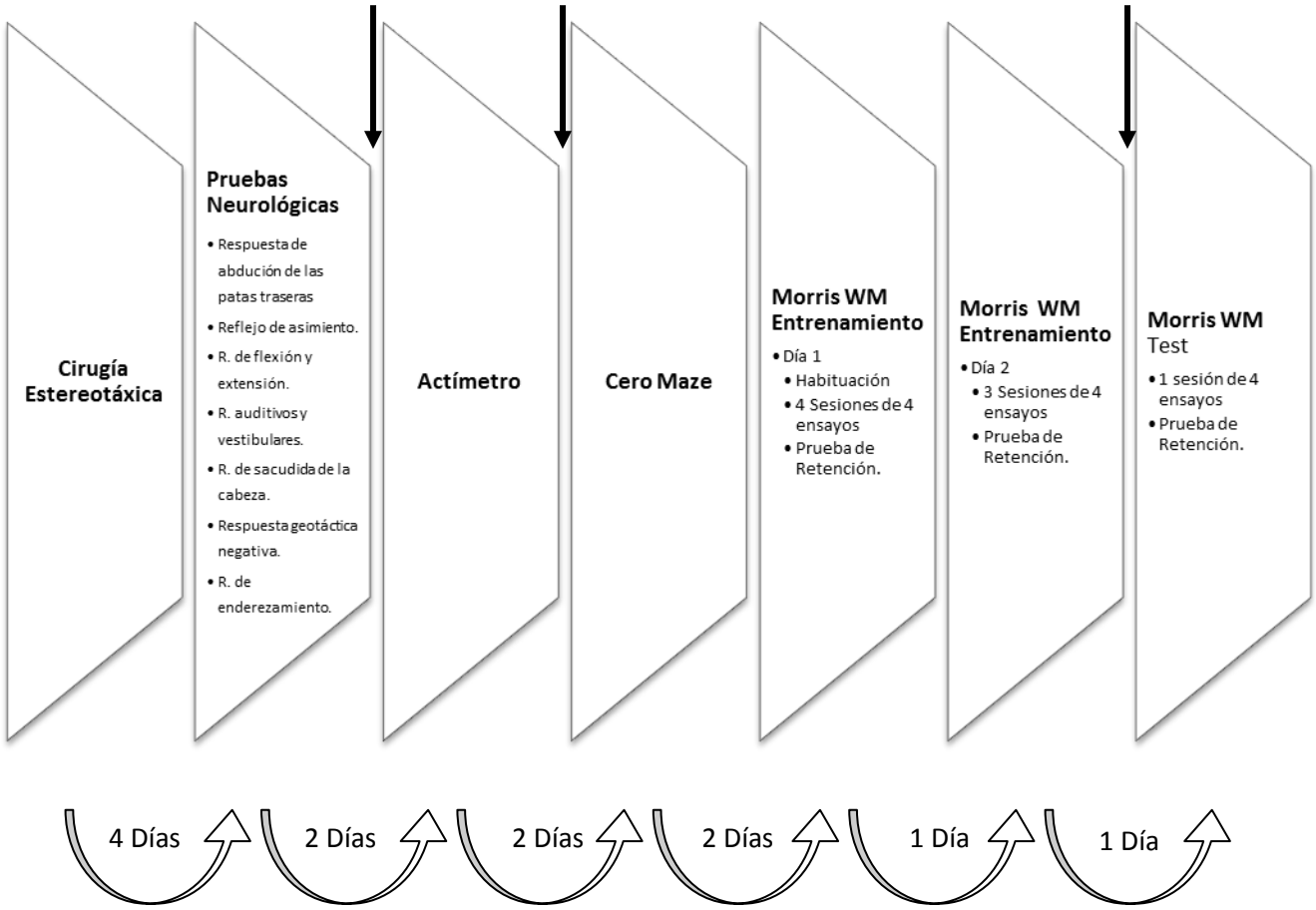


Figura 6: Línea de tiempo del protocolo experimental de los animales incluidos en el Artículo 8. Las flechas verticales representan los momentos de infusión del fármaco (en caso de los animales del grupo experimental) y suero (en el caso de animales "control"). Los periodos de tiempo entre cada prueba se indican en la parte inferior de la figura.

3.3. TÉCNICAS DE INACTIVACIÓN TEMPORAL

Tal y como se verá más adelante, en algunos de los experimentos se utilizaron modelos de inactivación temporal para evaluar los efectos de la inactivación cerebral en determinadas regiones cerebrales del sistema límbico y regiones corticales, que se creían implicadas en las fases de recuerdo y extinción en la orientación espacial en roedores (Artículos 2, 6, 7 y 8).

En todos los casos, al día siguiente de realizar las pruebas neurológicas, los animales se sometieron mediante cirugía estereotáxica al implante de cánulas intracerebrales de forma permanente, mediante las cuales se introdujeron las microcánulas de infusión de drogas en el momento pertinente para cada experimento. Para ello, tras ser anestesiados con Xilacina (5mg/kg) intramuscular y Ketamina (80 mg/kg) intraperitoneal, los animales se colocaron en el aparato estereotáxico (Narishinge, Japón ó Kopf, EEUU). Se realizó una incisión en la piel de la zona superior de la cabeza, y se ajustó al cráneo de forma que los puntos lambda y bregma quedasen en el mismo plano horizontal. Posteriormente se perforó el hueso realizando pequeñas incisiones circulares haciendo uso de una taladradora de uso dental (Minitor, Japón) para implantar cánulas en posición dependiente del área objeto de inactivación de manera bilateral en ambos hemisferios. Así, en el caso de inactivación de la región prelímbica de la corteza prefrontal medial (Artículos 3 y 6), las cánulas se colocaron según coordenadas desde el punto bregma: Antero-Posterior (AP) +3.1, Lateral (L) ±0.07, Dorsal-Ventral (DV) -3.0 mm (Paxinos & Watson, 2004) de forma que el bisel quedase en la porción dorsal del área prelímbica. En los supuestos en los que el área objeto de inactivación era el área CA1 del hipocampo dorsal (Art. 2, 7 y 8), las coordenadas fueron AP -3.6, L ±2.6 y DV -2.1 mm desde Bregma.

Las cánulas se fabricaron a partir de agujas estériles de 22 gauge de diámetro (Becton Dickinson S.A., España). Una vez colocadas las cánulas se horadaron dos aberturas más en posición anterior y posterior a los anteriores, donde se colocaron tornillos para aumentar la sujeción del cemento dental (Glaslonomer Cement, Shofu Inc., Reino Unido) con el que se formó una cobertura a modo de protección de las cánulas, dejando sobresalir del mismo solo los orificios superiores por los que posteriormente se insertarían las microcánulas de infusión.

Para una mayor sujeción y protección del recubrimiento se realizó una sutura superficial de la piel colocándola por encima del cemento dental en la parte caudal de la incisión

quirúrgica. Los animales se recuperaron de la operación bajo observación y cuidados postquirúrgicos hasta recobrar la consciencia. Posteriormente fueron alojados en jaulas individuales hasta su total recuperación.

3.4.OBTENCIÓN DE LOS TEJIDOS

Para todos los experimentos, una hora y media después de finalizada la última prueba conductual en el laberinto acuático de Morris a la que habían sido sometidos los animales (transfer o prueba de recuerdo en los Artículos 1-2, última ensayo de extinción en los Artículos 4-7 y último ensayo de adquisición en el Artículo 8), éstos fueron sacrificados por distintos métodos según la metodología de análisis a la que se destinaban.

Así, los animales experimentales y sus correspondientes controles con cuyos cerebros se realizó un análisis molecular mediante técnicas de actividad metabólica cerebral (histoquímica para la Citocromo C Oxidasa) o técnicas inmunohistoquímicas para la proteína c-Fos (Artículos 1,2-4-8) fueron sacrificados por decapitación.

En estos casos, el cerebro se extrajo rápidamente y se recubrió con un gel crioprotector (Jung, Alemania), congelándolos por inmersión en isopentano a -80º C durante dos minutos, para posteriormente mantenerlos refrigerados a -40º C, previniendo así el deterioro del tejido y la consiguiente pérdida de actividad enzimática. En momentos previos a la tinción mediante estas técnicas, los cerebros fueron seccionados coronalmente mediante un microtomo criostático (Microm International GmbH, modelo HM 505 E, Heidelberg, Alemania) en cortes de 30 µm de grosor que se colocaron en portaobjetos que previamente habían sido limpiados con alcohol de 100º (para la técnica histoquímica de la CO) o en portaobjetos gelatinizados (en el caso de la inmunohistoquímica c-Fos).

La misma técnica de sacrificio y conservación de los tejidos se utilizó en el caso de los cerebros analizados mediante Western Blot. Con este fín, previamente a su utilización, los cerebros se descongelaron y se dividieron mecánicamente en cuatro secciones que contenían el hipocampo, estriado, cortex prefrontal y el resto de las cortezas.

Por último, los animales del experimento 8 destinados a realizar técnicas de inmunohistoquímica para la detección del neuropéptido Y (NPY) fueron anestesiados con pentobarbital sódico (Sigma-Aldrich) inmediatamente después del último procedimiento en el MWM, y se les sacrificó mediante perfusión vascular con tampón fosfato salino (PBS) (10ml)

seguido de paraformaldehído al 4% (20ml). Seguidamente se extrajeron los cerebros para fijarlos de nuevo por inmersión en la misma solución, pasando posteriormente a una solución crioprotectora de sacarosa al 20%. Previamente a la tinción inmunohistoquímica se obtuvieron secciones coronales de 10µm a lo largo de su eje antero-posterior y se montaron, al igual que en el caso de c-Fos, en portaobjetos gelatinizados, que se almacenaron congelados a -40º C hasta su uso posterior.

3.5.TÉCNICAS DE BIOLOGÍA CELULAR Y MOLECULAR

3.5.1. HISTOQUÍMICA DE LA CITOCROMO OXIDASA

El protocolo utilizado para medir la actividad metabólica cerebral fue el descrito por Wong-Riley (1989) en su versión modificada propuesta por Gonzalez-Lima and Jones (1994). Las secciones congeladas se fijaron al portaobjetos durante 5 min en una solución de PBS con sacarosa (pH 7,6; 0,1M, 100g/l de tampón) con glutaraldehido al 0,5%. A continuación, las secciones fueron aclaradas en tres ocasiones, durante 5 min cada turno, en tampón fosfato con sacarosa, para así mantener la estructura de tejido y el equilibrio osmótico. Posteriormente, se preincubaron 4 min en solución TRIS (0.05M tampón Tris a pH 7.6 con 275mg/l de cloruro de cobalto, el cual aumenta la sensibilidad de la reacción de tinción, en tampón con 10% en volumen de sacarosa, 38% de ácido clorhídrico 0,1N, 5 ml/l de dimetilsulfóxido (DMSO).

A continuación se realizó un lavado en tampón fosfato (pH 7,6; 0,1M) para posteriormente incubar las secciones en oscuridad y agitación continua durante una hora en una solución de tinción que contiene, por 100 ml de PBS 0.1 M a pH 7.4: 15 mg de citocromo c (Sigma,St. Louis, MO, EEUU), 0,002 g de catalasa, 4g de sacarosa y 0,25 ml de DMSO. La diaminobencidina (DAB) actúa como un cromógeno que, tras haber sido oxidada por la CO endógena, se fija a la membrana mitocondrial dando lugar al color que observamos tras la tinción. Es necesario preparar esta solución de tinción en condiciones de baja iluminación ambiental, ya que la presencia de luz podría dar lugar a una tinción inespecífica debido a la oxidación espontánea de la DAB.

Posteriormente, y a temperatura ambiente, se detiene la reacción con un baño de tampón con formalina al 4% (Prolabo, España) con un 10% de sacarosa, durante 30 min.

Después de ser fijado, el tejido se deshidrató mediante baños de cinco minutos en una cadena de alcoholes de concentración creciente (30%, 50%, 70%, 95%, 95%, 100%, 100%).

Por último las secciones se aclararon en un baño de xileno (Prolabo, Barcelona, España) durante 10 minutos, y posteriormente se realizó el montaje con Entellán (Merck, Darmstadt, Alemania), para la conservación del tejido. Un ejemplo del resultado de la tinción se muestra en la FIGURA 7,A.

Con el objetivo de corregir las posibles variaciones de la tinción citocromo c oxidasa en los distintos sets de incubación se utilizaron una serie de secciones de diferentes grosores (10, 30, 50 y 70 μm) como estándares, obtenidas de homogeneizados cerebrales de rata de actividad CO conocida. Este homogeneizado se obtuvo tras la decapitación y extracción de los cerebros de 12 ratas Wistar macho adultas. Para ello, tras una inmersión en tampón fosfato de pH 7,6 a 4ºC, los cerebros extraídos fueron triturados hasta conseguir una masa homogénea que fue introducida en tubos de microcentífuga. Posteriormente su contenido fue centrifugado y congelado por inmersión lenta en isopentano a -70ºC, tras lo cual se almacenaron a -40ºC. Los homogeneizados que acompañaron durante todo el proceso a las secciones coronales se usaron para establecer comparaciones entre sets de incubación.

A través de las secciones de homogenado se convierte un dato cualitativo, como es la densidad de gris medio de la imagen, en un valor cuantitativo de unidades de actividad CO (μmol de citocromo c oxidado/min/g de tejido húmedo a 23ºC). Esta conversión se realiza mediante una ecuación de regresión entre la densidad óptica y la actividad enzimática determinada por espectrofotometría (Gonzalez-Lima & Jones, 1994).

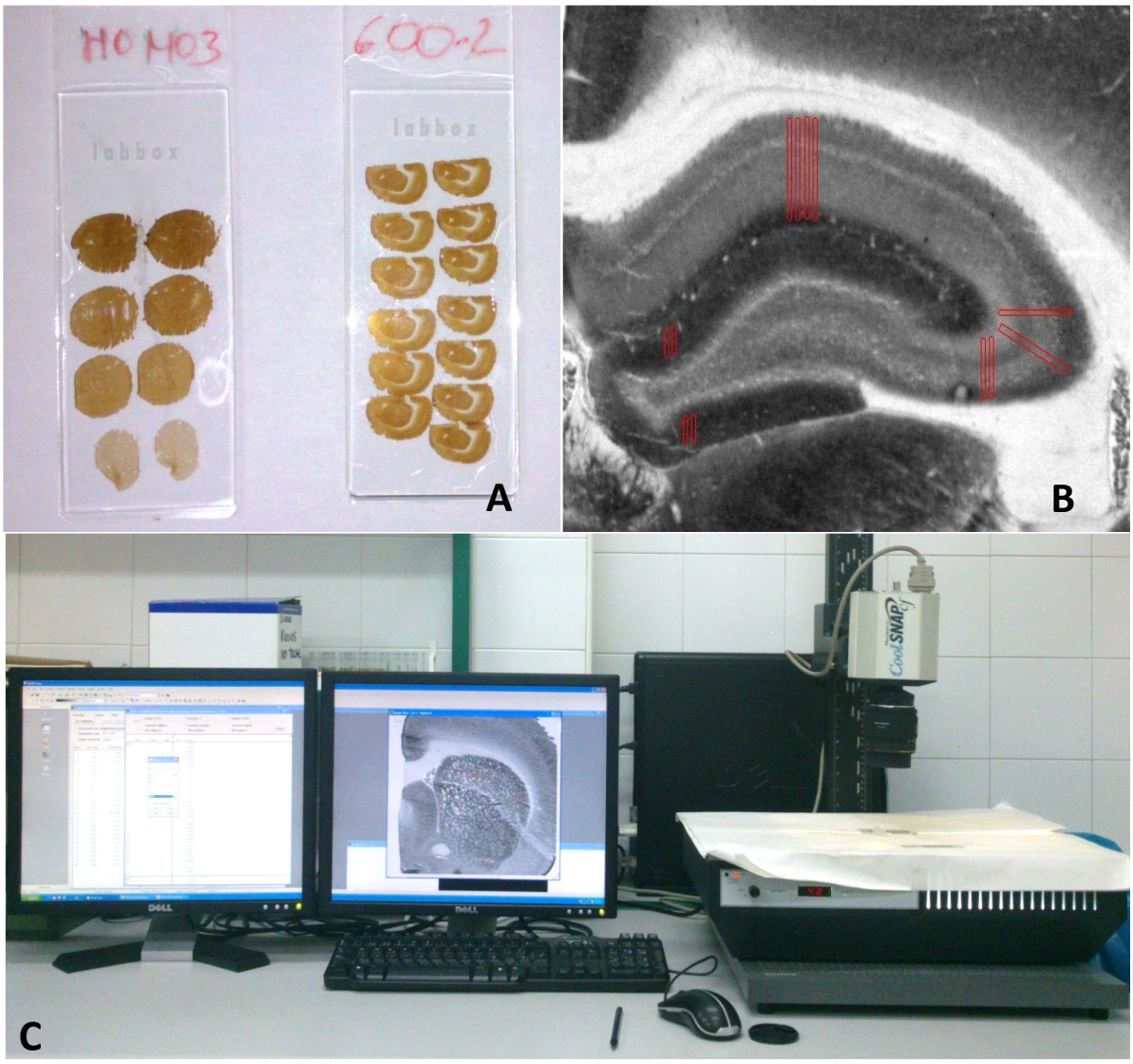


Figura 7: **A. A la izquierda se muestra una fotografía de la serie de secciones de distintos grosores (10,30, 50 y 70 µm) utilizadas como estándares para confeccionar la curva de regresión lineal que permite convertir los valores de medida de densidad óptica de las estructuras seleccionadas en valores de actividad citocromo oxidasa. A la izquierda se observan secciones coronales de cerebro de rata teñidas mediante esta técnica. B. Detalle de los cuadrados de muestreo utilizados para la medición mediante densitometría óptica de la actividad CO en el hipocampo dorsal. C: Imagen del sistema de análisis y software utilizado. A la izquierda se muestra en la pantalla la sección elegida, que se observa gracias a la cámara de alta sensibilidad que recoge la imagen del portaobjetos que se pretende cuantificar.**

Transformación de la actividad enzimática y cuantificación.

La cuantificación de la actividad del enzima CO se realizó mediante el estudio de la densitometría óptica de la tinción. Para ello se utilizó un sistema de análisis de imagen informatizado (MCID Elite Interfocus Linton, Inglaterra), tal como se muestra en la FIGURA 7 B y C).

En primer lugar se tomaron medidas densitométricas de los estándares que acompañaban a los sets de incubación, pudiendo así conformar la curva de regresión que permitiría comparar las distintas series de tinción. Seguidamente se seleccionaron las estructuras de interés de acuerdo al atlas histológico de secciones coronales de cerebro de rata de Paxinos and Watson (2004). Para cada estructura se tomaron 12 mediciones en total (cuatro medidas en tres secciones consecutivas), realizando cuadrados de tamaño ajustado al tamaño de cada estructura y orden pseudoaleatorio. Posteriormente se hallaron las medias de actividad CO por cada animal y estructura.

Las estructuras escogidas y coordenadas respecto al punto Bregma en las que fueron analizadas dependieron de los objetivos de cada experimento, por lo que se explican en detalle en cada uno de los mismos (Ver Artículos 1, 2, 4,6-8).

ANÁLISIS INMUNOHISTOQUÍMICO

ANÁLISIS DE EXPRESIÓN DEL GEN *c-fos*

Técnica Inmunohistoquímica

El marcaje de la proteína c-Fos (Artículo 5) comenzó con las secciones colocadas en portas gelatinizados, tal como hemos comentado anteriormente. Éstas recibieron una fijación de 30 min en paraformaldehído al 4% (0.1M, pH 7.4). Tras una incubación en peróxido de hidrógeno al 0.9% en tampón fosfato para eliminar la actividad peroxidasa endógena, se realizó un lavado adicional en PBS, y se cubrieron por goteo con la solución de incubación o solución de bloqueo, durante 30 min. La solución incluía Tritón X-100 al 10% (Sigma, USA) y seroalbúmina bovina al 3%. Pasado este tiempo las secciones se incubaron con el anticuerpo primario policlonal de conejo α-c-Fos (1:10.000, Santa Cruz Biotech, sc-52, USA) diluido en tampón fosfato salino con Triton X-100 al 10%. La incubación tuvo lugar en una cámara húmeda durante la noche, a 4ºC de temperatura. Seguidamente las secciones se aclararon con

PBS y se incubaron con el anticuerpo secundario, de cabra α-conejo biotinilado IgG (1:200, Pierce, EEUU), durante dos horas y a temperatura ambiente. Las secciones pasaron por tres lavados sucesivos con PBS y se trataron con una solución de complejo peroxidasa avidina–biotina (Vectastain ABC Ultrasentitive Elite Kit, Pierce, EEUU) durante una hora. La reacción se paró aclarando de nuevo las secciones en PBS. Una vez finalizado el proceso las secciones se deshidrataron pasando por una cadena de alcoholes de concentración creciente, aclaradas con xileno y montadas para su conservación posterior con medio hidrófobo (*Entellán*®, Merk, EEUU) y cubreobjetos de vidrio. Cada set de tinción incluyó secciones en las que el anticuerpo primario no fue añadido, como controles a posibles reacciones inespecíficas. En todos los experimentos los portaobjetos se codificaron de forma que el investigador que llevaba a cabo el experimento no conociese el grupo experimental al que pertenecía cada uno de los sujetos.

Cuantificación Celular

El número total de células inmunoreactivas positivas a c-Fos se cuantificó en tres secciones consecutivas separadas por 30µm. Las regiones a cuantificar, que se localizaron al igual que en el caso anterior, con ayuda del atlas de Paxinos and Watson (2004), se detallan en el Artículo 5. Las regiones cerebrales escogidas se enmarcaron manualemente con un rotulador apto para escritura en cristal, y el área total a cuantificar en las distintas regiones cerebrales se estimó usando el software de análisis de imagen (Jandel Scientific, San Rafael, CA, EEUU).

La cuantificación se llevó a cabo realizando un muestreo sistemático de cada región seleccionada utilizando marcos cuadrados superpuestos de un tamaño total de 0.0576 mm^2, aunque el número de marcos varió para adecuarse al tamaño total de la región a muestrear. En todos los casos, el porcentaje de área en la que se realizó el muestreo respecto al área total de las tres secciones seleccionadas fue superior al 10%.

La identificación de los núcleos positivos a c-Fos a tener en cuenta se basó en su apariencia de puntos homogéneamente oscuros con bordes bien definidos. Para ello, se empleó un microscopio (Olympus BH-2, Japón) unido a una cámara analógica (Sony XC-77, Japón) y un monitor de 300X de magnificación total. El número medio de células inmunoreactivas a c-Fos se calculó para cada sujeto y región y se dividió entre el total de marcos de muestreo en cada portaobjetos (denominada “área cuantificada”).

ANÁLISIS DE LA EXPRESIÓN DEL NEUROPÉPTIDO Y

Esta técnica inmunohistoquímica (Artículo 8) comenzó con las secciones coronales de tejido montadas en portas gelatinizados, tal y como se ha explicado anteriormente. Se realizó un doble marcaje fluorescente para NPY y para la proteína III beta-tubulina (Tuj-1) como marcador específico neuronal y contraste específico Hoechst 33342 (Sigma-Aldrich) como marcador nuclear, para observar no solo la cantidad de proteína, sino también su localización inter o intracelular.

Las secciones se bloquearon con 10% de sero albúmina fetal bovina (FSS) con 0.5% de tritón X-100 en PBS. Seguidamente, se incubaron con un anticuerpo policlonal α-NPY (1:1000, Sigma-Aldrich, St. Louis, MO, USA) durante la noche a 4ºC, lavados e incubados durante 90 min. a 23ºC con anticuerpo Alexa Fluor 488 (1:200, Invitrogen, UK). Seguidamente las secciones se bloquearon de nuevo con solución que contenía 1% de FSB, y se incubaron con anticuerpo α-Tuj-1 (1:1000, Covance, CA, EEUU). Después de un último aclarado con PBS se incubaron de nuevo con otro anticuerpo fluorescente, en este caso Alexa Fluor 594 (1:200, Invitrogen) a temperatura de 4ºC durante la noche. Los núcleos se pusieron de manifiesto con Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA). Para el montaje se utilizó medio fluorescente Dako (Dako North America, Carpinteria, EEUU).

Cuantificación de la inmunohistoquímica para NPY

Una vez el tejido había sido marcado para detectar lass proteínas de interés, el análisis de la fluorescencia emitida se reveló mediante el uso de un microscopio confocal LSM 710 Meta (Carl Zeiss, Oberkochen, Alemania), tal como se aprecia en la FIGURA 8.

La absorbancia media para cada grupo experimental se analizó mediante el software de imagen ImageJ. Específicamente, se tomaron cinco medidas por sección con un cuadrado de muestreo de 13,12μm^2 de tamaño, en al menos dos secciones consecutivas por región de interés.

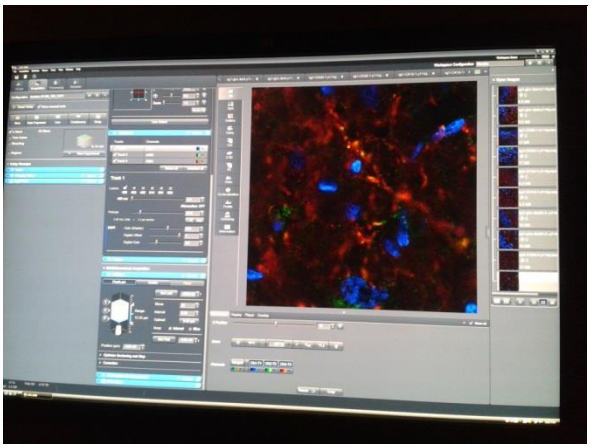


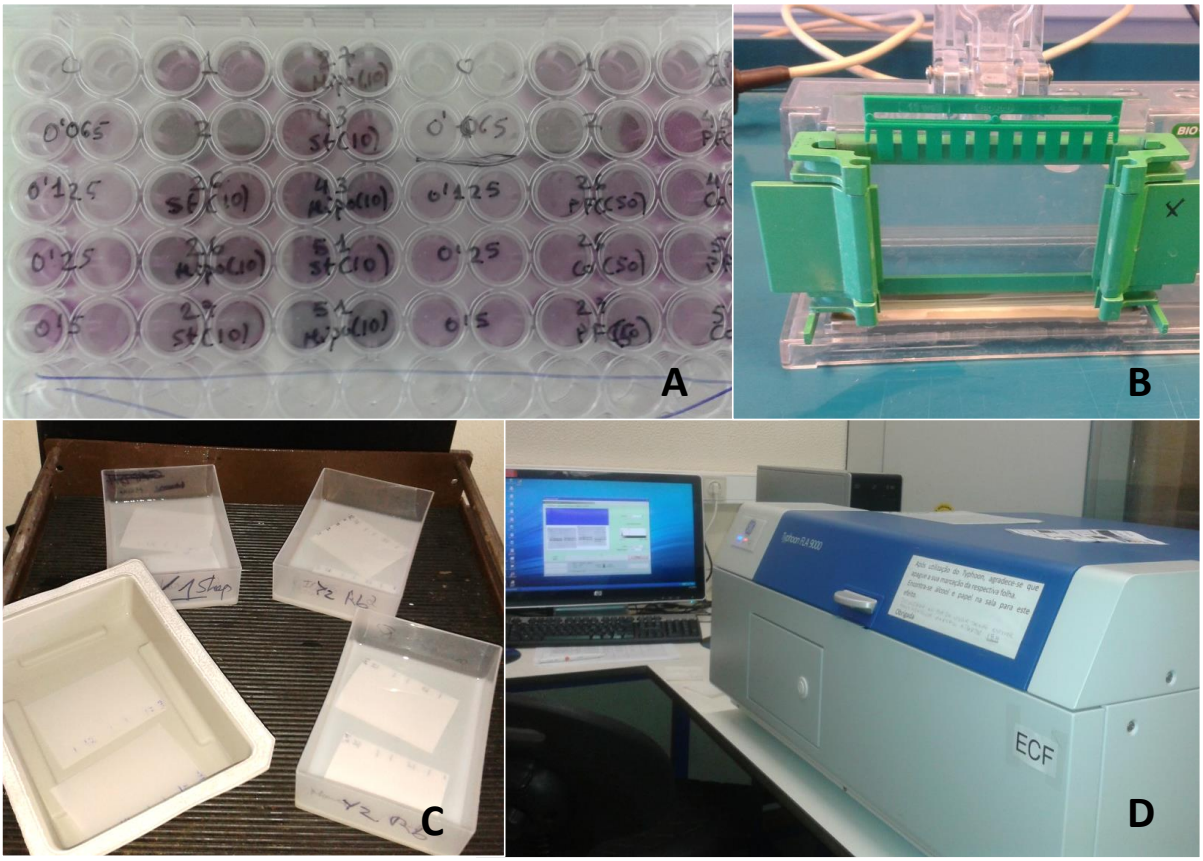
Figura 9: Detalle del microscopio láser confocal LSM 710 Meta utilizado para tomar las imágenes resultantes de la inmunohistoquímica α-NPY cerebral. La detección espectral continua a lo largo de todo el rango de longitud de onda permite detectar los tres anticuerpos unidos a distintos fluorocromos para el marcaje de Tuj-1 o marcador neuronal, NPY y Hoechst como marcador de núcleos celulares (mostrados como rojo, verde y azul respectivamente). La superposición de colores indica existencia del neuropéptido Y en células neuronales.

ANÁLISIS MEDIANTE WESTERN BLOT

El protocolo seguido para realizar la técnica de western blot, se describe brevemente a continuación. Después de homogeneizar las regiones cerebrales en las que había sido dividido el cerebro en los animales de ambos grupos (Hipocampo, estriado, córtex prefrontal y resto de cortezas), se determinó la concentración de proteína por alícuota mediante el uso de un kit de análisis de BCA (Pierce, Rockford, IL, EEUU) y las muestras de proteína de 25 a 60 µg se separaron en geles de electroforesis de sodio dodecil-sulfato poliacrilamida (SDS-PAGE) al 12%. Las proteínas se transfirieron a membranas de polifluoruro de vilideno PVDF (Millipore Iberica, Madrid, Spain). Después de bloqueadas, las membranas se incubaron una noche a 4ºC con el Ac policlonal anti-NPY Y_1R (1:1000, AbD Serotec, Oxforshire, UK) y anti-NPY Y_2R (1:200, Alomone Labs, Jerusalem, Israel). Posteriormente las membranas se incubaron durante una hora a temperatura ambiente con sus respectivos anticuerpos secundarios, para ser reveladas mediante una detección por quimioluninescencia, usando para ello EFC (ECF kit, Amersham). Las bandas resultantes se visualizaron en un sistema Typhoon 9000 (GE Healthcare Europe GmbH). (FIGURA 8).

Como control de la electroforesis se utilizó el anticuerpo monoclonal anti-β-actina (1:20.000, Sigma-Aldrich, St. Louis, MO, USA).

Figura 8: La imagen muestra alguna de las etapas de la técnica de Western Blot. A: Placa multipocillos para la cuantificación de proteína total en cada una de las muestras por el método de BCA; B: Separación de las bandas de proteínas mediante electroforesis en gel de agarosa; C: Bloqueo e incubación con distintos anticuerpos de las membranas de PVDF a las que habían sido transferidas las bandas de proteínas ya separadas desde el gel. Por último, D: Escaneado de las bandas reveladas mediante detección por quimiofluorescencia y visualizadas con un escáner y software Typhoon 9000.



Cuantificación

La medida de cuantificación de cada uno de los receptores NPY a analizar en cada región y animal se obtuvo mediante el análisis de la densidad óptica de las bandas, utilizando para ello el software ImageJ (NIH, Bethesda, MD, EEUU). Para ello se tuvo en

cuenta el total de proteína cargada, inferido de la cantidad de proteína beta-actina en cada muestra, que se empleó como control de carga.

3.6.ESTUDIO ESTADÍSTICO

Todos los datos derivados de los experimentos presentados en este trabajo fueron analizados mediante el software SigmaPlot 11.0 (Systat Software, San Jose, CA, EEUU), y los software SAS 9.4 PROC MIXED o SAS 9.4 PROC CALIS (SAS Institute Inc., EEUU). En todos los casos se consideró estadísticamente significativo un *p*-valor ≤0.05.

3.6.1. ANÁLISIS DE LOS PROCEDIMIENTOS CONDUCTUALES.

Memoria espacial

Adquisición.

Para evaluar la prueba de aprendizaje de orientación espacial de los roedores, se analizó, en primer lugar las latencias de escape de los a animales, mediante la prueba ANOVA unifactorial de medidas repetidas. Para ello se tomó como factor el día de aprendizaje . En caso de encontrar diferencias significativas, se empleó la prueba a posteriori test de Tukey HSD (*honest significant difference*) para comparar los distintos días de aprendizaje entre sí. Dada la alta actividad observada en los animales, se observó que éstos acudían inicialmente a la antigua localización de la plataforma, cambiando rápidamente de cuadrante para buscar otras opciones una vez comprobado que aquella ya no se encontraba en su lugar original. Por este motivo, solo se tuvo en cuenta la primera parte de las pruebas de retención en los posteriores ensayos estadísticos (Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2007; Spooner, Thomson, Hall, Morris, & Salter, 1994).

Posteriormente, para analizar el tiempo de permanencia en cada cuadrante durante la prueba de retención, se empleó un ANOVA unifactorial de medidas repetidas. En este caso se tomó el cuadrante como factor. En caso de diferencias significativas se empleó igualmente el test de Tukey o el test Holm-Sidak, para comparar las diferencias de los distintos cuadrantes entre sí. En los casos en los que fallaba la presunción de normalidad u homogeneidad de varianzas se llevó a cabo un test de Kruskall-Wallis (Artículo 5).

En el Artículo 6 las latencias de escape se analizaron mediante un modelo mixto de medidas repetidas (MMRM). En este sentido, aunque el test de Tukey constituye la aproximación tradicional para identificar diferencias entre pares de medias en diseños de medidas repetidas como el que nos ocupa, su uso ha creado controversia en ciertas ocasiones. Ya en los años 80 algunos autores, como Boik (1981), mostraron que incluso pequeñas desviaciones de la esfericidad pueden dar lugar a resultados sesgados en los test a posteriori de comparaciones múltiples.

Por este motivo, y siguiendo la recomendación de Vallejo, Moris, and Conejo (2006), para controlar la tasa de error correspondiente a la familia de comparaciones pareadas, aplicamos el procedimiento Bonferroni de rechazo secuencial de abajo-arriba de Hochberg (1988). Para ello, utilizamos el comando ESTIMATE en el procedimiento MIXED del SAS y la opción HOC en el procedimiento MULTITEST del SAS. El análisis del modelo mixto de medidas repetidas fue llevado a cabo ajustando múltiples patrones de covarianza con los parámetros estimados con el método de la máxima verosimilitud restringida, tal y como está implementado en la versión 9.4 del paquete estadístico SAS (2013).

En la presente Tesis Doctoral usamos sendas técnicas Bonferroni de rechazo secuencial para contrastar las hipótesis referidas a las comparaciones múltiples efectuadas entre los niveles de la variable tiempo (asumida discreta) y a la interacción del tiempo con los tratamientos, de resultar esta última distinta de cero. Específicamente, el procedimiento secuencial de arriba-abajo de Holm (1979) modificado por Shaffer (1986) y el procedimiento de abajo-arriba de Hochberg (1988). Estos métodos controlan adecuadamente la tasa de error de Tipo I para los contrastes pareados y de la interacción en diseños de medidas parcialmente repetidas con matrices de varianzas y covarianzas heterogéneas y desigual número de unidades experimentales dentro de cada grupo. Seguidamente describimos brevemente como proceden.

Mediante el procedimiento Holm-Shaffer uno comienza ordenando por rangos los valores ρ asociados con el estadístico de la prueba, $\rho_{(1)} \leq \rho_{(2)} \leq ... \leq \rho_{(c)}$. Estos valores p serán obtenidos utilizando pruebas estándar o pruebas robustas, en función de que se cumpla o no el supuesto de esfericidad multimuestral. A continuación, el valor ρ más pequeño es comparado con el valor crítico de Dunn, esto es, α/c, donde $c = q(q-1)/2$, q el número de niveles de la variable categórica tiempo y α denota la tasa de error a controlar; si $\rho_{(1)} \leq \alpha/c$ la hipótesis nula $H_{(1)}$ es rechazada, de lo contrario, detenemos el proceso y declaramos nulas todas las hipótesis,

$H_{(1)}$, $H_{(2)}$, ..., $H_{(c)}$. Si $H_{(1)}$ es rechazada, procedemos a comparar el siguiente valor ρ más largo, $\rho_{(2)}$, aquí en vez de dividir α por el número de pares de comparaciones restantes, c - 1, se divide por el número de pares de comparaciones que podrían ser iguales, condicionados al rechazo de la hipótesis previa, c^*_2; si $\rho_{(2)} \leq \alpha/c^*_2$, la hipótesis $H_{(2)}$ es rechazada, en caso contrario, detenemos el proceso. El procedimiento continúa del modo expuesto rechazando H_k (k = 1, 2, ..., c) si $\rho_{(k)} \leq \alpha/c^*_k$, dados k-1 rechazos previos.

A su vez, siguiendo el procedimiento paso a paso de Hochberg (1988) , se ordenan por rangos los diferentes valores ρ asociados con el estadístico utilizado para probar las hipótesis correspondientes a los diferentes contrastes; esto es, $\rho_{(1)} \leq \rho_{(2)} \leq ... \leq \rho_{(c)}$. Bajo este enfoque, se parte de la hipótesis que tiene la mayor probabilidad de ser retenida a la que tiene la menor, para ello se comienza testando la hipótesis asociada con el valor ρ más largo. Si $\rho_{(c)} \leq \alpha$, rechazamos todas las hipótesis y detenemos el proceso; por el contrario, si $\rho_{(c)} > \alpha$, retenemos $H_{(c)}$ y procedemos a probar $H_{(c-1)}$. Si $\rho_{(c-1)} \leq \alpha/2$, además de la hipótesis implicada rechazamos todas las demás; en caso contrario, $H_{(c-1)}$ es retenida. A continuación, $\rho_{(c-2)}$ es comparado con $\alpha/3$, y así se continúa el proceso hasta que alguna hipótesis resulte rechazada. De ser retenidas todas las hipótesis el proceso se finaliza comparando $\rho_{(1)}$ con α/c.

Recuerdo

Para evaluar el tiempo medio que los animales pasaron en los distintos cuadrantes durante la prueba de retención (en este caso Prueba de "Recuerdo") se aplicó un ANOVA unifactorial de medidas repetidas usando el cuadrante como factor. Cuando se encontraron diferencias significativas entre cuadrantes se aplicó un test de *Tukey* como prueba a *posteriori* (Artículos 1-3).

Extinción

Para evaluar la extinción se utilizó de nuevo un ANOVA unifactorial de medidas repetidas de forma independiente para cada grupo experimental usando el tiempo en el cuadrante de escape como factor (Artículos 5, 6).

En el Artículo 5 se usó un ANOVA de dos factores de medidas repetidas para evaluar las diferencias entre grupos en la distancia recorrida y en la velocidad durante la extinción.

Actividad horizontal espontánea y Conductas de tipo ansioso.

Para evaluar la actividad espontánea de los animales se analizó un tiempo total de 30 minutos, analizados en sectores de cinco minutos mediante un test T- de *Student,* para evaluar diferencias entre los grupos control y experimental. La misma prueba estadística fue utilizada para analizar las medias del tiempo que pasaron los animales en los brazos abiertos en el EZM, y la distancia total recorrida entre grupos (Artículo 8).

3.6.2. ANÁLISIS DE LOS RESULTADOS DE ESTUDIOS MOLECULARES Y CELULARES

Análisis de la actividad CO En los casos en los que se utilizó esta técnica de estudio (Artículos 1,3,4 y 6-8) , los datos de la cuantificación de la actividad CO para cada una de las regiones cerebrales a estudiar se aplicó un test t de *Student* para muestras independientes entre los grupos control y experimental en cada caso.

Para el análisis de la conectividad funcional de las distintas estructuras cerebrales implicadas se utilizó la correlación de Pearson entre las regiones de cada grupo a estudiar (Artículos 1 y 2) seguido de una corrección por el método Jacknife (Shao & Dongsheng, 1995) para evitar errores debidos a tamaños de muestras muy pequeños. Para controlar la tasa de error correspondiente a la familia de comparaciones pareadas, aplicamos sendos procedimientos de rechazo secuencial. En concreto, el procedimiento Bonferroni de rechazo secuencial de arriba-abajo de Holm-Shaffer y el procedimiento Bonferroni de rechazo secuencial de abajo-arriba de Hochberg.

En los Artículos 4 y 6 se utilizó un *Path analysis* para examinar las posibles redes de conectividad entre estructuras. En este caso, el análisis de la covarianza se llevó a cabo usando una estimación por máxima verosimilitud.

El ajuste global se realizó, como es usual, con la prueba de la razón de verosimilitud, distribuida según χ^2. Si la prueba de la razón de verosimitud no resulta significativa, se asume un ajuste global absoluto satisfactorio. Debido a que la hipótesis nula afirma que el modelo especificado ajusta los datos (es decir, las matrices de covarianza predichas y observadas no diferirían significativamente), se exigió que el estadístico χ^2 no resulte significativo ($p > 0.05$) para apoyar el modelo especificado. Con el propósito de verificar la mejora que se obtendría en el ajuste cuando se utiliza el modelo hipotetizado en lugar del modelo nulo, también fueron

contemplados tres índices de ajuste incremental. En concreto, el ajuste normado (NFI), el ajuste no normado (NNFI) y el índice de ajuste comparativo (CFI) cuyos valores en el entorno de 0.9 fueron interpretados como indicadores de un ajuste razonable para el modelo. También utilizamos la raíz del error cuadrático medio (RMSEA) con valores de menos de .08, indicando un ajuste aceptable del modelo hipotetizado. El análisis de los residuales normalizados puso de relieve que la matriz de covarianza predicha por el modelo no se aleja más de lo esperable por azar de la matriz de covarianza observada. La prueba estadística de los efectos del modelo se calculó dividiendo los coeficientes estandarizados por su correspondiente error estándar. A partir de este cálculo obtuvimos el valor *t* que nos permitió verificar la significación de las rutas (*path*) entre las variables estudiadas. En nuestra evaluación final de la fuerza de cada ruta dentro del modelo, consideramos estadísticamente significativos aquellos parámetros estimados cuyo valor de *t* resultó superior a ± 1.96.

Análisis de la expresión de c-Fos

El número de células inmunoreactivas a c-Fos (Artículo 5) en ambos grupos se estimó mediante un ANOVA de dos factores tomando para ello ambos grupos (control y experimental) y las áreas o núcleos en cada estructura cerebral como factores.

Al igual que en el caso de la memoria espacial, para controlar la tasa de error correspondiente a la familia de comparaciones pareadas, aplicamos el procedimiento Bonferroni de rechazo secuencial de abajo-arriba de Hochberg (1988). Para ello, utilizamos el comando ESTIMATE en el procedimiento MIXED del SAS y la opción HOC en el procedimiento MULTITEST del SAS. El análisis del modelo mixto de medidas repetidas se realizó mediante el procedimiento MIXED del SAS (2013) con los grados de libertad del numerador y denominador de la prueba F ajustados mediante la solución de Kenward y Roger.

Análisis de la expresión de NPY determinada por inmunohistoquímica

La expresión regional del neuropéptido Y en las distintas regiones de los grupos control y experimental se determinó mediante el uso de un test T de *Student*. En los casos en los que los supuestos de normalidad u homogeneidad de varianzas fallaban se utilizó un test no paramétrico U de Mann-Whitney.

Análisis estadístico de la concentración relativa de NPY determinada por Western blot

Al igual que en el caso anterior, la concentración de los receptores NPY Y_1R e Y_2R en los grupos control y experimental en cada región se determinó mediante el uso de un test T de *Student* o un test no paramétrico U de Mann-Whitney según los casos. Para ello se tuvieron en cuenta las medidas de densitometría de la intensidad de las bandas mediante el software ImageJ previamente mencionado. Para normalizar los datos se dividió la intensidad obtenida en las bandas de proteína de cada receptor entre la obtenida de las bandas de la proteína β-actina

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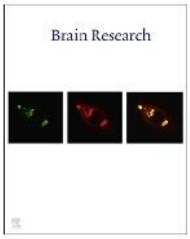

Research Report

Functional interactions between dentate gyrus, striatum and anterior thalamic nuclei on spatial memory retrieval

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ABSTRACT

The standard model of memory system consolidation supports the temporal reorganization of brain circuits underlying long-term memory storage, including interactions between the dorsal hippocampus and extra-hippocampal structures. In addition, several brain regions have been suggested to be involved in the retrieval of spatial memory. In particular, several authors reported a possible role of the ventral portion of the hippocampus together with the thalamus or the striatum in the persistence of this type of memory. Accordingly, the present study aimed to evaluate the contribution of different cortical and subcortical brain regions, and neural networks involved in spatial memory retrieval. For this purpose, we used cytochrome c oxidase quantitative histochemistry as a reliable method to measure brain oxidative metabolism. Animals were trained in a hidden platform task and tested for memory retention immediately after the last training session; one week after completing the task, they were also tested in a memory retrieval probe. Results showed that retrieval of the previously learned task was associated with increased levels of oxidative metabolism in the prefrontal cortex, the dorsal and ventral striatum, the

Abbreviations: AcC, nucleus accumbens core; AcSh, nucleus accumbens shell; Ba, amygdala, basal nucleus; Ce, amygdala, central nucleus; Me, amygdala, medial nucleus; AD, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; DS, dorsal striatum; Cg, cingulate cortex; CA1d, Cornus Ammonis 1 of the dorsal hippocampus; CA1v, Cornus Ammonis 1 of the ventral hippocampus; CA3d, Cornus Ammonis 3 of the dorsal hippocampus; CA3v, Cornus Ammonis 3 of the ventral hippocampus; CO, cytochrome c oxidase; DGd, dentate gyrus of the dorsal hippocampus; DGv, dentate gyrus of the ventral hippocampus; dH, dorsal hippocampus; Ent, entorhinal cortex; IL, iinfralimbic cortex; LM, lateral nucleus of the mammillary bodies; LS, lateral septum; MM, medial nucleus of the mammillary bodies; mPFC, medial prefrontal cortex; MS, Medial septum; MD, mediodorsal thalamic nucleus; MWM, Morris water maze; N, Naïve group; PAR, parietal cortex; PRh, perirhinal cortex; PFC, prefrontal cortex; PrL, prelimbic cortex; PM, premammillary nucleus; M1, primary motor cortex; R, Retrieval group; RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; SMR, spatial reference memory; SuM, supramammillary nucleus of the mammillary bodies

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anterodorsal thalamic nucleus and the dentate gyrus of the dorsal and ventral hippocampus. The analysis of functional interactions between brain regions suggest that the dorsal and ventral dentate gyrus could be involved in spatial memory retrieval. In addition, the results highlight the key role of the extended hippocampal system, thalamus and striatum in this process. Our study agrees with previous ones reporting interactions between the dorsal hippocampus and the prefrontal cortex during spatial memory retrieval. Furthermore, novel activation patterns of brain networks involving the aforementioned regions were found. These functional brain networks could underlie spatial memory retrieval evaluated in the Morris water maze task.

1. Introduction

The standard model of memory system consolidation supports the temporal reorganization of brain circuits underlying long-term memory storage (Bontempi et al., 1999; Frankland and Bontempi, 2005) by including interactions between the dorsal hippocampus and the medial prefrontal cortex (mPFC). According to this hypothesis, the initially hippocampus-dependent memories are later stored within hippocampal–cortical networks, and finally in the neocortex (McClelland et al., 1995, Squire and Alvarez, 1995; Frankland and Bontempi, 2005; Smith and Squire, 2009; Leon et al., 2010). This idea is consistent with the active role of the medial prefrontal cortex (mPFC) during memory retrieval, probably acting as a match and mismatch comparator that prevents the hippocampus from re-encoding existing memories (Frankland and Bontempi, 2005). Evidence from numerous studies indicates that different brain networks are activated during early or late stages of spatial learning involving memory acquisition (Conejo et al., 2004, Fidalgo et al., 2014) or consolidation (Deiana et al., 2011). However, brain networks involved in long-term memory retrieval as compared with those involved during memory acquisition or consolidation are not well known.

In addition to the dorsal hippocampus and the cortex, other brain regions have been suggested to be involved in spatial memory retrieval. In particular, it has been reported a possible role of particular thalamic nuclei (Loureiro et al., 2012a), the striatum (Iaria et al., 2003) or the ventral portion of the hippocampus (Loureiro et al., 2012b) in the persistence of a spatial memory. In this regard, Aggleton and Brown (1999) postulated an "extended hippocampal system" in which the thalamus together with the dorsal hippocampus would be required for successful performance of several memory tasks. Moreover, particular subdivisions of the striatum have been proposed as key regions in procedural, implicit or habit memory systems (Packard et al., 1989; Fidalgo et al., 2012a) and they have also been associated with behavioral flexibility (Palencia and Ragozzino, 2005; Ragozzino, 2007). On the other hand, the ventral portion of the hippocampus is usually considered to be associated with the modulation of stress, emotions, and affects (Moser and Moser, 1998; Bannerman et al., 2004) whereas spatial memory processes are almost exclusively associated with the dorsal hippocampus. However, recent studies have proposed a functional continuity along the hippocampus required for behavioral performance based on rapid place learning (Bast et al., 2009) that is also reported during retrieval of a recent spatial memory task, since studies in rodents showed activation in both the dorsal and ventral hippocampus during this task (Bontempi et al., 1999; Maviel et al., 2004).

In a previous paper (Conejo et al., 2010) we reported the functional brain networks related with the acquisition of spatial reference memory in the Morris water maze. In addition, we demonstrated the temporal dynamics of brain networks activated during the acquisition of spatial memory. In a recent paper (Conejo et al., 2013) we showed that inactivation of the dorsal hippocampus impaired long-term spatial memory retrieval. Moreover, functional brain networks between the mPFC and the dorsal hippocampus were differentially activated after unilateral or bilateral hippocampal inactivation. However, the role played by different cortical and subcortical brain regions in spatial memory retrieval and their potential functional interactions during this process have still not been addressed.

For this purpose, we used quantitative cytochrome c oxidase (CO) histochemistry as a reliable marker of brain metabolic capacity because CO activity represents an index of mitochondrial metabolic competence (Bertoni-Freddari et al., 2001) associated with energy demands of neurons after prolonged stimulation (Wong-Riley, 1989; Gonzalez-Lima and Jones, 1994). CO histochemistry has been successfully used in previous studies to map changes in brain metabolism involved in a variety of behavioral tasks in different animal species (Agin et al., 2001; Puga et al., 2007). Moreover, studies in rats reported changes in CO activity related to learning and memory tasks performed in the water maze (De la Torre et al., 1997; Villarreal et al., 2002; Riha et al., 2011; Fidalgo et al., 2012b; Conejo et al., 2013). CO histochemistry will be used to map changes in regional brain CO activity correlated with the retrieval of a previously learned spatial task. This method can be applied both to detect differences in the metabolic capacity of specific brain regions and to investigate their functional connectivity (Sakata et al., 2000). In this regard, brain regions that are functionally coupled and their coordinated changes can be expressed as changes in the strength of pairwise correlations of CO activity between brain regions, as previously reported (Sakata et al., 2000; Puga et al., 2007). Considering all of the above mentioned, the present study aimed to evaluate the contribution of different cortical and subcortical brain regions, and the neural networks involved in long-term spatial memory retrieval tested in the Morris water maze.

2. Results

2.1. Behavioral results

Animals did not show sensory or motor deficiencies evaluated with the neurological assessment battery. Therefore, no animals were discarded due to the presence of neurological signs.

2.1.1. Reference memory task

As shown in Fig. 1, the mean escape latencies of the experimental animals significantly decreased throughout acquisition sessions in the Retrieval group [$F_{4,36}=24.49$, $p<0.001$]. Post-hoc Tukey's tests showed differences in escape latencies between the first day versus the second day ($p<0.01$) and first day as compared to each one of the following training days ($p<0.001$), between the second and fourth day ($p=0.01$) and lastly between day 2 versus the last training day ($p<0.001$). Furthermore, the retention probe showed significant differences between the time spent within the four virtual maze quadrants [$F_{3,27}=51.88$, $p<0.001$]. Post-hoc analyses showed that the time spent in D quadrant differed from the other quadrants ($p<0.001$) and that quadrant A (next to the reinforced quadrant) differed from the escape quadrant C ($p<0.02$), although the time spent in all non-reinforced quadrants stayed below chance probability (25% of total time spent in each quadrant or 3.75 s). See Fig. 2.

We found no significant differences between Retrieval and Naïve groups in the mean velocity [$F_{1,99}=1.96$, $p=0.178$] and the mean distance swum [$F_{1,99}=0,46$, $p=0,51$] during the learning phase. However, significant differences across sessions were found for the distance swum [$F_{4,99}=25.08$, $p<0.001$], and an interaction was found between session and group in the mean swim velocity [$F_{4,99}=3.69$, $p<0.01$].

2.1.2. Long-term memory retrieval test

Retrieval test showed that animals preserved a preference for the previously reinforced quadrant seven days after the learning phase finished (Fig. 3). In this regard, the retention probe carried out a week after the last acquisition day showed differences between quadrants in the mean time spent during the retrieval test [$F_{3,27}=31.08$, $p<0.001$] and post-hoc analysis showed that the mean time spent in the escape quadrant (D) significantly differed from the rest of quadrants ($p<0.001$).

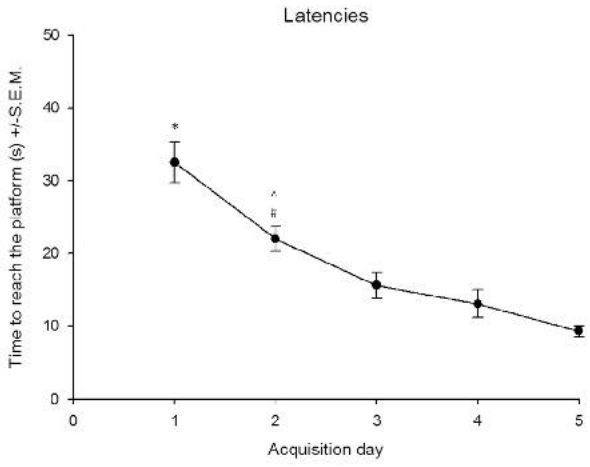

Fig. 1 – Mean escape latencies (±S.E.M.) to reach the hidden platform during the daily session over 5 days by the Retrieval group ($p<0.001$). Post-hoc Tukey tests showed differences between the day 1 as compared to the rest of days (*$p<0.002$), day 2 vs. day 5 (#$p<0.001$), and day 2 vs. day 4 (\widehat$p=0.01$).

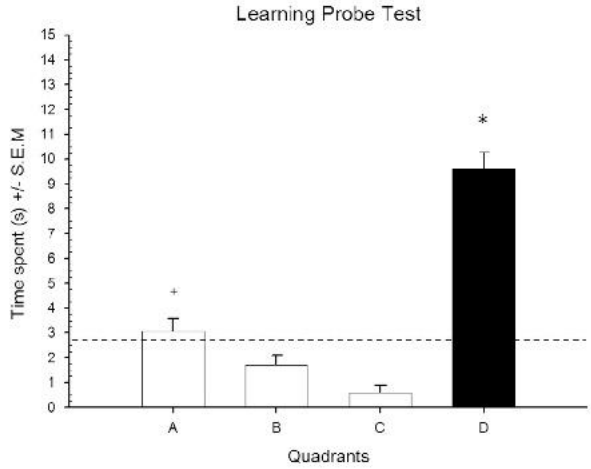

Fig. 2 – Memory retention probe. There were statistically significant differences ($p<0.001$) in the mean time spent in each of the four virtual quadrants during the first 15 s of the retention probe that followed the learning protocol. All pairwise multiple comparison procedures (Tukey's tests) showed differences between the escape quadrant (D) and quadrants A, B, and C (*$p<0.001$). Time spent in quadrant A (adjacent to the goal quadrant) differed from quadrant C (located opposite to quadrant A) (+$p<0.02$) although time spent in all non-reinforced quadrants was below chance (25% time in each quadrant), here represented by a broken line.

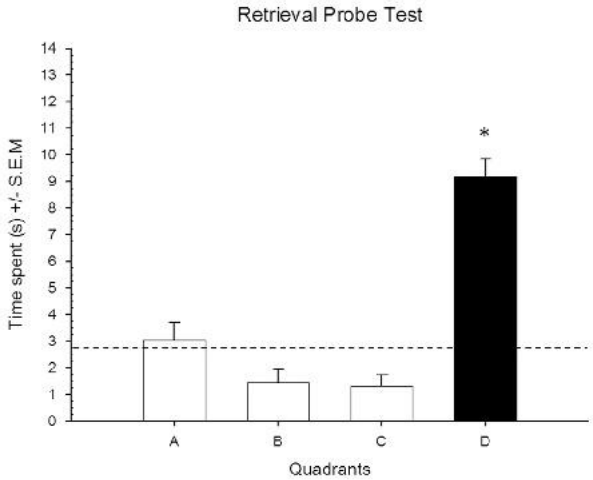

Fig. 3 – Memory retrieval probe. Preference for the escape quadrant was similar to the retention probe performed immediately after the memory acquisition phase. There were significant differences between quadrants ($p<0.001$). In particular, differences were found between the escape quadrant as compared with the rest of quadrants (*$p<0.001$) in all cases.

2.2. CO activity

2.2.1. Mean brain CO activity

Significant differences in CO activity between groups were found in the prefrontal cortex, particularly in the prelimbic [$t_{17}=2.678$, $p<0.02$], infralimbic [$t_{16}=3.252$, $p=0.01$], and cingulate areas [$t_{17}=-3.979$, $p<0.01$] as well as the motor cortex [$t_{16}=3.885$, $p<0.01$]. We found also significant group differences in the anterodorsal thalamus [$t_{17}=2.144$, $p<0.05$], nucleus accumbens core [$t_{17}=2.373$, $p=0.03$] and shell [$t_{17}=4.079$, $p<0.01$] as well as in the dorsal striatum [$t_{14}=3.122$, $p<0.01$]. Finally, group differences in CO activity were found in the dentate gyrus of the hippocampus at dorsal [$t_{18}=2.839$, $p \leq 0.01$] and ventral [$t_{15}=2.332$, $p<0.05$] levels. Increased metabolic activity was found in the experimental group as compared to the naïve group in all of the above mentioned areas. Table 1 shows the mean CO activity values measured in the selected brain regions of both experimental and naïve groups.

2.2.2. Interregional within group correlations of CO activity

Numerous interregional correlations were found in the experimental group (Fig. 4). After a jackknife procedure and Schaffer's sequentially rejective step-down Bonferroni, and Hochberg's sequentially rejective step-up Bonferroni corrections were carried out to detect true pairwise differences, highly significant cross-correlations of CO activity in the retrieval group revealed two different brain networks. The first one comprised the prefrontal cortical regions (infralimbic, prelimbic and cingulate cortices) and the primary motor cortex. In addition, the cingulate cortex showed positive correlations with both the nucleus accumbens core and shell, which had positive correlations between them as well. However, both the prelimbic and infralimbic cortices showed negative correlations with the dentate gyrus of the dorsal hippocampus, being the latter region positively correlated with the CA1 and CA3 areas of the dorsal hippocampus too. A second network in the experimental group included hippocampal ventral areas (CA1v, CA3v and DGv), as well as between anterior thalamic nuclei (AD, AV), being both structures correlated with the CA1 area of the ventral hippocampus. All correlations comprising the second network were positive.

On the other hand, the naïve group showed few positive correlations between brain regions, involving only the cingulate and prelimbic cortices of the prefrontal cortex, and between the CA1 and CA3 areas of the ventral hippocampus. As already mentioned, all correlations were considered statistically significant if $p \leq 0.05$.

3. Discussion

Our results show that animals in the Retrieval group trained in a spatial learning task, followed by a spatial memory

Table 1 – Cytochrome oxidase activity units (μmol/min/g tissue wet weight) measured in the selected brain regions of the groups. Data represent mean ± S.E.M.

Location	N	Retrieval	N	Naïve
Prelimbic cortex*	10	28.22±1.05	9	25.04±0.43
Infralimbic cortex**	10	27.29±0.85	8	23.97±0.41
Cingulate cortex**	10	28.69±0.78	9	25.04±0.44
Primary motor cortex 1**	10	29.77±0.78	8	25.69±0.65
Parietal cortex	10	32.53±2.01	10	29.30±0.83
Retrosplenial agranular cortex	8	34.35±1.23	9	31.72±0.67
Retrosplenial granular cortex	8	36.78±1.35	10	34.42±0.78
Entorhinal cortex	8	24,52±1,31	10	24.32±1.00
Perirhinal cortex	8	27.58±1.91	10	26.60±1.20
Anterodorsal thalamus*	9	51.68±1.52	10	47.82±1.02
Anteroventral thalamus	9	39.75±1.17	10	37.76±0.92
Mediodorsal thalamus	9	29.89±1.5	10	28,42±0,91
Lateral mammillary bodies	10	43.17±1.23	7	42.56±1.38
Medium mammillary body	9	34.95±0.95	7	34.30±0.70
Premammillary bodies	10	33.84±0.93	7	33.35±0.65
Supramammilllary body	10	27.72±0.57	7	27.48±0.92
Acumbens nucleus Core*	9	33.89±0.79	10	30.87±0.97
Acumbens nucleus Shell**	9	38.74±0.72	10	33.74±0.96
Dorsal striatum**	7	31.76±0.57	9	28.27±0.87
Lateral septum	9	27.04±0.91	10	25.38±1.06
Medial septum	9	25.35±0.65	10	24.83±1.08
CA1 dorsal hippocampus	10	30.14±1.75	10	26.72±0.87
CA3 dorsal hippocampus	10	27.90±1.53	10	24.94±0.65
Dentate gyrus (dorsal)*	10	40.64±2.00	10	34.35±0.96
CA1 ventral hippocampus	8	36.90±2.03	9	32.67±1.16
CA3 ventral hippocampus	8	37.24±1.90	9	34.08±1.45
Dentate gyrus (ventral)*	8	33.22±1.36	9	29.09±1.15
Basal amyddala	10	32.83±0.99	10	33.86±0.88
Central amygdala	9	32.22±0.97	10	31.33±0.66
Lateral amygdala	10	24.21±0.90	10	23.87±0.67
Medial amygdala	10	26.70±1.04	10	25.46±0.80

* $p<0.05$.
** $p<0.01$.

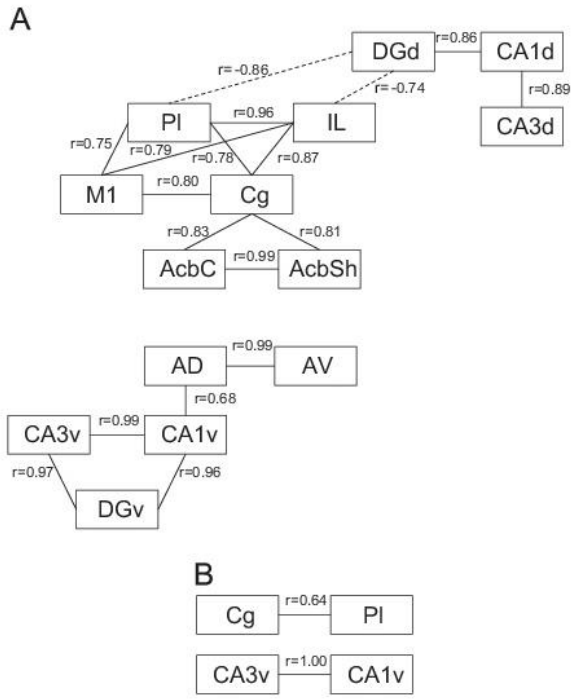

Fig. 4 – Schematic diagram showing the significant interregional correlations of CO activity calculated for the experimental (A) and control (B) group. Solid and broken lines represent respectively highly positive and negative pair-wise Pearson's correlations ($r>0.64$; $p<0.05$).

retention probe one week later were able to remember the location of the escape platform. Mastery of the spatial memory task acquisition was revealed both by the significant decrease of latencies and distance to reach the platform along training days, and the significantly higher amount of time spent in the target quadrant as compared with the rest of quadrants during the memory retention probe. In this regard, it should be noted that both groups (Retrieval and Naïve) swam an equivalent amount of time to account for the effects on brain metabolism of motor activity and emotional processes not specifically memory-related. This result was evidenced by the absence of differences between groups neither in the mean distance swum nor in mean swimming speed. Likewise, during the retention probe carried out seven days later, animals still spent more time in the previously reinforced quadrant. Therefore, we could consider that acquisition and retrieval of spatial memory have been successfully completed in our study.

Regarding the study of brain oxidative metabolism, our results showed higher CO activity in the Retrieval group (R) as compared with the Naïve (N) group in several regions analyzed, including the prefrontal cortex (cingulate, prelimbic and infralimbic areas), primary motor cortex, the dorsal and ventral striatum, the nucleus accumbens shell and core, and the dentate gyrus of the dorsal and ventral hippocampus. In addition, correlation studies revealed the activation of different brain networks in both groups. Thus, the Retrieval group showed a more intricate pattern, including two independent functional brain networks. The first one included interactions not only between the PFC, specifically the cingulate cortex, and the dorsal hippocampus (dH), as we had previously reported (Conejo et al., 2013) but also between the PFC and the striatum. A second network included the thalamic nuclei together with the ventral portion of the hippocampus. On the other hand, the Naïve group showed only short-scale brain networks between closely interconnected brain regions. These networks were limited to the prelimbic and infralimbic cortices of the PFC, and the CA1v-CA3v subfields of the ventral hippocampus independently.

Our findings are consistent with the prevalent idea that suggests the involvement of distributed or large-scale cortical-dorsal hippocampus networks in spatial memory (Bontempi et al., 1999; Frankland and Bontempi, 2005; Wang and Cai, 2008; Leon et al., 2010; Conejo et al., 2013). In addition to the involvement of this well-known hippocampal-prefrontal cortex circuit, there is broad agreement on the temporal reorganization of circuits underlying spatial memory (Bontempi et al., 1999; Maviel et al., 2004, Conejo et al., 2010). In other words, the neural networks involved in recent memory storage are supposed to differ from those underlying remote memory. This fact is supported by lesion studies suggesting that inactivation of specific cortical regions would alter remote memory without disrupting a recent one (Quillfeldt et al., 1996; Takehara et al., 2003; Frankland et al., 2004). Moreover, the prefrontal cortex has been largely known for playing an important role in navigational tasks. Specifically, the prelimbic and infralimbic areas have been related with spatial working memory (Ragozzino et al., 1998) and recent spatial memory (Wang and Cai, 2008) while the cingulate area is suggested to be involved in spatial or contextual discrimination tasks (Frankland and Bontempi, 2006; Frankland et al., 2006). The potential of the prefrontal cortex for the integration and synthesis of information from a large number of sources (Miller, 1996) may indicate its ability to process remote memories (Blum et al., 2006), similar to the role of the hippocampus to process recent memories (Frankland and Bontempi, 2005). Accordingly, inactivation of the prelimbic or anterior cingulate cortices prevents the recall of remote memories (Frankland et al., 2004; Maviel et al., 2004) and pharmacological studies involving the disconnection of hippocampal–PFC circuits reported impaired spatial learning (Wang and Cai, 2008).

In addition to the CO activity increase found in the dorsal and ventral portions of dentate gyrus, a negative correlation between the cingulate cortex and the dorsal DG was found in the Retrieval group after the task. In this regard, we had previously reported a progressive mPFC involvement in the mastery of a spatial reference memory task (Conejo et al., 2010) as shown by the highest mPFC activation during late acquisition stages. Moreover, the mPFC–dorsal hippocampus functional coupling proved to be consistent throughout the retrieval phase (Conejo et al., 2013). Taken together, this data would support the hypothesis stated by Frankland and Bontempi (2005) who suggested that the mPFC could play an active role during memory recall, by preventing the hippocampus to re-encode existing memories. Although the above-mentioned relationship between mPFC and dorsal hippocampus has been largely addressed, the specific role played by the DG in the process is not well known. In this regard, many studies focused on hippocampal subfields demonstrated that the DG may participate in working memory tasks (Emerich and Walsh,

1989; Gilbert et al., 2001) and those tasks that require the use of information that is trial-specific during memory retrieval in spatial learning tasks (Talpos et al., 2010). The latter type of tasks were used in our study, since rats were received a single memory retrieval trial a week after training in the spatial learning task. As regards to the role played by the DG in spatial memory, several studies showed that selective lesions of the DG in rodents disrupt performance of spatial working memory tasks (McLamb et al., 1988) and specifically impair spatial reference memory tasks (Nanry et al., 1989; Xavier et al., 1999; Okada and Okaichi, 2009; Morris et al., 2012).

Similarly, we found increased oxidative metabolism in the dorsal striatum and the nucleus accumbens of experimental animals in agreement with previous studies (Miranda et al., 2006; Miyoshi et al., 2012) reporting that the dorsal striatum together with the dorsal hippocampus are required for spatial navigation in the MWM. According to these authors, during navigation, the spatial relationships among environmental stimuli are processed in the hippocampus to establish a kind of map of the spatial context (Telensky et al., 2011) meanwhile the dorsal striatum uses this information to provide the best motor action plan to navigate to a target location. On the other hand, the dorsal striatum would be involved in the selection of motor actions required for navigation that can be based on contextual or cued information (Opris and Bruce, 2005; Da Cunha et al., 2009). Regarding spatial information retrieval, there is evidence from studies using functional magnetic resonance imaging in humans showing that participants considered as more accurate spatial navigators displayed higher hippocampal and caudate activation when following a well-learned route to an unseen goal in a familiar environment (Hartley et al., 2003). Therefore, the dorsal striatum could be required to use spatial information previously mapped by the hippocampus and integrated by the mPFC to guide navigation to the goal place, although further studies will be necessary to properly assess its specific contribution during spatial reference memory retrieval.

Finally, the anterior and mediodorsal thalamic nuclei have been involved in memory retrieval processes (Staudigl et al., 2012). According to this author, the activity of the mediodorsal thalamic nucleus and the PFC would be synchronized during human memory retrieval, suggesting that these brain regions could send an early memory signal to the cortex, which may trigger subsequent memory search and decision process. In this regard, contextual fear memory retrieval in mice is also reported to be supported by a functional connectome in which the thalamus is involved as part of a thalamic–hippocampal–cortical network (Wheeler et al., 2013), as shown by the expression of the immediate early gene c-fos. Our data are also in agreement with previous findings demonstrating that lesions of the anterior thalamic nuclei impair both spatial (Warburton and Aggleton, 1999; van Groen et al., 2002, Lopez et al., 2009; Lim et al., 2014) and non-spatial (Wolff et al., 2006) memory processes in rats, in a way similar to hippocampal lesions. Furthermore, disconnection studies demonstrated previously the functional link between the aforementioned brain regions in spatial memory tasks (Warburton et al., 2001; Henry et al., 2004). Also, the so-called "head direction system" cells found in the anterior thalamic nuclei represent the direction in which the animal's head is facing, and it seems to be associated with the integrity of the anterior thalamic nuclei (Shinder and Taube, 2011). In our study, CO activity of the selected thalamic nuclei was positively correlated with the CA1v subfield of the ventral hippocampus in the Retrieval group. Therefore, we cannot rule out the possibility that their functional interaction with the ventral hippocampus could be particularly involved in spatial orientation processes. Regarding the ventral hippocampus, increased metabolic activity was measured in the DGv of the experimental group. Traditionally, the dorsal and ventral hippocampi are believed to have different functions on basis of their connectivity in different brain networks (Fanselow and Dong, 2010; Pennartz et al., 2011). This functional differentiation is found also in the human hippocampus, which implies an anterior–posterior functional division (Iaria et al., 2007; Doeller et al., 2008). The ventral hippocampus (homologous to the anterior hippocampus in humans) is thought to be more related to emotions and body states (Fanselow and Dong, 2010) whereas conversely the dorsal hippocampus (posterior part in humans) is associated with visuo-spatial and cognitive skills, including spatial learning (Morris et al., 1982, Moser and Moser, 1998). Therefore, the ventral hippocampal activation found could be attributed to the stress associated with the water mask task. However, no differences between groups were found in our study in regions related to escape under stress such as the perirhinal cortex and the basolateral amygdala (Villarreal et al., 2002). In this regard, it seems feasible that the ventral hippocampus would play a different role in spatial reference memory retrieval task. These results agree with a recent study (Ruediger et al., 2012) reporting a key role of this area in goal-oriented learning and searching behavior. As regards to spatial reference memory retrieval, our data support previous studies using immediate-early gene expression, 2-deoxyglucose uptake (Bontempi et al., 1999; Maviel et al., 2004) and lesion studies (Loureiro et al., 2012b) that demonstrated an engagement between dorsal and ventral parts of the hippocampus in SMR retrieval, measured between one and five days after learning.

4. Experimental procedures

4.1. *Subjects*

Male Wistar rats (University of Oviedo animal facility, Spain) weighing 220–270 g at the start of the experiment were used. Animals were kept under standard rearing conditions (light period: 08:00–20:00 h, room temperature 23±2 °C) with ad libitum access to food and tap water. Experimental procedures and manipulations of the animals were approved by the University of Oviedo bioethics committee to guarantee that European Directive 2010/63/UE and Spanish regulations (RD 1201/2005) on the use and care of experimental animals were followed.

4.2. *Behavioral procedure*

Rats were trained in a water maze (diameter: 1.5 m) to evaluate spatial memory (Morris, 1984).The maze was filled with tap water at constant temperature (21±1 °C) throughout the experiment. Visual cues were available in the test room, including different pictures on the room walls, a bookshelf,

colored objects made of paper fixed on two poster panels surrounding the pool and a closed window. Two halogen spot lights facing the walls and placed in the floor illuminated the room. An automated video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands) was used to record the swim paths and escape latencies of each animal in the water maze.

All animals were first tested in a neurological assessment battery to discard possible motor and sensory deficits. The neurological battery used included the following tests: abduction response of hind limbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response and righting reflex (Bures et al., 1976). Rats were handled daily during 5 days. Behavioral testing was carried out during the morning between 09:30 and 14:00 h.

The animals were randomly distributed into two groups, Retrieval (R, $n=10$) and Naïve (N, $n=10$). The Naïve group underwent the same behavioral protocol in the Morris water maze (i.e: habituation, reference memory task, memory retention test, single refreshing trial and long-term memory retrieval test) and swam during an equivalent period of time as compared to the Retrieval group rats, but in the absence of a platform in the swimming pool throughout testing period.

4.2.1. *Habituation*

During the habituation, rats were trained in a visually-guided learning task to find a visible platform. The water maze was virtually divided into four equal quadrants (A, B, C, and D). The rats received two sessions with 1 h interval between them. In each session, they were released facing the tank walls from the central part of each quadrant following a pseudorandom sequence, four times per session. Between sessions, rats were returned to their home cages. The escape platform was placed in the center of the quadrant D, 2 cm above the surface of the water during the habituation phase. Rats were allowed to swim during 60 s to locate the platform in each trial, or gently guided to it after that period of time. They remained there for 15 s and later placed in a plastic bucket during 15 s until the next trial.

4.2.2. *Reference memory task*

Animals were trained during five consecutive days in a hidden platform task and tested for retention test immediately after the last acquisition trial.

During the acquisition phase, Retrieval group animals were trained in a daily session of four trials, when they were released from the central border of each of the quadrants in a pseudorandom order to search for a hidden escape platform beneath the water surface (1.5 cm). Therefore, they followed a hidden platform learning task procedure, in which the platform was kept in the same quadrant (escape quadrant, D) along the acquisition stage, and rats were required to find it using spatial cues available in the room following training. As explained above, rats were allowed to swim during 60 s and left in the platform during 15s.

4.2.3. *Memory retention test*

After the last training day, rats were submitted to a memory retention test in a single probe trial. During this trial, the platform was removed from the maze, and rats were released from the opposite quadrant. They were allowed to swim during 60 s. After this period, animals were picked up from the pool and placed again in the plastic bucket. In order to prevent extinction of the previously learned task, all animals underwent an additional trial in which the platform was presented again in its original place, and the animals received a single trial of 60 s. In this case, animals were released from the same point (quadrant B).

Memory retention was evaluated measuring the amount of time spent in the escape quadrant as compared to the rest of quadrants.

Although the probe trial lasted 60 s, only the first 15 s were analyzed due to the high activity levels of the rats. It was observed that most animals tended initially to swim in the correct quadrant, but then quickly started to search in the rest of quadrants if they have failed to locate the absent platform (Spooner et al., 1994; Conejo et al., 2007).

4.2.4. *Long-term memory retrieval test*

One week after the last acquisition trial, an additional probe trial was carried out under the same conditions mentioned in the previous section.

4.3. *Cytochrome oxidase hystochemistry*

Ninety minutes after completion of the spatial reference memory retrieval task, brains were removed and tissue was quickly frozen in isopentane at −70 °C (Sigma-Aldrich, Madrid, Spain) and stored at −40 °C. Later, 30 μm-thick coronal sections were obtained from the brain tissue using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). Brain sections were processed for quantitative CO histochemistry as previously described (Conejo et al., 2010).

We used a modified version of the method originally described by Wong-Riley based on the quantitative CO histochemical method developed by Gonzalez-Lima and Jones (Gonzalez-Lima and Jones, 1994). Batches of sections obtained from brain tissue homogenate from Wistar rats were included in each CO staining bath together with brain sections. Batch standards of brain homogenate were previously analyzed by spectrophotometrical methods to measure mean of CO activity. Each homogenate standard slide contained a set of sections of different thicknesses (10, 30, 50 and 70 μm) in order to assess the linearity of the histochemical reaction as related to CO enzyme content in the tissue. Brain sections and homogenate standards were incubated during 5 min with a 0.5% glutaraldehyde solution, rinsed in phosphate buffer and preincubated 5 min in a solution containing in 50 mM Tris buffer with 0.0275% cobalt chloride, 0.5% DMSO, and 10% sucrose. After a 5-min rinse in PBS, sections were incubated for 60 min at 37 °C in a CO staining solution containing 0.05% diaminobenzidine tetrahydrochloride, 0.0075% cytochrome c from horse heart (Sigma, Barcelona, Spain), 0.002% catalase (Sigma), 5% sucrose, and 0.25% DMSO sulfoxide in PBS (0.1 M; pH 7.4). The reaction was stopped by immersing the sections in formalin solution for 30 min at room temperature with 10% sucrose and 4% formalin. Sections were then dehydrated in graded concentrations of ethanol, cleared with xylene, and preserved with Entellan (Merck, Darmstadt, Germany).

4.4. Densitometric quantification

A computer-controlled image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) composed of a high precision illuminator, a digital camera and a computer with specific image analysis software was used to perform image analysis of the stained slides. The mean relative optical density was measured using four non-overlapping measures obtained in three consecutive sections. Calibration of optical density measures for CO activity units was performed using the stained homogenate standards for each staining batch. For each staining batch the software calculated a linear regression between optical density and CO activity, using the measured optical density attributed to each section of brain tissue homogenate. We then checked that homogenate section thickness was directly correlated with increased optical density of the histochemical reaction. To account for inter-batch variability, we used the calculated linear regression equation to estimate CO activity from OD measures in the brain sections. Average relative optical density measured in each brain region was converted into CO activity units (1 unit: 1 μmol of cytochrome c oxidized/min/g tissue wet weight at 23 °C) using the calculated regression curve in each homogenate standard. These measures were averaged to obtain one mean per region for each animal in cortical regions, including cingulate (Cg), prelimbic (PrL), and infralimbic cortex (IL) of the medial prefrontal cortex and primary motor cortex (M1), all of them measured 3.70 mm from Bregma; granular (RSG) and agranular (RSA) retrosplenial cortices at −4.52 mm; parietal (PAR) −3.80 mm, entorhinal (Ent), and perirhinal (PRh) cortices at −4.52 mm. The following subcortical regions were also analyzed: medio dorsal (MD) anterodorsal (AD) and anteroventral (AV) thalamic nuclei, all of them measured at −1.40 mm; lateral (LS) and medial septum (MS) at 0.20 mm, dorsal striatum (DS), accumbens core (AcC) and shell (AcSh) nucleus, measured at 1.00 mm; dorsal hippocampus including CA1, CA3 and dentate gyrus of the dorsal (CA1d, CA3d, DGd areas) at −3.30 mm; and ventral hippocampus (CA1v, CA3v, DGv areas) at −4.52 mm from Bregma. Medial (Me), basal (Ba), lateral (La) and central (Ce) amygdaloid nuclei measured at −3.14 mm from Bregma; medial (MM), lateral (LM) and supramammillary nuclei (SuM) of the mammillary bodies measured at −4.52 mm, as well as the premammillary nucleus (PM) at −4.16 mm. The selected brains regions were located and identified following the Paxinos and Watson's atlas (Paxinos and Watson, 2004).

4.5. Data analysis

All data were analyzed by SigmaStat 3.2 software (Systat Software, Chicago, USA) and were expressed as mean ± S.E.M. The results were considered statistically significant when $p<0.05$.

4.5.1. Behavioral data

Mean escape latencies and total distance swum were automatically recorded for each daily session (four trials per session) in the water maze, and data were analyzed using one-way repeated measures ANOVA, with training day as the repeated-measures factor. During the retention and retrieval probe trials, the mean time spent in the different quadrants was analyzed using one-way repeated measures ANOVA design with quadrant as factor. When significant differences were found, tests for multiple comparisons (Tukey's tests) were used to identify the group or groups that significantly differed from the others.

Two-way repeated measures ANOVAs were applied to evaluate differences in the mean distance swum and mean velocity in Naïve and Retrieval groups.

4.5.2. Mean CO activity and CO activity correlations

Group differences in regional mean CO activity were evaluated by an unpaired t-test in each one of the brain regions measured.

Functional connectivity between brain regions was analyzed by calculating pairwise correlations in CO activity between brain regions in each experimental group. Pearson's product–moment correlation coefficients between pairs of brain regions in each experimental and control groups were calculated. In order to minimize possible variations in the intensity of the CO staining not resulting from the experimental manipulation, CO activity values were normalized by dividing the measured activity of each brain region by the average CO activity value for all regions measured in each animal. In addition, a "jackknife" procedure was used (Shao and Dongsheng, 1995), in order to avoid type I errors caused by the high number of possible comparisons and the brain regions measured. This procedure involves the calculation of all possible pairwise correlations resulting from removing one subject each time, and then considering only those correlations that remain significant ($p<0.05$) across all possible iterations.

Additionally, a Schaffer's sequentially rejective step-down Bonferroni, and Hochberg's sequentially rejective step-up Bonferroni procedures were carried out to detect true pairwise differences.

5. Conclusions

Together, these results highlight the previously unknown key role of the dentate gyrus in memory retrieval of a spatial reference memory task, including its ventral portion. In addition, our study supports the contribution of the thalamus, striatum and the extended hippocampal system to spatial memory retrieval. Our results agree with previous studies showing an interaction between the dorsal hippocampus and the prefrontal cortex during this process. Finally, novel activation patterns of brain networks involved in spatial memory retrieval were found.

Acknowledgments

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Artículo 2


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Hippocampal Inactivation with TTX Impairs Long-Term Spatial Memory Retrieval and Modifies Brain Metabolic Activity

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Abstract

Functional inactivation techniques enable studying the hippocampal involvement in each phase of spatial memory formation in the rat. In this study, we applied tetrodotoxin unilaterally or bilaterally into the dorsal hippocampus to evaluate the role of this brain structure in retrieval of memories acquired 28 days before in the Morris water maze. We combined hippocampal inactivation with the assessment of brain metabolism using cytochrome oxidase histochemistry. Several brain regions were considered, including the hippocampus and other related structures. Results showed that both unilateral and bilateral hippocampal inactivation impaired spatial memory retrieval. Hence, whereas subjects with bilateral hippocampal inactivation showed a circular swim pattern at the side walls of the pool, unilateral inactivation favoured swimming in the quadrants adjacent to the target one. Analysis of cytochrome oxidase activity disclosed regional differences according to the degree of hippocampal functional blockade. In comparison to control group, animals with bilateral inactivation showed increased CO activity in CA1 and CA3 areas of the hippocampus during retrieval, while the activity of the dentate gyrus substantially decreased. However, unilateral inactivated animals showed decreased CO activity in Ammon's horn and the dentate gyrus. This study demonstrated that retrieval recruits differentially the hippocampal subregions and the balance between them is altered with hippocampal functional lesions.

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Introduction

Solving the memory puzzle involves understanding the hippocampal role in spatial behaviour, clarifying the particular contribution of both hippocampi and its interaction with other brain structures. Several studies described the effect of unilateral hippocampal interventions on memory tasks. In this regard, it seems well established that partial hippocampal inactivation with tetrodotoxin (TTX) or lidocaine caused severe memory problems in different hippocampal-dependent tasks, like the Morris water maze, rotation arenas or passive avoidance tasks, by altering those processes engaged in memory formation [1], [2], [3], [4]. In the last years, it was shown that the hippocampus can be necessary for retrieving memories acquired days or weeks before [5], [6], [7]. However, the comparison of functional unilateral versus bilateral inactivation in long periods after acquisition has not been explored yet.

The assessment of brain activity during such experimental manipulations using cytochrome oxidase (CO) histochemistry has shed light on the interactions between the hippocampus and other related structures [8], [9], [10]. These results could provide evidence about the role of different brain structures engaged to any of the phases of memory formation during the learning experience. In this way, hippocampo-cortical functional integrated circuit seem relevant for successful performance and retrieval of spatial memory [11], [12], [13], [14], [15], [16].

CO is a mitochondrial enzyme that catalyzes the transfer of electrons to oxygen generating ATP via the coupled process of oxidative phosphorylation [17]. CO activity reflects changes in the brain metabolic capacity induced by energy requirements, and CO activity is regulated by and closely correlated with brain functional activity [18], [19].

Several authors demonstrated CO changes in memory circuits associated with spatial memory after several experimental manipulations. Hence, it was applied to discern how different structures modify their metabolic demands in subjects solving working memory tasks [20] or under other experimental manipulations [9], [21].

However, it is not clear how the hippocampal system and related structures functionally interact when the hippocampus is unilaterally or bilaterally inactive and the subject is forced to recall spatial information learned several weeks before with an intact brain. Similarly, it is unknown how hippocampal inactivation may affect the functional interrelationships between the hippocampus and prefrontal cortex, and therefore affect the spatial behavior. Here we applied CO histochemistry to determine the brain metabolism in rodents that have to retrieve long-term memories in the Morris water maze under unilateral or bilateral hippocampal

reversible inactivation. In the same way, interregional CO activity correlations among medial prefrontal cortex and dorsal hippocampus are also used to determine functional changes in the neural networks therein following cerebral inactivation.

Materials and Methods

Animals

Thirty male adult Wistar rats (300–350 g) from the breeding colony of the University of Oviedo (Oviedo, Spain) were used in this study. They were housed under standard conditions (12-h light/dark cycle with lights on from 08:00–20:00h), at constant room temperature of 21±2 °C with ad libitum access to food and water. All experimental procedures carried out with animals were approved by a bioethics committee of the University of Oviedo and strictly followed the European Communities Council Directive (2010/63/UE) and the Spanish legislation (R.D. 1201/2005) for the care and use of experimental animals.

Surgery

Rats were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.m.) and given additional doses of ketamine i.p. as needed to maintain deep anaesthesia. Subjects were placed in a stereotaxic frame (Narishinge, Tokio, Japan) and the scalp was incised and retracted. The skull was exposed and adjusted until bregma and lambda were on the same horizontal plane. After small burr holes were drilled, stainless-steel cannulae (26 gauge) were implanted bilaterally or unilaterally in the dorsal hippocampus (coordinates relative to bregma: AP –3.5 mm, ML ±2.5 mm, DV –2.00 mm from dura) according to Paxinos and Watson's Atlas [22]. Cannulae were secured using dental cement and anchoring screws.

Apparatus

Animals were trained in the Morris water maze, using a circular water tank made of black fibreglass (diameter = 1.5 m and height = 75 cm) placed 50 cm above the floor [23]. The pool 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 23±1°C during the entire test period. The experimental room had numerous visual cues on the walls such as posters, plastic dishes, and a shelf. The swimming pool was indirectly illuminated by two halogen spotlights (500 W) located on the floor and facing the walls. The Morris water maze was divided virtually into four quadrants, according to the cardinal points (N, S, E, W) and swimming paths were recorded and analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

Behavioural procedure

Habituation. Rats were allowed to recover for 7 days during which they were handled daily. On day one, each rat received two habituation sessions spaced 1 h apart. Rats were randomly released four times per session, facing the pool wall from one of the four compass locations around the pool. Subjects were returned to their home cages between sessions. The escape platform used on the first day was painted white and stood up 2 cm above the water surface. Rats were allowed to freely swim to locate the escape platform or placed on it if 60s had elapsed. They remained on the platform for 15 s. Then they were introduced into a black plastic bucket for 30 s. The water was stirred between trials in order to remove olfactory traces of previous swim patterns [24].

Training phase. After the habituation phase, each animal received a single four-trial session during five consecutive days, days 2 to 6. The platform remained in the same position as during habituation. In each trial, the subjects were released randomly from one of four compass locations and had to search for a hidden platform that remained in the same position during the whole training period. On day 6, after completing the last trial of the training phase, each rat was subjected to a probe trial. The escape platform was removed and subjects were introduced during 30 s from the quadrant opposite to the target quadrant.

Intracerebral Injections. Tetrodotoxin (TTX), a highly selective voltage-gated sodium channel blocker, was used to temporally inactivate the dorsal hippocampus. Twenty-eight days after finishing the training rats received 1 µl of saline or 5 ng of TTX in 1 µl of saline. During infusions, rats were placed on the experimenter's lap, where grooming or excessive motion were limited. An injection cannula (32 G) protruding 2 mm from the guide cannula was inserted into the hippocampus. The injection solution was delivered during 90 s using a Hamilton syringe connected to the injection cannula with a short piece of polyethylene tubing. The injection cannula was left in place for an additional 1 min to achieve a proper diffusion of the drug from its tip. Tissue inactivation lasts approximately 3 h [25].

Subjects were randomly assigned to any of the three groups: bilateral TTX injections (BIL; n = 10), right unilateral TTX injections (RU; n = 10), and saline injections (CTR; n = 10). Rats were subsequently returned to their home cages, and any abnormalities in movement were examined for 30 min before they were placed into the maze for the remote memory probe.

Remote Memory Probe. The remote memory probe began 45 min after the intracerebral injection. Subjects were released from the quadrant opposite to the target quadrant and allowed to swim for 30 s. Time spent in each quadrant and total distance swum were recorded and analyzed later using the video-tracking system. Additionally, the pool was also conceptually divided into a central circular area and two concentric annular areas (inner, middle and outer areas, respectively). The total number of visits and swimming time in these rings were used to evaluate the exploratory activity of each group.

Quantitative Cytochrome Oxidase Histochemistry

Ninety minutes after the behavioral procedures, rats were decapitated and their brains quickly frozen in isopentane. Coronal brain sections (30 µm thick) were obtained using a cryostat microtome (Microm HM-505E, Heidelberg, Germany) and processed for CO histochemistry according to the method described by Gonzalez-Lima and Jones [26]. A total of twelve measurements (four readings in three consecutive coronal sections) were taken per brain region. These measurements were averaged to obtain one mean value per region for each animal and were expressed as arbitrary units of optical density (OD). In order to quantify enzymatic activity and to control staining variability across different staining baths, slides including sets of tissue homogenate standards obtained from adult male Wistar rat brains were included in the study. These standards were cut at different thicknesses (10, 30, 40, and 60 µm) and included in each staining bath with the rest of slides. Previously, mean cytochrome oxidase (CO) activity of the homogenate was spectrophotometrically assessed. Therefore, sets of sections from rat brain homogenate of known CO activity were used as calibration standards in each CO staining bath. Series of coronal sections from each brain together with a complete set of standards were used to perform CO histochemistry.

Briefly, slides were lightly fixed for 5 min with a 1.5% glutaraldehyde, rinsed three times in phosphate buffer and preincubated in a solution containing cobalt chloride and dimethylsulfoxide dissolved in Tris buffer. Once the sections had been rinsed in phosphate buffer (pH 7.6; 0.1 M), they were incubated in darkness for 1 h at 37°C in a solution containing diaminobenzidine, sucrose, cytochrome c and catalase (Sigma-Aldrich, Spain) dissolved in phosphate buffer (pH 7.6; 0.1 M), which was continuously stirred. The slides were rinsed three times with cold phosphate buffer, and then dehydrated and coverslipped with Entellan (Merck, Darmstadt, Germany).

Regression curves between section thickness and known CO activity measured in each set of standards were calculated for each incubation bath. Finally, average regional optical density measured in each brain region was converted into CO activity units (micromoles of cytochrome c oxidized/min/g tissue wet weight at 23°C) using the calculated regression curve in each homogenate standard. CO histochemical staining intensity in each brain region of interest was measured densitometrically and converted to CO units using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) composed of a high precision illuminator, a digital camera and a computer with specific image analysis software. CO activity in both the right and left hemispheres of the selected brain regions (located in the cortex, diencephalon and amygdala) were previously measured in every subject. However, no significant differences between right and left hemispheres were found. Therefore, we decided to show only these brain regions in the right hemisphere. Eight brain regions were measured unilaterally in each subject. In addition, the prefrontal cortex and dorsal hippocampus were measured bilaterally. The dorsal part of the hippocampus (CA1, CA3 and DG areas) was measured approximately between –4.30 and –4.40 mm anterioposterior from bregma (Paxinos & Watson's rat brain atlas) in order to avoid possible direct effects of TTX diffusion from the injection site at –3.5 mm. The actual extension of the TTX area of influence at the injection site was estimated in previous pilot studies to be on average less than 1.5 mm in diameter.

Six animals, four from BIL group and two from RU group were discarded after the histology since cannulae did not reach the hippocampus. According to this, the final number of subjects per group was: CTR n = 10, RU n = 8, BIL n = 6.

Statistical Analysis

Behavioural Data. Mean escape latencies during the training phase were analysed using two-way repeated measures ANOVA (group x days). Similarly, two-way repeated measures ANOVAs (group x quadrant) were used to evaluate differences between groups in mean time spent in the different quadrants during the retention and remote memory probes. In addition, the mean number of visits and time spent in the previously mentioned circular concentric areas in the remote memory probe were analyzed with two-way repeated-measures ANOVAs (group x area). Finally, the total distance swum during the remote memory probe was evaluated with one-way ANOVA. Tukey's HSD post-hoc tests were applied when significant ANOVA results were found.

CO activity. Differences in CO activity between experimental groups in each brain region were evaluated by one-way ANOVA. Tukey's post hoc tests were used when ANOVA indicated significant group differences. In order to evaluate possible changes in hippocampal functional connectivity caused by TTX injections, regional CO activity data were analyzed using pair-wise correlations between the hippocampal areas in each experimental group. The analysis of interregional correlations was done by calculating Pearson product-moment correlations. CO activity values were normalized by dividing the measured activity of each structure by the average CO activity value of the hippocampal areas measured for each animal. This was done to reduce variation in the intensity of the CO staining not resulting from experimental manipulation. In addition, in order to avoid errors derived from calculation of multiple correlations using small sample sizes we used a 'jackknife' procedure [27] based on the calculation of all possible pair-wise correlations resulting from removing one subject each time and taking into consideration only those correlations that remain significant ($p<0.01$) across all possible combinations. Statistical analysis was performed using statistical analysis software (SigmaStat 3.5, Systat Software, San Jose, California, USA).

Results

Behavioural Results

Groups did not differ in their latency to find the hidden platform ($F_{2,21} = 0.23$; $p>0.05$) but there was a significant main effect of days ($F_{4,84} = 74.9$; $p<0.001$) and no interaction ($F_{8,84} = 0.37$; $p>0.05$). Tukey HSD test revealed that subjects learned the task, since latencies decreased significantly across sessions in the five training days ($p<0.05$) (Fig. 1). Additionally, groups did not differ during the retention probe ($F_{2,21} = 1.57$, $p>0.05$) but there was significant main effect of quadrant ($F_{3,63} = 23.2$; $p<0.001$). Post hoc analysis showed that subjects remembered the position of the hidden platform since they spent more time swimming into the target quadrant ($p<0.01$) (Fig. 2).

When subjects received saline or TTX unilaterally or bilaterally, the data analysis of the remote memory probe showed an interaction between group and quadrant ($F_{6,63} = 13.1$; $p<0.001$). Post hoc analysis revealed that CTR animals remembered the platform location twenty eight days later, spending more time swimming in the escape quadrant ($p<0.001$). However, RU and BIL groups did not search for the missing platform in the correct quadrant. Hence, RU group showed a significant trend to swim in quadrant C ($p<0.001$), whereas BIL group showed no preference for any quadrant (Fig. 2).

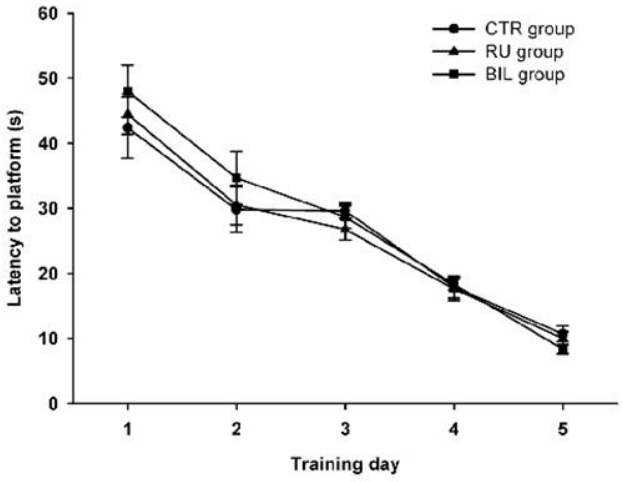

Figure 1. Learning Curves. Similar mean escape latencies across training days in the water maze of the three experimental groups. Data are presented as mean ± S.E.M.
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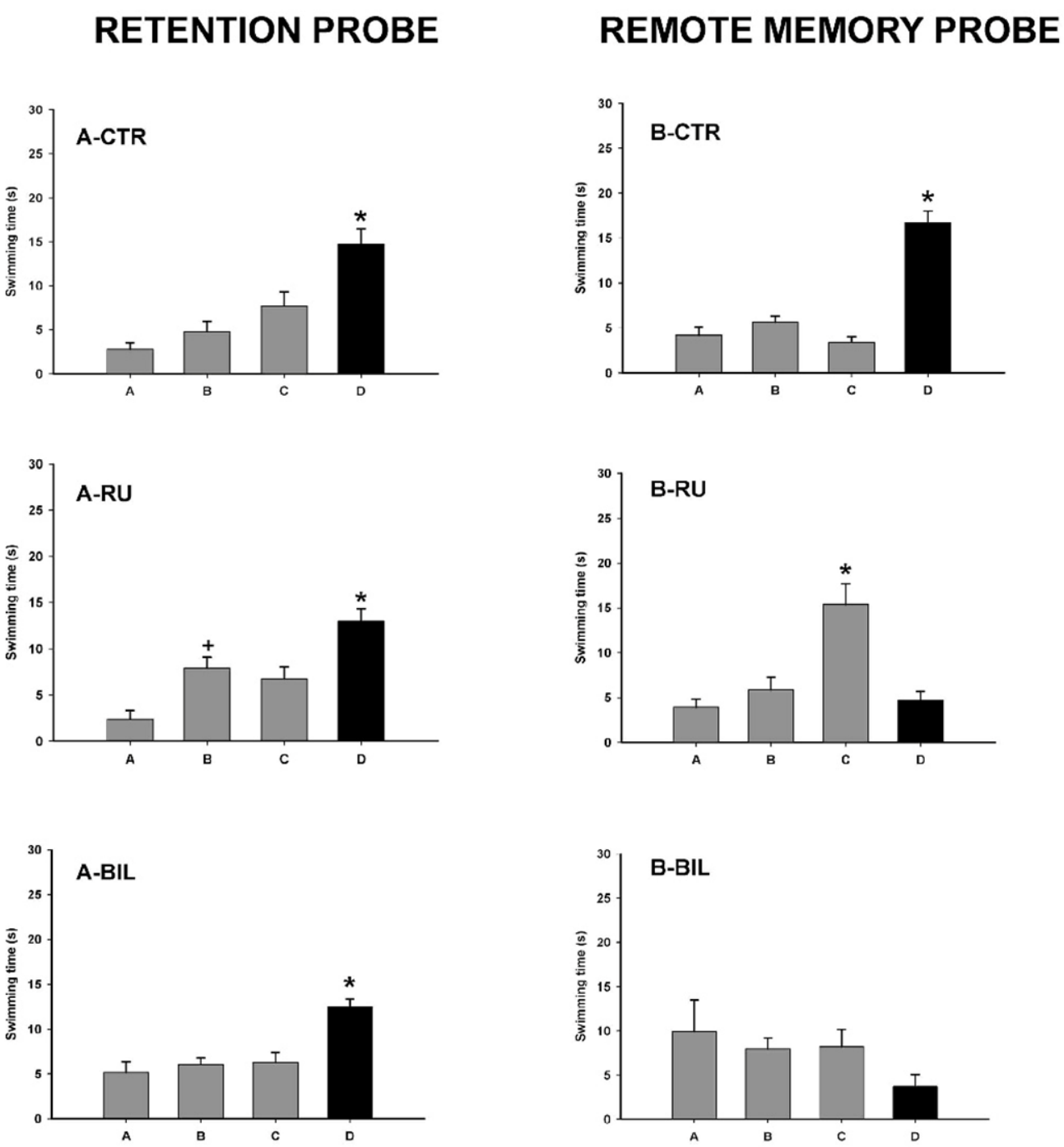

Figure 2. Retention probe and remote memory probe. Mean time spent in the different quadrants during retention probe (left column) and after TTX injection (remote memory probe, right column) in the different experimental groups. Bars represent mean swim latencies in the different quadrants of the water maze during the probes. D = target quadrant, C = opposite, A = counter-clockwise, B = clockwise. *$p<0.01$, significantly different as compared to the rest of quadrants, $^{+}p<0.05$, significantly different as compared to quadrant A. CTR: control, RU: right, and BIL: bilateral groups.

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Analysis of the number of visits to the predefined concentric circular areas showed significant effects of group ($F_{2,21} = 3.87$; $p<0.05$) and circular area ($F_{2,42} = 40.7$; $p<0.001$), and no interaction ($F_{4,42} = 0.9$; $p = 0.4$). Post hoc test showed a strong tendency in RU and BIL groups to cross more frequently the limits of the rings than CTR group ($p = 0.06$). No significant group differences were found in the total distance swum ($F_{2,21} = p>0.05$).

Mean Brain CO Activity

Quantification of CO activity in the dorsal hippocampus showed differences between groups in CA1 area (right:

$F_{2,21} = 121.3$; $p<0.001$ and left: $F_{2,21} = 196.6$; $p<0.001$) and CA3 area (right: $F_{2,21} = 71.3$; $p<0.001$ and left: $F_{2,21} = 23.2$; $p<0.001$). Post hoc analysis showed that BIL group had significantly higher CO activity in the CA1 and CA3 areas ($p<0.001$) in both hemispheres. Moreover, CO activity was higher in the CTR group compared to RU group in CA1 and CA3 areas of both hemispheres ($p<0.01$).

Regarding the dentate gyrus (DG), ANOVA disclosed significant differences between groups in the right DG ($F_{2,21} = 36.7$; $p<0.001$) and left DG ($F_{2,21} = 13.8$; $p<0.001$). In the right DG, CTR group showed higher CO activity compared to the other groups ($p<0.05$), and BIL group displayed higher CO activity as compared to RU ($p<0.05$). In the left DG, CTR and BIL groups exhibited higher CO activity than RU group. Mean regional CO activity measured in the experimental groups is summarized in Table 1. We found group differences in only cingulate area, with BIL group had higher CO activity in left hemisphere ($F_{2,21} = 9.3$; $p<0.001$). See Table 2.

As regards to the rest of brain regions quantified, group differences emerged in the lateral mammillary nucleus and the entorhinal cortex ($F_{2,21} = 17.7$; $p<0.001$ and $F_{2,21} = 27.2$; $p<0.001$ respectively). Post hoc test showed higher CO activity levels in all experimental groups (RU and BIL) as compared with the CTR group ($p<0.05$). Activity differences also appeared in the dorsal thalamic nucleus ($F_{2,21} = 7.7$; $p<0.01$), the perirhinal cortex ($F_{2,21} = 26.7$; $p<0.001$) and the basolateral amygdala ($F_{2,21} = 6.44$; $p<0.01$). Post hoc test revealed that BIL group had higher CO activity as compared to the rest of groups in all those regions ($p<0.05$). See Table 3 for additional brain regions quantified.

Interregional within-group correlations of hippocampal CO activity

Significant regional correlations were found in particular areas of the right and left hippocampus in the different experimental groups (Fig. 3). A negative cross-correlation between the right CA1 area and the right DG was found in the CTR group. The BIL group showed positive correlations among the left and right DG and the right CA3 area. However, the RU group had significant correlations limited to the left hippocampus (Fig. 3).

Table 1. Mean CO activity measured in hippocampal regions.

	CTR	BIL	RU
Left Hippocampus			
CA1 area	31.6±1.0^{+}	42.4±0.9*	13.7±0.8
CA3 area	30.5±1.7^{+}	38.2±1.8*	18.6±2.2
Dentate gyrus	38.8±2.6	33.4±1.8	23.7±1.1*
Right Hippocampus			
CA1 area	29.6±1.1^{+}	41.6±0.7*	15.8±1.2
CA3 area	31.8±1.0^{+}	40.1±0.9*	18.6±1.3
Dentate gyrus	36.4±1.3^{+}	31.7±0.8*	22.0±1.1

*$p\leq0.01$, significantly different from the rest of groups (Tukey's tests), $^{+}p\leq0.01$, significantly different as compared to RU group.
doi:10.1371/journal.pone.0064749.t001

Table 2. Mean CO activity measured in prefrontal areas.

	CTR	BIL	RU
Left Prefrontal Cortex			
Prelimbic Area	26.2±1.1	25.1±0.8	23.8±0.9
Infralimbic Area	21.4±0.9	22.3±0.1	20.6±1.8
Cingulate Area	25.0±0.7	26.4±1.3	27.5±0.9
Right Prefrontal Cortex			
Prelimbic Area	24.5±0.6	25.0±1.2	21.6±0.6
Infralimbic Area	23.1±0.6	25.1±0.6	21.1±1.0
Cingulate Area	22.7±0.4	26.8±0.7*	22.2±0.9

*$p\leq0.01$, significantly different from the rest of groups (Tukey's tests).
doi:10.1371/journal.pone.0064749.t002

Discussion

Unilateral Inactivation Impaired Retrieval as Much as Bilateral Inactivation

This study showed that dorsal unilateral and bilateral hippocampal inactivation has similar effects on retrieval of memories acquired 4 weeks before. The time period used to evaluate remote memory was based on previous studies using one month (28 days) to evaluate long-term or remote memory after hippocampal inactivation or lesion [28], [29], [30]. Both treatments impaired performance in the remote probe test in the Morris water maze. Subjects did not remember the position of the hidden platform. This result agrees with previous works reporting the hippocampal involvement in retrieval of spatial memories acquired several weeks before in the Morris water maze [5], [6], [7]. Therefore, our results agree with recent evidence about hippocampal recruitment during spatial memory retrieval [30].

Despite the disturbance of spatial memory in both groups, it is noteworthy that the unilateral and bilateral inactivation altered spatial memory in a different way. Hence, whereas bilateral treatment subjects distributed the searching around the pool, unilateral inactivated subjects showed a marked preference for the

Table 3. Mean CO activity (±S.E.M.) measured in selected brain regions of the different experimental groups.

	CTR	BIL	RU
Cortex			
Entorhinal	14.4±0.3*	19.9±0.8	18.5±0.6
Perirhinal	13.1±0.3	18.1±0.4*	14.4±0.6
Diencephalon			
Anterodorsal thalamic nucleus	30.6±0.5	34.1±0.5*	31.9±0.7
Anteroventral thalamic nucleus	24.6±0.4	21.6±0.8	23.2±1.0
Medial mammillary nucleus	30.2±0.5	31.1±1.2	30.5±1.1
Lateral mammillary nucleus	27.4±0.6*	31.5±0.4	31.2±0.4
Amygdala			
Lateral nucleus	14.8±0.6	16.6±0.8	14.6±0.8
Basolateral nucleus	21.3±1.1	25.9±0.1*	20.7±0.7

*$p\leq0.01$, $^{+}p\leq0.05$ significantly different from the rest of groups (Tukey's tests).
doi:10.1371/journal.pone.0064749.t003

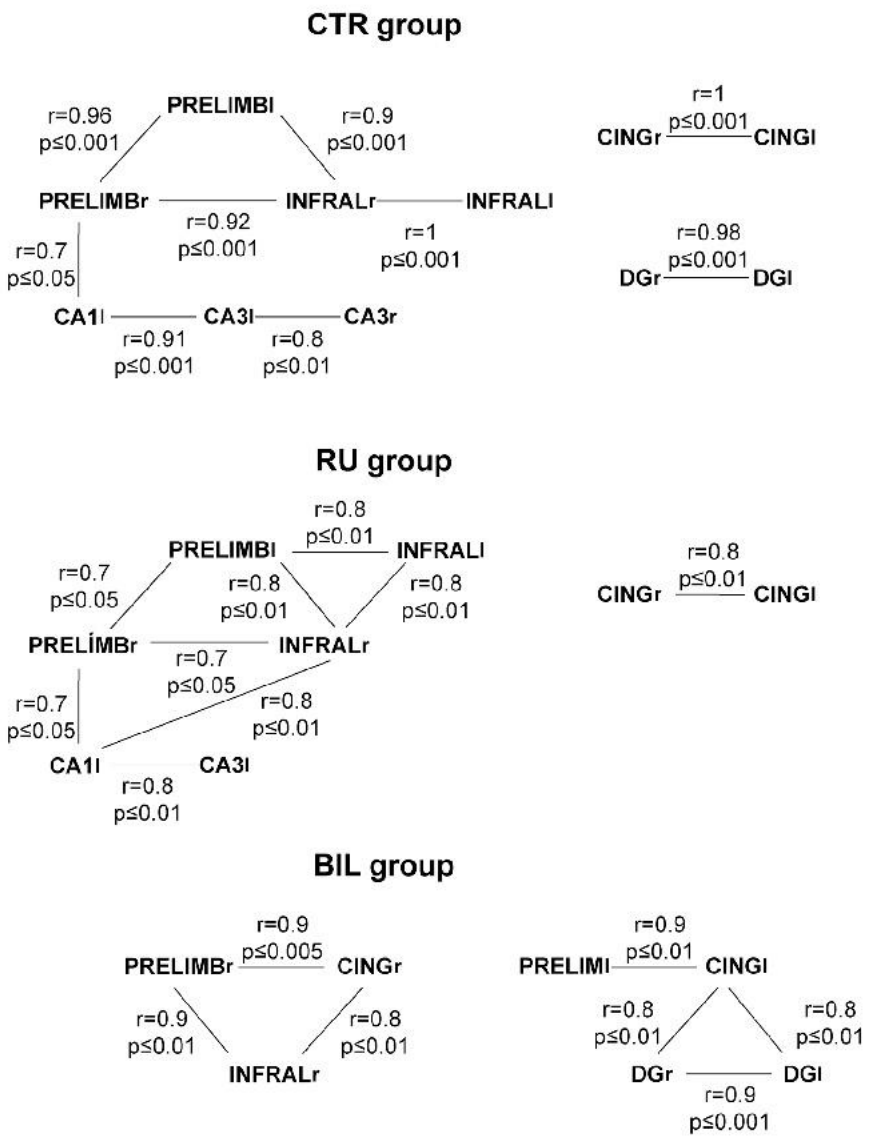

Figure 3. Interregional within-group correlations of CO activity. Schematic diagram showing significant correlations in CO activity among right (R) or left (l) between hippocampal and prefrontal regions calculated for the different experimental groups. Abbreviations: prelimbic (PRL) and infralimbic (IL) cortex, cingulate cortex (CG), hippocampal dentate gyrus (DG) and subfields (CA1 and CA3).
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lateral quadrant. This probably shows that unilateral treated subjects preserve some memories although inaccurate about the goal, similarly to the alterations manifested by rats that received hippocampal inactivation after training, knowing how but not where [31].

The effects of unilateral hippocampal inactivation on behaviour are to some extent controversial. Unilateral blockades not always impair hippocampal-dependent behaviours. In order to understand this effect we probably need to pay attention both to the task used and the memory phase affected by the treatment. Therefore, in very spatial-demanding tasks like the Morris water maze or active place avoidance arenas, unilateral inactivation alters all phases of memory formation, as shown by different studies carried out during the last 20 years [1], [3], [32]. However, the same interventions do not consistently alter memories in hippocampal-dependent tasks where orientation demands are low. This is the case of passive avoidance tasks, where orientation and navigation in this environment in limited and demands are more related to context recognition [4], [8], [33].

On the other hand, it is necessary to consider the memory phase interrupted during hippocampal inactivation. Retrieval was demonstrated to be more prone to interference than other phases of memory formation. As Moser and Moser [2] showed, the amount of hippocampal tissue required for retrieval is higher than that needed for acquisition.

Other phases of memory formation were also tested under unilateral and bilateral hippocampal interventions and similar results were found. Hence, when intrahippocampal injections of TTX were applied to block consolidation, unilateral and bilateral treatments did not differ [34]. So, although unilateral blockade theoretically leaves the contralateral hippocampus intact to hold a memory, one hippocampus cannot be enough to support and adequately process spatial memories. We have to consider that cognitive alterations after unilateral blockade could be caused by a plausible interference between the inactivated and untreated

hippocampi. In this respect, it is well known that each hippocampus sends and receives fibres from the contralateral hippocampus [35], and unilateral lesion of one hippocampus can disturb physiological processes in the contralateral side [36], [37].

It is also possible that the spatial memory was lateralized to the right hippocampus [38], and as a consequence of this, right hippocampal inactivation impaired spatial memory retrieval. However, this point is not clear. Right and left hippocampal inactivation showed, in fact, subtle behavioural effects [38] while other authors did not detect them [1]. Moreover, the role of each hippocampus in spatial behaviour is also matter of debate in humans. Hence, unilateral epileptic focus in the medial temporal lobe or unilateral hippocampal removal is enough to prevent spatial learning in virtual reality tasks, and this can be independent of the side of the brain involved [39], [40].

Hippocampal Blockade Modifies Metabolic Activity in Several Structures Involved in Spatial Orientation

Cytochrome oxidase histochemistry (CO) was used to assess brain energy metabolism of several brain structures that could be involved in the solution of this task. Previous works showed that CO activity can reflect metabolic changes linked to learning and memory processes [8], [10], [21], [41].

Our study proved that DG, CA3 and CA1 manifested different metabolic activity according to the treatment received. CTR group displayed positive correlations between right and left DG areas and between ipsilateral CA areas. Also contralateral CA3 areas showed positive correlations between them. This pattern is altered as the hippocampal activity is blocked. DG and CA3 regions were proposed to process the geometry of the environment [42], being essential mossy fibre inputs to CA3 for encoding spatial information [43]. Furthermore, unlike the other groups, bilaterally inactivated animals showed dissociation regarding CO activity found in different regions of the hippocampus. The animals with bilateral inactivation showed increased activity in CA1 and CA3 areas during retrieval, while the CO activity of the dentate gyrus largely decreased. It may be that CA1/CA3 areas and the dentate gyrus have opposing functions during different phases of spatial memory processing. Some authors [44], [45] have demonstrated that the perforant path input to CA3 area is critical for memory retrieval processes (related to a pattern completion mechanism) whereas the dentate gyrus is critical for memory encoding processes (as probably related to spatial pattern separation mechanisms). This means that impaired learning or general memory deficits found in an animal never being able to perform a task are not indicative of impaired pattern completion [46]. The different CO activity observed between Ammon's horn areas and the dentate gyrus may be indicative of this dissociation, since during memory retrieval, spatial pattern completion is essential in order to recover the full stored information, but pattern separation, which occurs at the time of encoding and storage, is not essential, and for this reason the dentate gyrus appears to be inhibited during expression/retrieval.

Since the hippocampus is needed for an adequate orientation, partial bilateral and unilateral inactivation caused alterations in other structures that develop an important role in the orientation system of the brain. Hence, patterns of correlations slightly changes in RU group and is very altered in BIL group. This loss of positive correlations supports the hypothesis that TTX impaired the network involved in retrieval of spatial memories. Note that the comparison of different correlations between hippocampal components provides information about the neural net that underlies the behavioural processes studied. In this regard, it was demonstrated that analyses at the level of neural networks were more sensitive to understand brain dysfunctions than attending only to the parts that integrate the system [47].

We also did pay attention to the changes of metabolic activity in the groups of study. Our work showed that an impaired behavioural performance did match with an increase of the brain activity in the entorhinal cortex and lateral mammillary nucleus revealed by CO histochemistry. CTR group showed reduced CO activity in the entorhinal cortex in comparison with all treated groups. It is well known that the entorhinal cortex is profusely connected with the hippocampal system and contains cells which are suggested to be specialized in the coding of spatial information [48]. Moreover, lesions of the dorsolateral area of the entorhinal cortex were reported to impair retrieval of spatial memories acquired one week before [49]. Since the hippocampal system physiology is disrupted by TTX injections, this could trigger an increase in the activity of those brain structures involved in retrieval of memories. An alternative hypothesis suggests that unsuccessful attempts of finding out the position of the platform would increase the exploratory activity and the CO metabolism in the entorhinal cortex. As shown before, exploratory activity can regulate the activity of the entorhinal cortex. Matrov et al. [50] reported that the rats that displayed high rates of exploratory activity increased their oxidative metabolism in the entorhinal cortex. As we described with respect to the frequency of visiting the different ring-segments of the MWM, inactivated groups changed segment more frequently than controls, although no differences were found in the total distance covered.

Similar metabolic patterns were displayed in other brain regions involved in spatial orientation. The lateral mammillary bodies and anterodorsal thalamic nucleus are known to take part of the Papez circuit and the head direction system [51] which contributes to the processing of both allocentric and geometric cues [52]. Moreover, the lateral mammillary nucleus directly projects to the anterodorsal thalamic nucleus via the mammillothalamic tract [53]. Accordingly, lesions of the mammillothalamic tract impair allocentric and egocentric spatial navigation in the water maze [54]. Previous studies demonstrated that CO activity changes in the lateral mammillary bodies after learning in a spatial working memory task [20], [55]. In our work, BIL and RU groups showed an increased activity when compared with the CTR group. Regarding the anterodorsal thalamic nucleus, we found a higher CO activity in BIL group in comparison with the CTR group. Although the anterodorsal thalamic nucleus receives a major projection from the subiculum, the main output of the hippocampus, hippocampal lesions was reported not to disrupt head direction cell signals [56]. However, it is well known that the above-mentioned structures are part of the Papez circuit and during learning and memory processes these regions interact changing their metabolism [8]. So it would not be unusual that hippocampal inactivation produced changes in CO activity in these linked structures.

It is also necessary to point out that the BIL group increased its CO activity in many other several brain regions related to memory circuits. Hence bilaterally inactivated subjects increased CO activity in the perirhinal cortex, a brain structure that has been related to object recognition [57], [58] and discrimination [59], as well as spatial memory retrieval [60]. As Ramos [60] demonstrated, rats with perirhinal inactivation were impaired in retrieving spatial memories that were well acquired before the intervention. The activity in the cingulate cortex is also increased in BIL in comparison with CTR and RU groups. This brain structure links cortical and limbic structures and it was reported to be involved in spatial memory in rats [61], [62]. Finally, other structures like prelimbic and infralimbic cortices did not reflect any change in

their CO activity, and probably shows that they were not directly involved or detected by CO histochemistry after the retrieval of spatial information required in our experiment. As reported before, infralimbic and prelimbic cortices are important in attentional processes and flexibility of behaviour [63] but they are also involved in memory extinction or consolidation of fear memories [64] that perhaps were not engaged in the retrieval phase of our spatial memory task. In agreement with our results, a recent study of remote spatial memory retrieval using both functional inactivation techniques and c-fos expression confirmed that only the cingulate cortex and not the prelimbic or infralimbic cortices is required for remote memory retrieval [30].

In conclusion, this experiment showed that retrieval of spatial memories depends on the integrity of the hippocampal system even several weeks after the initial training. However, since hippocampal inactivation altered metabolic activity in regions functionally related with the hippocampus, other regions could underlie the behavioural deficits registered. Moreover, inactivation of one hippocampus causes the same effect as bilateral blockade of this brain structure, an effect that has been reported in other hippocampal-dependent tasks [3].

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Author Contributions

Conceived and designed the experiments: NMC JMC JLA. Performed the experiments: NMC JMC HGP. Analyzed the data: NMC HGP. Contributed reagents/materials/analysis tools: HGP MMC JLA. Wrote the paper: NMC JMC JLA.

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Artículo 3


Sausalito
GOLDEN GATE
Presidio
Fort Pt.
Black Pt.
Yellow Bluff
Pt. Diablo
Lime Pt.
Alcatraz I.
SAN FRANCI

Enviado A: Neuroscience

TEMPORAL INACTIVATION OF PRELIMBIC CORTEX DISRUPTS SPATIAL MEMORY RETRIEVAL TASK IN THE MORRIS WATER MAZE

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KEYWORDS: Prelimbic, muscimol, spatial memory retrieval, Morris Water Maze

ABREVIATIONS

IL: Infralimbic area

mPFC: Media prefrontal Cortex

MWM: Morris water maze

PL: Prelimbic area

RSM: Reference spatial memory

ABSTRACT

The prefrontal cortex has been repeatedly associated with reference spatial memory acquisition and retrieval. Specifically, the hipocampal-prefrontal cortex circuit has been reported to experience activation changes throughout the spatial memory process. The temporal functional contribution dynamic of the associated brain structures has being supported by the observed increased involvements of the prefrontal cortex at the end of the acquisition. However the individual role of different prefrontal cortex areas in the process and specifically the prelimbic area at this stage is still controversial. Therefore, the aim of our study was to determine the prelimbic area or the prefrontal cortex contribution to the retrieval of a previously acquired hidden platform task in the Morris Water Maze. For that purpose, male Wistar rats underwent a temporal bilateral inactivation, using the GABA-$_A$ antagonist, Muscimol, infused at the retrieval test time. A saline-infused group was used as a control group. Results showed that both groups of animals properly learned the task, but when the experimental group was under the muscimol effect, retrieval was significantly disrupted.

1. INTRODUCTION

The prefrontal cortex has been repeatedly associated with reference spatial memory acquisition and retrieval. The standard theory for memory consolidation supports a temporal reorganization of the brain circuits underlying long-term memory storage (Bontempi et al., 1999, Frankland and Bontempi, 2005) in which the medial prefrontal cortex and the hippocampus would interact to hold the cognitive process. Specifically, the hipocampal-prefrontal cortex circuit has been reported to experience activation changes throughout the spatial memory process, highlighting an important contribution of the prefrontal cortex at the end of the acquisition (Conejo et al., 2010a). As reported in this previous work, the functional brain networks related with the acquisition of spatial reference memory in the Morris water maze changed, showing a temporal dynamics during the acquisition of spatial memory (Conejo et al., 2010a) so that late stages seem to be associated with an increase metabolic activity in this region

. Regarding retrieval of this spatial orientation (Conejo et al., 2013) we showed that inactivation of the dorsal hippocampus impaired long-term spatial memory retrieval. Moreover, functional brain networks between the mPFC and the dorsal hippocampus were differentially activated after unilateral or bilateral hippocampal inactivation. Additionally, both the prelimbic and the infralimbic areas or the prefrontal cortex were found to be involved at late stages of a spatial memory extinction in the Morris Water Maze, once the extinction learning have being consolidated (Mendez-Couz et al., 2014). In the same line, the temporal inactivation of this area did not impair the extinction memory task when the activation was carried out before the extinction procedure (Mendez-Couz et al., 2015b), but altered brain metabolic activity in related areas and changed functional networks were found after the temporal activation. One of the prevalent hypothesis supports the notion of the memory being processed at the first moments at the hippocampal-cortical networks to be finally more cortical-dependents, so that during retrieval the mPFC would act as a miss-match comparator, preventing the hipocampal activity to re-encode already existing memories (Frankland and Bontempi, 2005). All the afromentioned studies point to an important role of the mPFC and particularly the prelimbic region for the consolidation and retrieval of the spatial memory. However, the specific role played by the prelimbic area and its temporal-dependent contribution remains controversial.

Taking the above into account, the objective of the present study is to assess the participation of the prelimbic region of the prefrontal cortex in the brain networks underlying spatial memory retrieval, and its relationship with previously related brain areas thought the retrieval of a previously acquired task evaluated in the Morris Water maze. For this purpose, selective temporal inactivation of the prelimbic region will be performed by intracerebral administration of the GABA-$_A$ agonist muscimol prior to a spatial reference memory retrieval evaluated in the Morris water maze (MWM).

2. MATERIAL AND METHODS

2.1. ANIMALS

A total of 30 male Wistar rats (*Rattus norvegicus*) between 260-330g were used. The animals were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain). They were housed under standard conditions (12-h light/dark cycle with lights on from 08:00–20:00 h), at constant room temperature of 23±2ºC with ad libitum access to food and water. All experimental procedures carried out with animals were approved by a local veterinary committee from the University of Oviedo vivarium and subsequent handling strictly followed the European Communities Council Directive 86/609 and RD 1201/2005.

2.2. SURGERY

Rats were stereotaxically implanted bilateral cannulae in the prelimbic region of the prefrontal cortex (coordinates from bregma: AP +3.1, L ±0.07, DV -3.0 mm). Animals recovered from surgery during 7 days prior to the habituation day in the Morris water maze (MWM).

At that point some rats were separated and used to check the surgery protocol. Animals were infused with 0,5µl of methyl blue staining. Ninety minutes after finishing the experimental manipulations, all rats were decapitated and their brains quickly frozen in isopentane. Coronal brain sections (30 µm thick) were obtained using a cryostat microtome (Microm HM-505E, Heidelberg, Germany) and processed for gallocianin-chrome alum stain. (Figure 1)

2.3. BEHAVIOURAL PROCEDURE

Before starting the experimental procedure rats were handled daily during 5 days to reduce anxiety related to manipulation procedures, after the surgical intervention and recory, animals were tested in a neurological assessment battery in order to discard possible motor and sensory deficits. The neurological tests used include: abduction response of hind limbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response and righting reflex (Bures et al., 1976).

Animals were trained during six consecutive days in a hidden platform task and tested for spatial memory immediately after the last training. During the habituation phase that lasted for one day rats were placed gently in the water facing the pool walls in a pseudorandom order. A cued escape platform (placed 2 cm above the water level) was located in the same quadrant during 2 sessions of four consecutive trials. Rats were allowed to swim until the escape platform was found or guided there by the experimenter's hand after 60 s had elapsed and remained 15 s on the platform. Rats rested for 30 s between trials. The following 5 days rats were training in spatial reference memory task. Each day animals received a four-trial session in a hidden platform task (2 cm beneath the water surface) in a constant reinforced quadrant. After the last learning trial animals underwent a learning Transfer test. Animals were randomly divided into Muscimol (Mu; n=12) or Saline (Sa; n=12) groups . Seven days later an infusion of 0,5µl of muscimol or saline vehicle respectively were administered bilaterally in the PL 30 min before the memory retrieval transfer test. Escape latencies and time spent in each quadrant were measured and analyzed later using a video-tracking system (Ethovision XT, Noldus Information Technologies, The Netherlands). A time line of the experiment is represented in Figure 2.

2.4. STATICTICS

All data were analyzed by SigmaPlot 11 software (Systat Software, Chicago, USA) and were expressed as mean ± S.E.M. The results were considered statistically significant when $p < 0.05$.

A repeated measures on-way ANOVA test was used to analyze the mean latencies to reach the platform along training days. A Tukey's test was used as a post-hoc test to see which day differed from the others.

The same test was used to test the quadrant preference during the retention memory test carried out immediately after the last learning session .In this case, the Holm-Sidak method was applied as post-hoc test.

A two ways repeated measures ANOVA was applied to elucidate differences between groups in the mean time spent in different quadrants during the retrieval test performed one week after finishing the acquisition phase. Group and quadrant were taken as factors. The same test as in the case before was used as all pairwise multiple comparison.

RESULTS

2.5. BEHAVIORAL RESULTS

Animals did not show sensory or motor deficiencies evaluated with the neurological assessment battery. Therefore, no animals were discarded due to the presence of neurological signs.

2.5.1. Reference memory task.

As shown in figure 3, the mean escape latencies of the experimental animals significantly decreased throughout acquisition sessions ($p<0.001$). Post-hoc Tukey's tests showed differences in escape latencies between the first day *versus* the rest of them ($p<0.05$). Furthermore, the retention probe showed significant differences between the time spent within the four virtual maze quadrants [$F_{3,27}= 51.88$, $p<0.001$]. Post-hoc analyses showed that the time spent in D quadrant differed from the other quadrants ($p<0.001$).

2.5.2. Long-term memory retrieval test

Retrieval test showed that saline animals preserved a preference for the previously reinforced quadrant seven days after the learning phase finished (Fig. 4). In this regard, the retention probe carried out a week after the last acquisition day showed differences between quadrants in the mean time spent during the retrieval test [$F_{3,27}= 31.08$, $p<0.001$] and post-hoc analysis showed that the mean time spent in the escape quadrant (D) differed from the others ($p<0.001$). The two ways ANOVA with groups and quadrants as factors showed differences between groups in the mean time spent at the different quadrants. All pairwise comparisons showed differences at the D quadrant ($p<0.05$).

3. DISCUSION

Our results show that both animals in the Muscimol and their control Vehicle saline group trained in a spatial learning task, followed by a spatial memory retention probe one week later were able to acquire the hiden platform or reference spatial memory task in the Morris Water maze, but after the bilateral infusion of the GABA-A antagonist muscimol into the prelimbic area of the prefrontal cortex, the retrieval of the task was disrupted in comparison to the Saline-infused control group. Mastery of the spatial memory task acquisition was revealed both by the significant decrease of latencies and distance to reach the platform along training days, and the significantly higher amount of time spent in the target quadrant as compared with the rest of quadrants during the memory retention probe. Likewise, during the retention probe carried out seven days later, saline animals still spent more time in the previously reinforced quadrant but those under prelimbic area inactivation were unable to remember the previously reinforced quadrant.

It is known that the prefrontal cortex plays an important role in memory and specifically in spatial memory processes. Spatial disturbance of spatial memory took place in the muscimol group was up to the point that subjects distributed the searching around the pool, not even having a preference for the lateral quadrants. This would indicate that bilateral treated subjects do not even preserve some memories although inaccurate about the goal, similarly to that occurring when the hippocampus is inactivated just after training, leaving the animals to know how the task should be done but not the exact location of the platform (they "how" but no "where") (Micheau et al., 2004). This is also the case of memory retrieval tasks occurring in the Morris water maze under an unilateral inactivation of the dorsal hippocampus previously to the retrieval task (Conejo et al., 2013). In our study, the searching procedure was equally distributed around the pool. This behavior has being previously reported after bilateral inactivation of CA1 field of the dorsal hippocampus in SRM retrieval carried out in the MWM (Conejo et al., 2013).

It is important to note that effects of prefrontal cortex inactivation depend both on the task used and the memory phase that is affected by the drug infusion. Attending to the task , the prefrontal cortex has been largely known for playing an important role in navigational aspects. Specifically, the prelimbic and infralimbic areas have been related with spatial working and recent memory (Ragozzino et al., 1998, Wang and Cai, 2008) while the cingulate area is suggested to be involved in spatial or contextual discrimination tasks (Frankland and Bontempi, 2006, Frankland et al., 2006). Foccusing in the memory phase, it is known that particular regions could be differentially involved at different time points throughout a particular task by changing its interactions with other regions, like the well-known case of the hippocampus in spatial memory (McIntosh, 2004). The potential of the prefrontal cortex for the integration and synthesis of information from a large

number of sources may indicate its ability to process remote memories (Miller, 1996),as the hippocampus process recent memories (Frankland and Bontempi, 2005). The different involvement of the prelimbic area of the prefrontal cortex throughout spatial memory phases has being reported both at late stages of acquisition (Conejo et al., 2010a) as well as at late stages of the extinction process, in which higher c-Fos expression both in the prelimbic and infralimbic regions of the prefrontal cortex was reported (Mendez-Couz et al., 2014). Regarding spatial memory, it is known to participate together with the prelimbic and cingulate areas in the hipocampal-prefrontal cortex neural networks that underlie retrieval of the previously learned spatial task (Conejo et al., 2013, Mendez-Couz et al., 2015a).

Our findings are consistent with the prevalent idea that suggests the involvement of distributed or large-scale cortical-dorsal hippocampus networks in spatial memory (Bontempi et al., 1999, Frankland and Bontempi, 2005, Wang and Cai, 2008, Leon et al., 2010, Conejo et al., 2013). In addition to the involvement of this well-known hippocampal-prefrontal cortex circuit, there is broad agreement on the temporal reorganization of circuits underlying spatial memory (Bontempi et al., 1999, Maviel et al., 2004, Conejo et al., 2010b). That is to say, the neural networks involved in recent memory storage are supposed to differ from those underlying remote memory. This fact is supported by lesion studies suggesting that inactivation of specific cortical regions would alter remote memory without disrupting a recent one (Quillfeldt et al., 1996, Takehara et al., 2003, Frankland et al., 2004).

Accordingly, inactivation of the prelimbic or anterior cingulate cortices prevents the recall of remote memories (Frankland et al., 2004, Maviel et al., 2004) and disconnection of hippocampal-PFC circuits reported impaired spatial learning (Wang and Cai, 2008). In this regard, the prelimbic area inactivation did not affect acquisition or reversal learning of different discrimination tests, but it selectively impaired learning when rats had to inhibit one strategy and shift to using a new strategy, this would support the key role of this area in the behavioural flexibility necessary to successfully use a new strategy. However, comparable to orbitofrontal cortex inactivation, strategy-switching deficits following prelimbic inactivation resulted from a perseverance of the previously relevant strategy, See Ragozzino (2007) for a review.

CONCLUSIONS

In conclusion, this study shows that the temporal inactivation of the prelimbic area of the prefrontal cortex disrupts the retrieval of a reference spatial memory acquired one week before. Taken together, the interpretation of our results supports current theories of a temporal dynamics in

to the hippocampal-cortical networks system supporting spatial memory, highlighting the less know role prelimbic area of the prefrontal cortex on spatial reference memory retrieval assessed in the Morris water maze.

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FIGURE LEYENDS

Figure 1: Time line of the experiment.

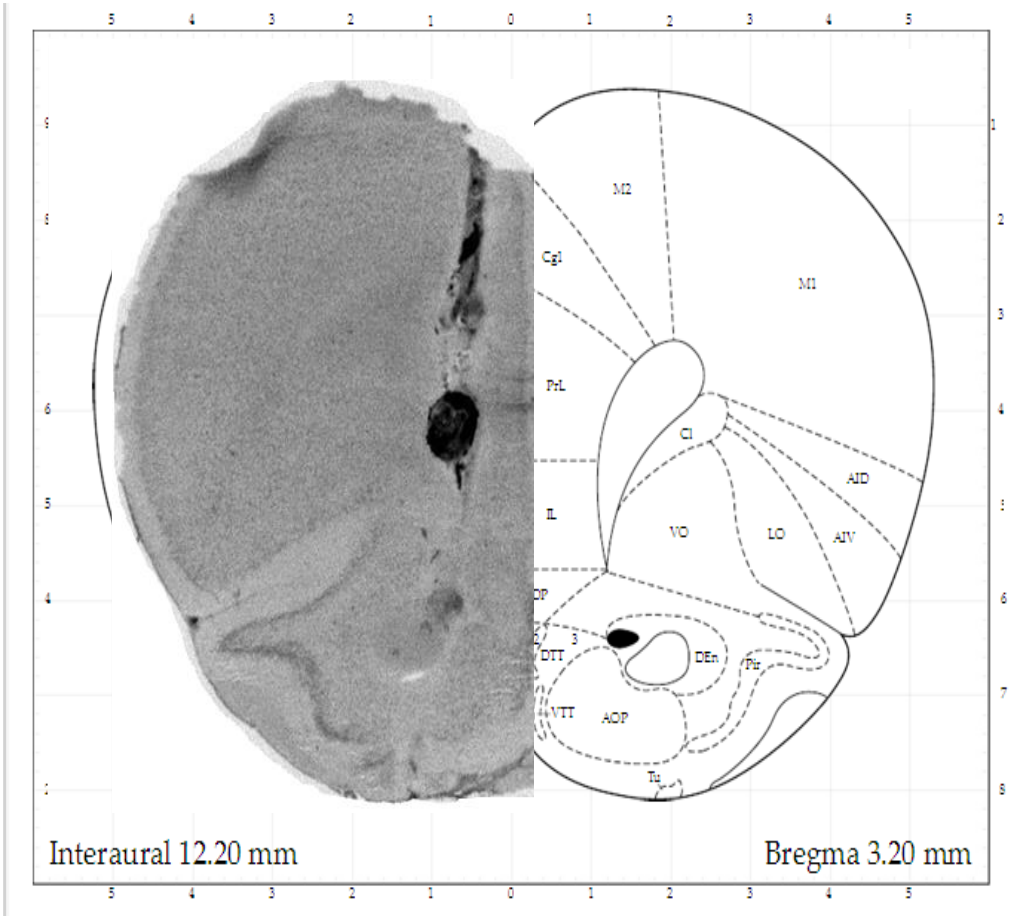
Figure 2: Microphotograph showing the site of infusion in the prelimbic cortex. Adapted from Paxinos and Watson (2004).

Figure 3: Mean escape latencies (±S.E.M.) measured in the experimental group. * Significantly different as compared to the following days ($p<0.05$, Tukey's post hoc test).

Figure 4: Mean time spent in each quadrant (±S.E.M.). *Significantly different for the escape quadrant as compared to the rest of quadrants (Tukey's post hoc test).

Figure 5: Muscimol vs Saline group at the retrieval transfer test. Differences between groups were found at the previously reinforced quadrant. ($p<0.05$)

FIGURE 1:


M2
Cg1
M1
PrL
Cl
AID
IL
VO
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DEn
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VTT
AOP
Tu
Interaural 12.20 mm
Bregma 3.20 mm

Figure 2:

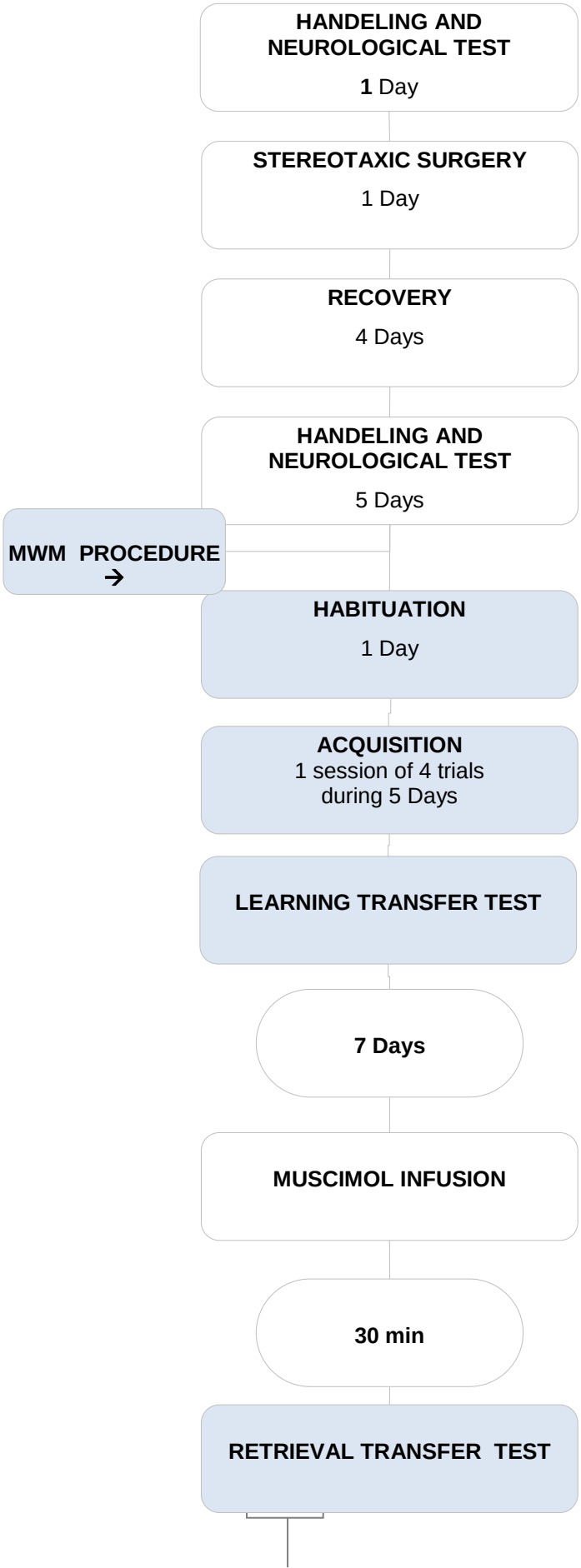
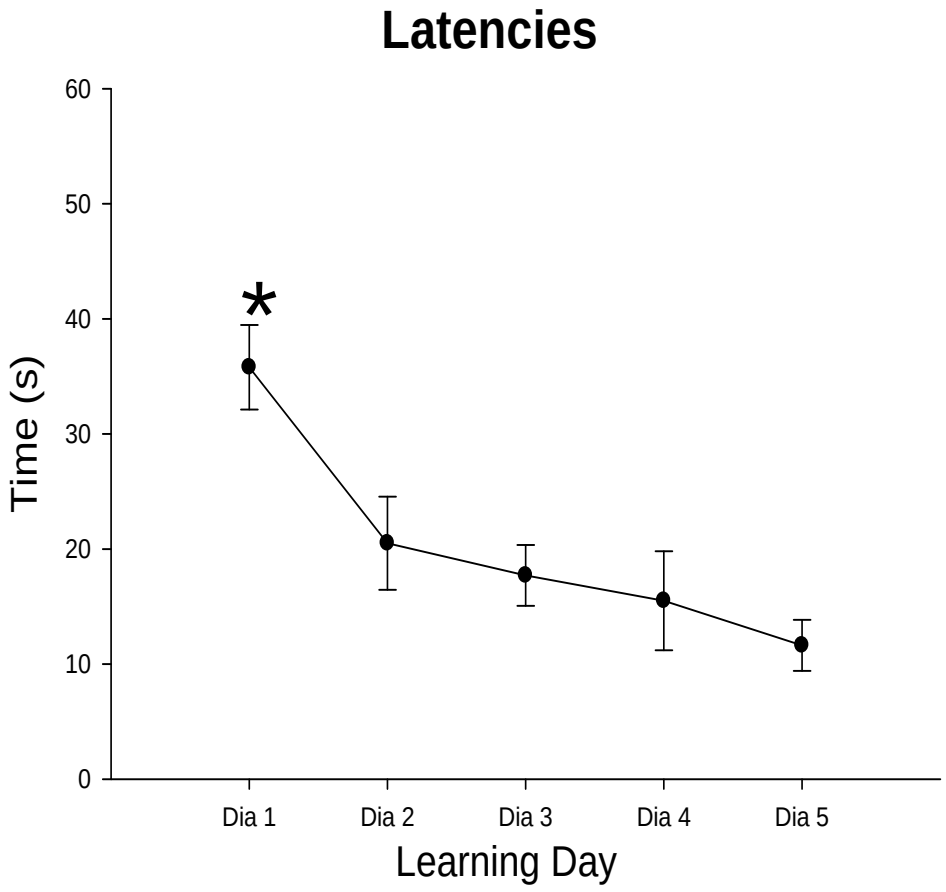

HANDELING AND NEUROLOGICAL TEST
1 Day
STEREOTAXIC SURGERY
1 Day
RECOVERY
4 Days
HANDELING AND NEUROLOGICAL TEST
5 Days
MWM PROCEDURE →
HABITUATION
1 Day
ACQUISITION
1 session of 4 trials during 5 Days
LEARNING TRANSFER TEST
7 Days
MUSCIMOL INFUSION
30 min
RETRIEVAL TRANSFER TEST

Figure 3:


Latencies
60
50
40
30
20
10
0
Time (s)
*
Dia 1
Dia 2
Dia 3
Dia 4
Dia 5
Learning Day

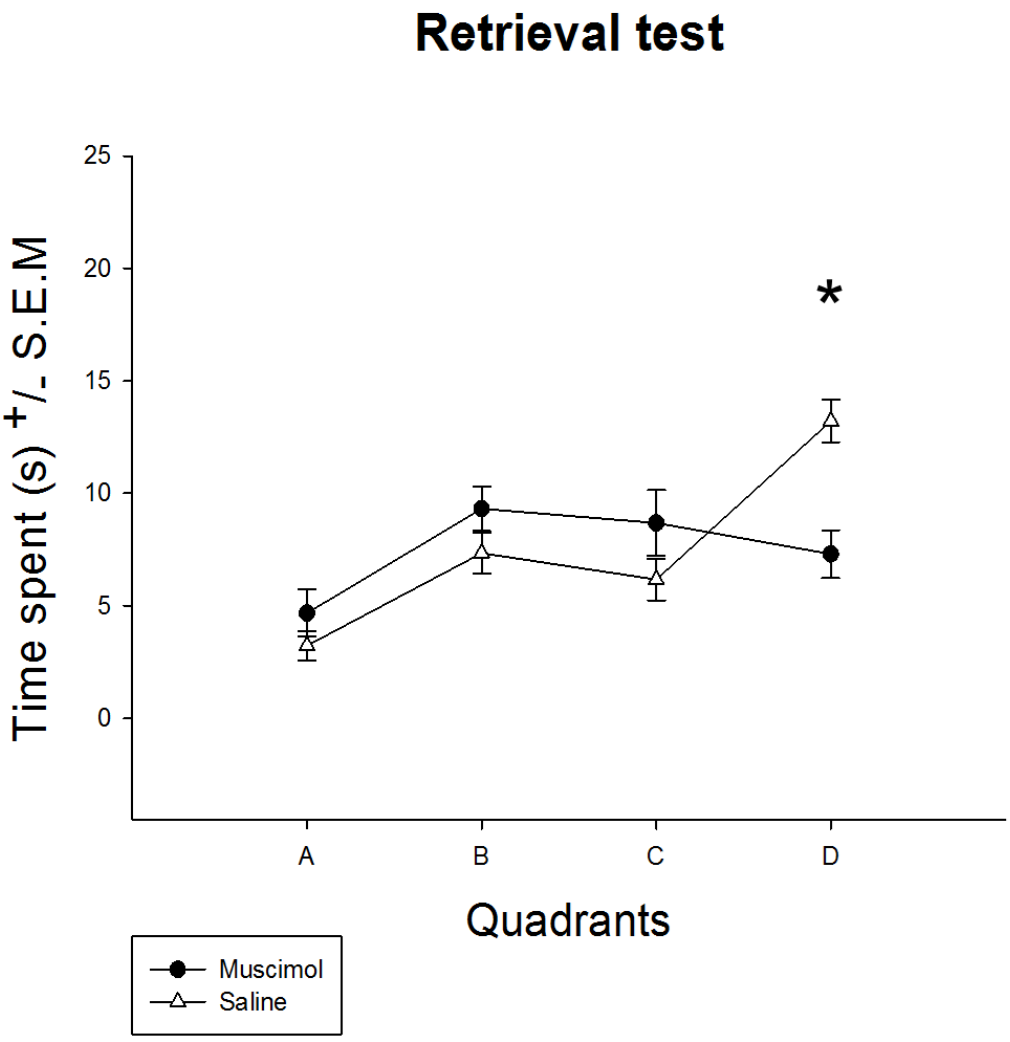

Retrieval test
Time spent (s) +/- S.E.M
25
20
15
10
5
0
*
A
B
C
D
Quadrants
Muscimol
Saline

Figure 4:

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Artículo 4


Sausalito
Yellow Bluff
Pt. Diablo
GOLDEN GATE
Fort Pt.
Black Pt.
South Rock
SAN FRANCI

ENVIADO A: Hippocampus.

SPATIAL MEMORY EXTINCTION DIFFERENTIALLY AFFECTS DORSAL AND VENTRAL HIPPOCAMPAL METABOLIC ACTIVITY AND ASSOCIATED FUNCTIONAL BRAIN NETWORKS

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ABBREVIATIONS

AcC: Nucleus accumbens Core

AcSh: Nucleus accumbens Shell

AD: Anterodorsal thalamic nucleus

AV: Anteroventral thalamic nucleus

Ba: Basal amygdaloid nucleus

CA1d: Cornu Ammonis 1 subfield of the dorsal hippocampus

CA1v: CornusAmmonis 1 subfield of the ventral hippocampus

CA3d: Cornu Ammonis 3 subfield of the dorsal hippocampus

CA3v: Cornu Ammonis 3 subfield of the ventral hippocampus

Ce: Central amygdaloid nucleus

CFI: Comparative Fit Index

CO: Cytochrome oxidase

DGd: Dorsal portion of the Dentate Gyrus

DGv: Ventral portion of the Dentate Gyrus

Ent: Entorhinal cortex

EX: Extinction group.

IL: Infralimbic region of the prefrontal cortex

La: Lateral amygdaloid nucleus

LM: lateral nucleus of the mammillary bodies

LS: Lateral septum

M1: Primary motor cortex

MB: Mammillary bodies

MD: Medio-dorsal thalamic nucleus

MeA: Medial amygdaloid nucleus

MM: Medial mammillary nuclei

mPFC: Medial prefrontal cortex

MS: Medial septum

N: Naïve group

NNFI: Non-Normed Fit Index

PL: prelimbic region of the prefrontal cortex

PM: Premammillary nucleus

PRh: Perirhinal cortex

RMSEA: Root-Mean-Square Error

RSA: Agranular retrosplenial cortex

RSG: Granular retrosplenial cortex

SE: Standard Error of the Mean

SuM: Supramammillary nucleus

ABSTRACT

Previous studies showed the involvement of brain regions associated with both spatial learning and conditioning in spatial memory extinction, although the specific role of the dorsal and ventral hippocampus and the extended hippocampal system including the mammillary bodies in the process is still controversial. The present study aimed to identify the involvement of the dorsal and ventral hippocampus, associated cortical regions, the amygdaloid nuclei, and the mammillary bodies in the extinction of a spatial memory task. In order to address these issues, quantitative cytochrome c oxidase histochemistry was applied as a metabolic brain mapping method. Rats were trained in a reference memory standard task using the Morris water maze, followed by an extinction protocol of the previously acquired memory task. Results show that rats learned successfully the spatial memory task as shown by the progressive decrease in measured latencies to reach the escape platform and the results obtained in the probe test. Spatial memory was subsequently extinguished as shown by the descending preference for the previously reinforced location. A control naïve group was added to ensure that brain metabolic changes were specifically related with performance in the spatial memory extinction task. Extinction of the original spatial learning task significantly altered the metabolic activity in the dorsal and ventral hippocampus, the amygdala and the mammillary bodies. Moreover, the ventral hippocampus, the lateral mammillary bodies and the retrosplenial cortex were differentially recruited in the spatial memory extinction task, as shown by group differences in brain metabolic networks. These findings provide new insights on the brain regions and functional brain networks underlying spatial memory, and specifically spatial memory extinction.

1. INTRODUCTION

Although the neural substrates of spatial memory acquisition, consolidation or retrieval have been thoroughly studied, the neural basis underlying the posterior extinction process remains controversial. The extinction process occurs when a former adaptive response is no longer effective in conducting to reinforcement, so the animal will gradually cease to emit this behavior (Rescorla and Wagner, 1972). In the case of the spatial memory assessed in a Morris Water maze (MWM), the escape platform itself would act as a rewarding stimulus, being responsible for the progressive improvement in the performance of the memory task along learning trials tested in the Morris water maze (Schulz et al., 2007, Huston et al., 2009, Huston et al., 2012). The prevalent

theory dictates that the same classical rules that govern instrumental learning are on charge of learning and extinction in the water maze (Huston et al., 2009). Therefore, both acquisition and extinction of spatial learning in the MWM and conventional classical and operant conditioning would share similar brain processes underlying spatial and non-spatial associative learning (Sanchez-Moreno et al., 1999, Prados et al., 2003, Prados et al., 2008). In this scenario, brain structures and functional networks underlying the latter processes would be similar. However, we have previously reported that brain networks and regions underlying spatial memory would differ when measured at late stages of spatial memory extinction (Mendez-Couz et al., 2014).

In a previous study using brain c-Fos protein expression, we demonstrated the involvement of the prefrontal cortex, amygdala and diencephalic structures like the mammillary bodies at late stages of a spatial memory extinction task performed in the water maze (Mendez-Couz et al., 2014). Although c-Fos immunohistochemistry can be useful to study changes in neuronal plasticity induced by spatial learning (Tischmeyer and Grimm, 1999, Vann et al., 2000, Mendez-Lopez et al., 2009, Pothuizen et al., 2009, Vanelzakker et al., 2011) it is known that c-Fos protein induction is transient and rapid, so that after cellular stimulation, c-Fos protein expression returns to basal levels after several hours (Sharp et al., 1993). In this context, c-fos protein expression would be associated with the last stage of the extinction process, that may reflect the new inhibitory learning taking place at that time, but we cannot preclude the possibility of other regions being involved throughout the extinction process.

Although the discrete involvement of those regions could play a key role in the process, it is well known that solving the memory puzzle as a whole involves understanding not only the discrete structures that might be participating, but also the functional interactions among them. That is to say, we should be seeking to elucidate the functional brain networks involved at memory systems level, even though this might represent the most difficult approach (Kandel and Pittenger, 1999).

We have previously reported the involvement of the amygdala, the mammillary bodies and the prefrontal cortex at late stages of this task (Mendez-Couz et al., 2014). However, prelimbic area inactivation did not impair acquisition of the extinction task (Mendez-Couz et al., 2015b) although changes in extinction-associated functional brain networks were found. The latter study also suggested the involvement of spatial memory-related structures such as the hippocampus, the

retrosplenial cortex, the mammillary bodies (Aggleton and Pearce, 2001) and the amygdala (Conejo et al, 2010) at early stages of this task. Therefore, the neural substrates of spatial memory extinction remain still a controversial issue.

Taking the aforementioned into account, we sought in the present study to characterize the involvement of brain structures related to spatial memory extinction in the water maze and their temporal dynamics. For this purpose, we used cytochrome c oxidase (CO) histochemistry as a reliable marker of brain oxidative metabolism, since it represents an index of mitochondrial metabolic competence (Bertoni-Freddari et al., 2001), being particularly useful in our case, as contrary to the c-Fos technique used, CO activity is associated with energy demands of neurons after prolonged stimulation (Gonzalez-Lima and Cada, 1994, Villarreal et al., 2002). Among other techniques that provide accurate quantification of CO activity like spectroscopy or biochemical approaches, we consider quantitative histochemistry to be the most appropriate method because it provides accurate anatomical localization. Therefore, CO activity mapping by quantitative histochemistry appears to be the most useful and reliable method to detect changes in brain metabolism induced by this training paradigm.

2. MATERIAL AND METHODS.

2.1. ANIMALS

Subjects were a total of twenty-one male adult Wistar rats (*Rattus norvegicus)* weighing between 260-310 g. They were randomly divided into Basal Extinction (BE, n=11) and Naïve groups (N, n=10).

The animals were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain). They were housed in a temperature controlled-room (23±2 °C). Lighting was kept on a 12-h light/dark cycle with lights on from 08:00–20:00 h. Water and food was available with *ad libitum* access throughout the experiment. All experimental procedures carried out with animals were approved by a local ethical committee from the University of Oviedo vivarium and in accordance with the European Communities Council Directive 2010/63/UE and the Spanish legislation on care and use of animals for experimentation (RD 1201/2005).

2.2. APARATUS

Animals were trained in a Morris water maze. The maze was a circular water tank , measuring 1.5 m in diameter by 75 cm in height, (Morris, 1984) .The pool was filled with tap water and a escape platform was hidden beneath the water surface. The water temperature was kept at 20±1 °C during the entire training period. The pool was surrounded by numerous visual patterns that acted as allocentric cues as previously described (Mendez-Couz et al., 2015b). Trials were recorded and later analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

2.3. BEHAVIOURAL PROCEDURE

Before the beginning of the experimental procedure, rats were handled daily during 5 days to reduce anxiety related to manipulation procedures. MWM procedure was carried out following a reference spatial memory protocol, after which rats underwent an extinction of spatial memory procedure adapted by(Rossato et al., 2006) as previously reported (Mendez-Couz et al., 2014, 2015b). The spatial memory tasks were performed between 09:30 and 14:00 h.

2.3.1. Habituation Phase

Rats received two sessions spaced 1 h apart in the habituation phase. During each session, animals were released from the central part of each quadrant facing the pool wall, following a pseudorandom sequence, four times each session. Rats were returned to their home cages between sessions. The maze was virtually divided into four equal quadrants (A, B, C, and D) and the escape platform was located in the center of quadrant D, and above the water level. Rats were allowed to swim up to 60 s to locate the platform in each trial, or guided to it after that time period. They were remained there for 15 s and then they rest in a black plastic bucket during 15 s until the next trial.

2.3.2. Reference memory task

Animals were trained during five consecutive days in a hidden platform task and tested for retention test immediately after the last acquisition trial. During the acquisition phase, EX group animals received a daily session of four trials, in which they were released from the central border of each quadrant following a pseudorandom order to search for a hidden escape platform. The

platform was kept in the same quadrant (escape quadrant, D) along the acquisition procedure, and rats were required to find it using spatial cues available in the room following training. Rats were allowed to swim during 60 s to reach the platform or gently guided to it after that time; they were 15 s on the platform and then they rested during 30 s in a black plastic bucket within trials.

2.3.3. Retention probe

After the last training day, rats were submitted to a retention test in a single probe trial. For this purpose, the platform was removed from the maze, and rats were released from the opposite quadrant. They were allowed to swim during 60 s. In order to prevent premature extinction of the previously learned task, all animals received an additional acquisition trial in which the platform was available again in its original place.

2.3.4. Memory Extinction task

Extinction group animals received four extinction sessions the day after the learning retention probe. Each session consisted in four single non-reinforced trials of 60 s each, resting 30 s in a plastic bucket in-between trial. Period of time within sessions for the same animal was 30 min.

The control Naïve group was added to the experiment to ensure that changes in brain activity would be specifically learning-related. These animals swam for an equivalent amount of time as compared to the trained group, including every phase followed by trained animals, (i.e. habituation, reference memory training, retention probe, post-retention trial and extinction protocol). In contrast to the EX group, the escape platform was absent during the whole behavioral protocol.

2.4. CYTOCHROME OXIDASE HYSTOCHEMISTRY

Ninety minutes after finishing the behavioral tasks, all animals were decapitated. Their brains were removed and then frozen in isopentane at −70 °C (Sigma–Aldrich, Madrid, Spain) and stored at −40 °C to preserve the enzyme activity. Brains were subsequently cut at 30 µm-thick coronal sections using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). These sections were mounted on slides and stored at −40 °C until processing with quantitative CO histochemistry. Brain tissue of one EX group animal and a few sections from animals of several

groups could not be used as a result of defective tissue processing, although the final number of sections available for histochemistry per group was equal or higher than seven in all cases.

A modified version of the method based on the quantitative CO histochemical method developed by Gonzalez-Lima and Jones (Gonzalez-Lima and Jones, 1994), was used. Staining variability across different baths was controlled by sets of tissue standards. These standards were obtained from Wistar rat brain homogenates of known CO activity, that had been determined spectrophotometrically at different thicknesses (10, 30, 50 and 70 μm). The standards were included with each batch of slides, as was previously explained (Conejo et al., 2013, Mendez-Couz et al., 2015a). Succinctly, slides were fixed for 5 min with a 0.5% glutaraldehyde solution, rinsed in phosphate buffer and preincubated 5 min in a solution containing 0.05 M Tris buffer pH 7.6 with 275 mg/l cobalt chloride 10% (w/v) sucrose and 5 ml dimethylsulfoxide. After a new wash in phosphate buffer (pH 7.6; 0.1 M) they were incubated at 37 °C for 1 h in a solution containing 50 mg 3,3'-diaminobenzidine, 15 mg cytochrome c (Sigma, St. Louis, MO, USA) and 4 g sucrose per 100 ml phosphate buffer (pH 7.4; 0.1 M). The reaction was stopped by fixing the tissue in buffered formalin (10% w/v sucrose and 4% formaline) for 30 min. Slides were subsequently dehydrated, cleared with xylene and coverslipped. CO histochemical staining intensity was measured by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) composed of a high precision illuminator, a digital camera and an with specific image analysis software. Four measurements in three following sections of relative optical density were taken per region, that is to say, twelve in total. In order to account for possible staining variations between brain sections from different staining batches and establish comparisons, measurements were also taken from CO-stained brain homogenate standards. Regression curves between section thickness and known CO activity, previously measured by spectrophotometric assay in each set of standards, were calculated for each one. Finally, average relative optical density measured in each brain region was transformed into CO activity units (1 unit: 1 μmol of cytochrome c oxidized/min/g tissue wet weight at 23 °C) using the previously calculated regression curve in each homogenate standard. Mean CO activity was calculated for each brain region and animal. Selected brain regions included the prelimbic (PrL), and infralimbic (IL) areas of the medial prefrontal cortex and the primary motor cortex (M1), all of them measured at $^{+}/_{-}$ 3.70 mm from Bregma; the granular (RSG) and agranular (RSA) retrosplenial cortices at $^{+}/_{-}$ -4.52 mm; the parietal (PAR) $^{+}/_{-}$ -3.80 mm, entorhinal (Ent), and perirhinal (PRh) cortices at $^{+}/_{-}$ -4.52 mm. Additionally, the following subcortical regions were also measured: the

mediodorsal (MD) anterodorsal (AD) and anteroventral (AV) thalamic nuclei measured at $^{+}/_{-}$ -1.40 mm; the lateral (LS) and medial septum (MS) at $^{+}/_{-}$ 0.20 mm, the nucleus accumbens core (AcC) and shell (AcSh) measured at $^{+}/_{-}$ 1.00 mm; hippocampal subfields including CA1, CA3 and dentate gyrus of the dorsal (CA1d, CA3d, DGd) and ventral parts (CA1v, CA3v, DGv) measured respectively at $^{+}/_{-}$ -3.30 mm and $^{+}/_{-}$ -4.52 mm from Bregma. The medial (Me), basal (Ba), lateral (La) and central (Ce) amygdaloid nuclei measured at $^{+}/_{-}$ -3.14 mm from Bregma; the medial (MM), lateral (LM) and supramammillary (SuM) nuclei of the mammillary bodies measured at $^{+}/_{-}$ -4.52 mm, as well as the premammillary nucleus (PM) at $^{+}/_{-}$ -4.16 mm. The selected brains regions were anatomically defined according to the Paxinos and Watson (2004) atlas.

2.5. DATA ANALYSIS

Statistical analysis was performed in Sigmastat 3.2 software (Systat software, Chicago, USA) and SAS 9.4 PROC CALIS (SAS Institute Inc, Cary, NC, USA).

2.5.1. Behavioral procedures

Repeated measures ANOVA were performed to analyze escape latencies across training sessions, with the average time used to reach the platform in each session as main factor. If the previously performed equal variance test failed, the Kruskal Wallis non-parametric test was carried out. When statistical differences were found, Tukey tests were used as multiple comparison procedures to isolate the session or sessions that significantly differed from the rest of sessions. Similarly, one-way ANOVA was applied to evaluate differences in the time spent in the four quadrants during the memory retention test. Tukey tests were also applied as post hoc tests when ANOVA results were significant.

Then, the same test was carried out to analyze the extinction process throughout the extinction procedure. In this case, the average time spent in the escape quadrant per session was used as factor, and a Holm-Sidak method was applied to evaluate differences between sessions.

All differences were considered statistically significant when $p<0.05$.

2.5.2. CO activity and functional brain networks

Student's *t*-tests were applied to evaluate significant CO activity differences between groups measured in each brain region.

In order to examine the functional relationships between observed brain regions (areas), exploratory path analyses were conducted. The analysis of covariance structure was performed using the maximum likelihood estimation. The model χ^2/χ^2 df ratio was used as a preliminary measure of overall fit, with conventional values for an acceptable fit being <2. Since the null hypothesis was that the specified model would fit the data (i.e., the predicted and observed covariance matrices would not differ significantly), non-significant χ^2 *p*-values were required as evidence to support the specified model. Comparative indices of fit reflected the improvement in fit obtained when using the hypothesized model instead of the null or baseline model. Therefore, a Non-Normed Fit Index (NNFI) > 0.9 and a Comparative Fit Index (CFI) > 0.9 were interpreted as indicating a reasonable fit for the model. We also used the Root Mean Square Error of Approximation (RMSEA), an index of the error between the model and the observed data, with values <0.05 or <0.08 indicating excellent and modest approximations, respectively. Convergence of the estimated covariance matrix with the sample covariance matrix was assessed by inspection of the matrix of normalized residuals. Parameter estimates for manifest variable equations were calculated and construed as indicating the strength of each individual path within the model. In post hoc assessments of the strength of each path within the model, we considered as significant those standardized parameter estimates in which the absolute *t*-values were >1.96.

3. RESULTS

3.1. BEHAVIOUR

3.1.1. Reference memory task and memory retention test

Animals successfully performed the hidden platform task, as shown by the escape latency results. The amount of time required to reach the escape platform decreased significantly across training sessions between Extinction and Naïve groups ($p \leq 0.001$). Specifically, there were significant differences in escape latencies between the first, fourth and fifth day, as well as between second day and the last two days. Lastly, significant differences were also found between the third day and the last training day [Tukey tests ($p \leq 0.05$)].

This result was confirmed by the learning transfer test performed immediately after the last learning trial. Differences were found between the time spent in the four quadrants ($F_{3,9}$ =9.71 p<0.01), showing differences among the one where the platform had been placed (D) and the rest of quadrants [Tukey test (p<0.05)].

3.1.2. Memory extinction task

Across the extinction procedure, the time spent in the preciously reinforced quadrant significantly decreased among sessions ($F_{3,39}$; p≤0.01). Post hoc analysis showed that subjects extinguished the previously learned task since they spent less time swimming into the target quadrant between the first and the penultimate session as well as among the first and the last extinction session [Holm-Sidak method (p<0.001)], similarly, there were differences between the second extinction session and the last one (p<0.01). See figure 1.

3.2. CO ACTIVITY AND FUNCITIONAL BRAIN NETWORKS

We found regional differences in CO activity means between naive and extinction group. Specifically, CO activity generally increased in the experimental versus the naïve group in the following brain regions: Ca1d (t_{18}= 2.26; p<0.05), parietal cortex (t_{17}=3.11; p<0.01), DGv (t_{15}=2.31; p<0.05), medial amygdaloid nucleus (t_{17}= 2.69; p<0.02), central amygdaloid nucleus (t17=2.26; p<0.05), retrosplenial granular (t_{14}=3.81; p<0.01) and agranular (t_{14}=2.21; p<0.05) cortices. However, the experimental group showed a lower CO activation in the medial (t_{14}=-2.66; p<0.02) and lateral (t_{14}=-5.04; p<0.01) nuclei of the mammillary bodies as compared to the same regions in the naïve group. See figure 2.

Functional brain network analysis revealed the activation of different networks in the extinction and Naïve groups. The first one showed a more intricate network, with higher frequency of positive correlations in CO activity involving the dorsal hippocampus, the retrosplenial cortex, the central and lateral amygdaloid nuclei and diencephalic regions like the lateral mammillary nucleus (Figure 3). However, the Naïve group showed negative CO activity correlations among the amygdala and hippocampal subfields like the dorsal CA1 and ventral DG, and also between the retrosplenial cortex and amygdala nuclei or between the central amygdaloid nucleus and the mammillary bodies. (See figure 3).

4. DISCUSSION

The results suggest that brain regions in the dorsal and ventral hippocampus, the parietal and retrosplenial cortices, the amygdala and the mammillary bodies are actively engaged during spatial memory extinction. Based on the different patterns and correlations of CO activity found, particular brain regions may mediate different processes taking place during spatial memory extinction. The dorsal CA1 and CA3 hippocampal subfields, the ventral dentate gyrus, the retrosplenial cortices and the medial mammillary bodies were recruited as active metabolic brain networks to complete spatial memory extinction, although the latter brain regions do not seem to play a key role at later stages of the task (Mendez-Couz et al., 2014).

These findings agree with the broad agreement of distributed hippocampal-cortical circuits involved in spatial memory (Bontempi et al., 1999, Frankland and Bontempi, 2005, Leon et al., 2010) and the temporal reorganization of networks underlying spatial memory formation (Maviel et al., 2004, Frankland and Bontempi, 2005, Conejo et al., 2010, Conejo et al., 2013, Mendez-Couz et al., 2015a).

Although a previous c-Fos expression study did not find changes in the dorsal or ventral hippocampal subfields at late stages of spatial memory extinction (Mendez-Couz et al., 2014), the involvement of both hippocampal subdivisions at early stages of the task is not entirely surprising, given the wealth of evidence implicating the hippocampus in spatial memory acquisition (Bontempi et al., 1999, Conejo et al., 2007, Conejo et al., 2010, Loureiro et al., 2012, Miyoshi et al., 2012, Fidalgo et al., 2014). According to the prevalent theory, extinction of a particular behavior may be understood as an active learning process involving formation of new memories, although the original memory trace would be preserved (Bouton et al., 2006). Indeed, some authors suggest that the molecular mechanisms underlying the acquisition or consolidation of a memory extinction task would be similar to those described during the acquisition of the original task (Lattal et al., 2003, Szapiro et al., 2003). This aspect of hippocampal system function may account for the higher CO activity values found in the dorsal CA1 hippocampal subfield of the extinction group as compared to the naïve group.

Specifically, we found higher CO activity in the dorsal CA1 hippocampal subfield, and direct CO activity correlations between the CA1 subfield and the retrosplenial cortex. According to the standard theory of memory consolidation, memories are initially stored in the hippocampus to

later become part of the neocortical circuits, thus becoming independent of the hippocampus (Alvarez and Squire, 1994, Squire and Alvarez, 1995). This theory has been successfully modeled in animals, in which, following damage to the hippocampus, memories acquired shortly before the damage are lost, meanwhile those acquired in the distant past remain intact (Milner et al., 1998). Therefore, our results showing cortico-hippocampal functional correlations after the extinction procedure would agree with this theory of memory consolidation.

The ventral dentate gyrus also showed higher CO activation in the extinction group as compared to the naïve group. The ventral hippocampus, together with the parahippocampus and the amygdala have been related to learning processes under stressful conditions (Villarreal et al., 2002) a result that could perfectly match our data if we take into account the absence of the escape platform in the water maze, which forces the animal to acquire a new learning strategy. In this regard, low-frequency stimulation of the ventral hippocampus seems to facilitate the extinction of contextual fear memories (Cleren et al., 2013). It is also remarkable that both the medial and central amygdala nuclei showed a pattern of increased CO activity too, which would agree with this view. Although the amygdala complex remains active at late stages of the extinction task (Mendez-Couz et al., 2014) neither the dorsal nor ventral hippocampus seem to maintain their activation, as revealed by the much more time-limited c-Fos protein induction (Mendez-Couz et al., 2014). This fact would suggest a time window at early stages of the acquisition of the extinction task in which the hippocampus would play a key role; meanwhile, new memories become more independent of the hippocampus to be processed in neocortical circuits over time. This would be also consistent with the standard model of memory consolidation. In this regard, the prefrontal cortex has been associated with later stages of spatial learning in the Morris water maze (Conejo et al., 2010), and specifically with the extinction of spatial memory, as revealed both by an increased brain c-Fos protein expression in the prelimbic and infralimbic areas (Mendez-Couz et al., 2014) and by a reorganization of underlying brain networks when this task is performed under prelimbic area inactivation (Mendez-Couz et al., 2015b). Strikingly, no differences were found in CO activation between groups. The lack of significant group differences in the mPFC region is consistent with previous findings, since prelimbic area inactivation in rats did not affect the acquisition of the spatial extinction task. This would support the idea of a later involvement of the mPFC, as it is known to act as modulator in long-term extinction (Milad and Quirk, 2002) and extinction maintenance (Herry and Garcia, 2002, 2003).

The finding that the retrosplenial cortex showed increased CO activity as well as interactions with the dorsal CA1 field in the extinction group as compared to the naïve group may seem logical if we take into account both the connectivity with the hippocampus and the anterior thalamic nuclei (Van Groen and Wyss, 2003, Aggleton et al., 2012) and results of lesion studies that strongly suggest a role in spatial memory (Vann and Aggleton, 2002). Specifically, the effect of those lesions was related with prior experience of the rat in the water maze (Cain et al., 2006). Additionally, as regards to the water maze reference memory task, rats already learnt the platform location with reference to several distal cues by using a cognitive map in a flexible way (Tolman, 1948, O'Keefe and Nadel, 1978, Morris, 1981). Some authors suggest that the retrosplenial cortex could be linked to location determination when visual cues are use flexibly (Hindley et al., 2014), which could be the current situation once the rats are aware of the absence of the previously present reinforcing stimulus, that requires the use of new escape strategy. In this case, the previously acquired cognitive map could be used during the new extinction task.

As expected, a direct CO activity correlation between dorsal hippocampus and both central and lateral amygdaloid nuclei was found. Our results enhance previous studies that demonstrated the implication of the amygdala throughout the extinction process. In Pavlovian fear conditioning, a model of fear and anxiety disorders extensively used, it is well known that the basolateral amygdala plays a pivotal role in the consolidation of memories related to fear and emotions (McGaugh, 2002), moreover, the basolateral amygdala is known to be involved both in the formation and extinction of fear memory (Akirav and Maroun, 2007). In this regard, together with the medial and central amygdala, we found differences in the basolateral nucleus at late stages of the special extinction process (Mendez-Couz et al., 2014), however, only the medial and central amygdaloid nuclei showed significant differences in metabolic activity between groups. A basolateral amygdala lesion study showed that expression of conditioned fear was blocked, but the extinction of the acquired memory occurred (Anglada-Figueroa and Quirk, 2005).

On the other hand, the extinction group shows CO activity correlations between the dorsal hippocampus and the amygdala, since both lateral and central amygdala nuclei show interactions with the CA3 subfield of the dorsal hippocampus, meanwhile those connections are formed with the ventral hippocampus in the naïve group.

According to previous immediate early gene expression results, the mammillary bodies showed different metabolic activity between groups, showing a higher CO activity in the control group as compared to the extinction animals. Association between the mammillary bodies and the hippocampus would be not entirely surprising given the existing functional and neuroanatomical connections (mainly via the postcommisural fornix) between them, although the lateral and medial nuclei are connected to the same structures but to different subregions among them, therefore they form a couple of parallel systems (Vann and Aggleton, 2004) that could account for their different implication in this task .

Thus, the mammillary bodies have been traditionally associated in humans with memory, owing to the amnesic nature of Korsakoff syndrome related to mammillary body damage (Mayes et al., 1988, Tanaka et al., 1997, Hildebrandt et al., 2001). Furthermore, the direct connection via fornix between these nuclei and the hippocampus has focused most research on spatial learning (Sziklas and Petrides, 1993, Santin et al., 2003, Conejo et al., 2004, Mendez-Lopez et al., 2009, Vann, 2010). Currently , most works referring to the role of the mammillary bodies in memory highlight the importance of hippocampal inputs to the nuclei, and this region is frequently described as part of the extended hippocampal system (Aggleton and Brown, 1999). However, it is known that medial and lateral nuclei differ in several aspects like morphology (Veazey et al., 1982), electrophysiological properties (Stackman and Taube, 1998, Sharp and Turner-Williams, 2005, Sharp et al., 2006) and connections. The lateral mammillary bodies are related to spatial navigation since they have both head-direction cells and angular direction cells (Stackman and Taube, 1998), but its relevance on memory is still a matter of debate, since bilateral LM lesions caused mild impairments in a T-maze spatial alternation task or working memory tasks (Vann, 2005). These nuclei showed lower levels of c-Fos expression after a spatial memory extinction task (Mendez-Couz et al., 2014), and a lower CO activity as compared to the control groups when this task was performed under inactivation of the prelimbic cortex (Mendez-Couz et al., 2015b).

Remarkably, not only the LM showed differences among groups, but also the medial mammillary nucleus. It is believed that the medial mammillary bodies are related to the theta-rhythm system, because MM theta activity is driven by the CA1 hippocampal subfield (Kocsis and Vertes, 1994). This fact that could explain the strong connection between these structures found in the extinction group as compared to the control group. This is in line with Kirk and Mackay (2003) results suggesting that the MM act as a relay of the hippocampal theta, projecting to the

diencephalon and again to the hippocampus, which may account for successful encoding . This may clarify why these nuclei appear to have an important role at the beginning of the extinction task acquisition, meanwhile only the lateral mammillary bodies showed up at later stages (Mendez-Couz et al., 2014). Moreover, the MM and its projections to the anterior thalamic nucleus are claimed to be required for hippocampal and retrosplenial cortex normal function, as demonstrated by lesion studies related to rapid allocentric encoding impairment [see (Vann, 2010) for a review], highlighting those lesion effects at initial stages of learning. According to this review, the classical theory in which the hippocampal inputs to the diencephalon via the fornix could be revised, including the diencephalon indirect projections to the hippocampal formation for an integrated memory. Further inactivation studies could shed light into the specific role of both lateral and medial mammillary nuclei on the extinction of spatial memory.

CONCLUSIONS

The above, along with the present study, adds to a growing literature supporting the notion that the dorsal hippocampus –diencephalic circuit does play a temporal dependent role in the spatial reference memory processes, and is specifically involved in the extinction of spatial learning in the Morris water maze.

Additionally, the dentate gyrus of the ventral hippocampus, the retrosplenial cortex and the medial mammillary bodies were recruited for the completion of the task, and new metabolic brain networks underlying performance of this task were enlightened. Further research is required for the elucidation of the temporal involvement and specific role of the brain networks that underlie spatial memory extinction.

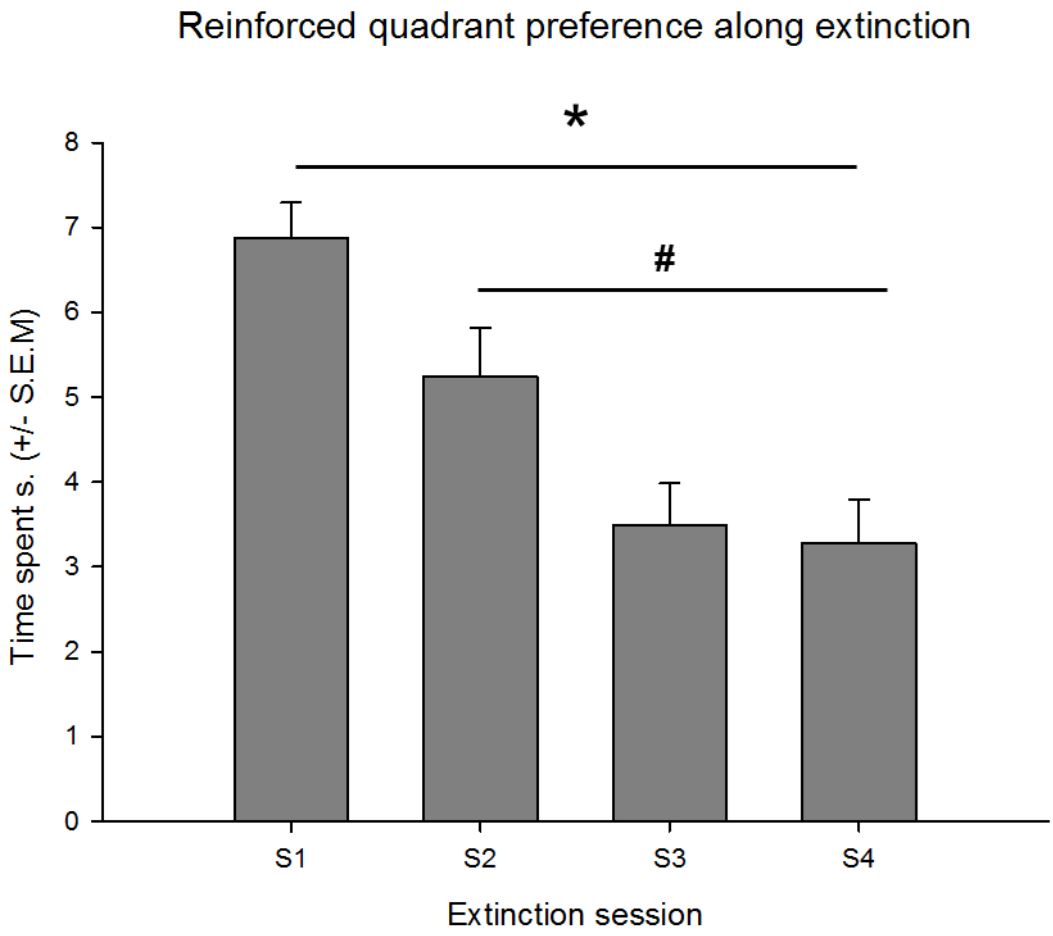
FIGURE LEGENDS.

Figure 1: Time spent during the first 15 s in the previously reinforced escape quadrant during the extinction test, with no platform available (mean ±S.E.M.). One-way repeated measures ANOVA applied to the mean time spent in the escape quadrant resulted in significant differences between sessions. Post hoc analysis by Tukey's tests revealed differences between sessions one and three and between sessions one and four (*p<0.01). There were also differences between session two and four (#p<0.05.

Figure 2. Representative images of CO histochemical stain performed in extinction and naïve groups showing the dorsal and ventral hippocampus, the mammillary bodies and the amygdala. The relative optical density of each region was measured taking three non-overlapping readings in each section, in three consecutive sections by using a square-shaped sampling window adjusted for each region size (MCID, InterFocus Imaging Ltd., Linton, England).

Figure 3. Path analysis of C.O. activity revealed several connections between the mammillary bodies, the hippocampus, amygdala nuclei and the retrosplenial cortex. Abbreviations: Retrosplenial granular (RSG) and parietal (PAR) cortex; dorsal (CA1d), (CA3d) and ventral hippocampus (DGv) fields; medial (MM) and lateral (ML) nuclei of the mammillary bodies; lateral (LaA), and central (CeA) amygdala nuclei. (p<0.05).

Figure 1.


Reinforced quadrant preference along extinction
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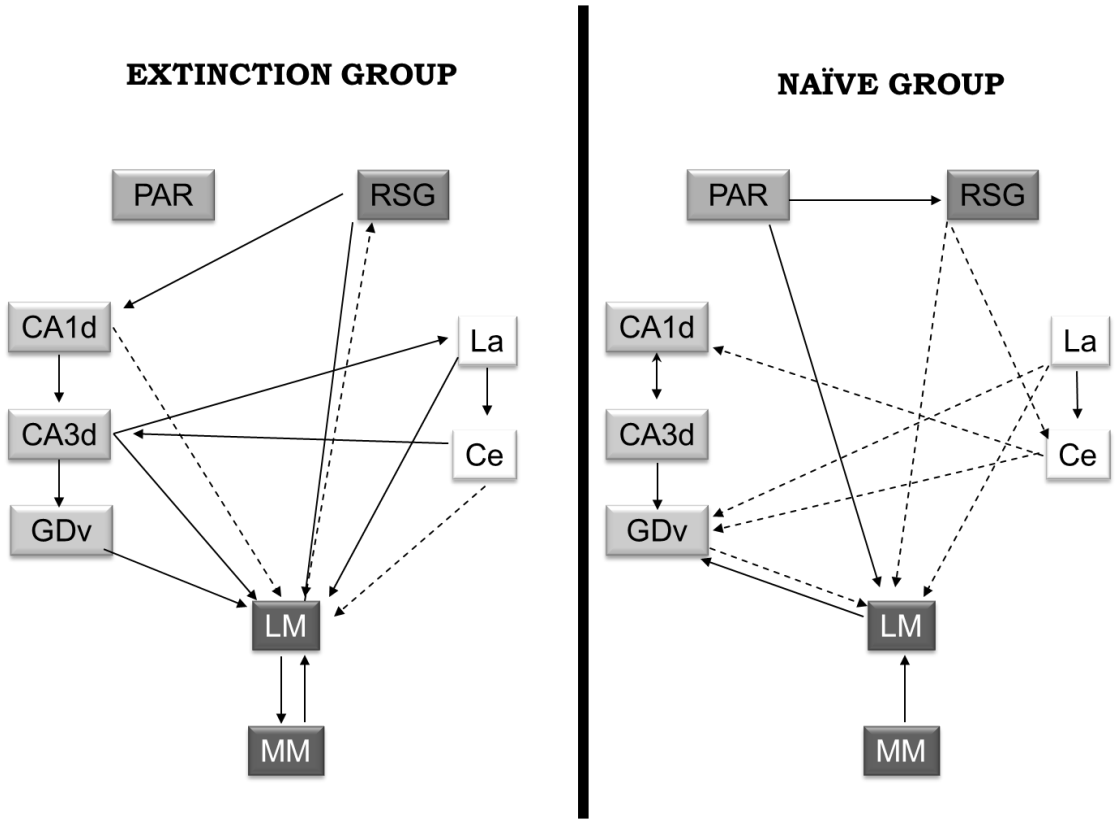
Extinction session

Figure 2.

Figure 3.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NAÏVE GROUP
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Artículo 5

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Research report

Spatial memory extinction: A c-Fos protein mapping study

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HIGHLIGHTS

- The medial prefrontal cortex is required for spatial memory extinction.
- The amygdala complex was activated after spatial memory extinction.
- The lateral mammillary nucleus is involved in spatial memory extinction.

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ABSTRACT

While the neuronal basis of spatial memory consolidation has been thoroughly studied, the substrates mediating the process of extinction remain largely unknown. This study aimed to evaluate the functional contribution of selected brain regions during the extinction of a previously acquired spatial memory task in the Morris water maze. For that purpose, we used adult male Wistar rats trained in a spatial reference memory task. Learning-related changes in c-Fos inmunoreactive cells after training were evaluated in cortical and subcortical regions. Results show that removal of the hidden platform in the water maze induced extinction of the previously reinforced escape behavior after 16 trials, without spontaneous recovery 24 h later. Extinction was related with significantly higher numbers of c-Fos positive nuclei in amygdala nuclei and prefrontal cortex. On the other hand, the lateral mammillary bodies showed higher number of c-Fos positive cells than the control group. Therefore, in contrast with the results obtained in studies of classical conditioning, we show the involvement of diencephalic structures mediating this kind of learning. In summary, our findings suggest that medial prefrontal cortex, the amygdala complex and diencephalic structures like the lateral mammillary nuclei are relevant for the extinction of spatial memory.

1. Introduction

Most adaptive behavior in complex organisms is learned behavior driven by the availability of positive or negative reinforcers. However, withdrawal of positive reinforcers leads to the extinction of the previously learned behavior. Over trials of extinction, the organism learns that the former adaptive response is no longer effective in conducting to reinforcement and it will gradually desist to emit this behavior. During standard Pavlovian tasks, repeated presentations of the conditioned stimulus (CS), but in the absence of the unconditioned stimulus (US) previously paired with the CS results in the extinction of the previously conditioned response. The effect of this procedure had been assumed to decrease the CS's ability to evoke the conditioned response established during early phases, when both stimuli (CS and US) were paired [1,2]. See also the review by Delamater [3].

According to Huston et al. [4], learning and extinction processes in the water maze follow the classical rules that govern

Abbreviations: Ba, amygdala basal nucleus; Ce, amygdala central nucleus; Me, amygdala medial nucleus; ANOVA, analysis of variance; CG, cingulate cortex; CS, conditioned stimulus; N, control naïve group; CA1d, cornu ammonis 1 of the dorsal hippocampus; CA1v, cornu ammonis 1 of the ventral hippocampus; CA3d, cornu ammonis 3 of the dorsal hippocampus; CA3v, cornu ammonis 3 of the ventral hippocampus; df, degrees of freedom; DGd, dentate gyrus of the dorsal hippocampus; DGv, dentate gyrus of the ventral hippocampus; EX, extinction group; EXR, extinction plus retention probe group; FWE, familywise error rate; IEGs, immediate early expression gene; IL, infralimbic cortex; LM, mammillary bodies lateral nucleus; MM, mammillary bodies medial nucleus; MWM, Morris water maze; PBS-T, PBS solution containing triton; PBS, phosphate-buffered saline; PL, prelimbic cortex; RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; US, unconditioned stimulus.

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instrumental learning. In fact, previous research suggests that acquisition and extinction of spatial learning evaluated in the Morris water maze (MWM) follow the same laws that determine conventional classical and operant conditioning, so that similar processes would underlie spatial and non-spatial associative learning [5–7].

Similarly, several researchers [8,9] suggest that the molecular mechanisms underlying the acquisition and/or consolidation of extinction memory are similar to those described for the acquisition and/or consolidation of the original contextual fear. In fact, extinction may be understood as new learning involving new memory formation, although preserving the original memory trace and it would be also associated with decreased responding in memory tasks [10]. Former studies have tried to explain neural basis underlying this process in terms of conditioning [8,11–13].

Although a number of previous studies have set the basis of conditioned response extinction processes, little is known about the same process when it follows spatial learning. In this regard, the study of brain circuits involved in memory consolidation has revealed the existence of connections between cortical and subcortical structures, that were previously described [14–19]. In addition, several studies focused on spatial memory extinction, although most of them were conducted almost exclusively at a behavioral level [6,7,20–22]. More recently, a few studies involving the neural basis of extinction [4,23–26] have been published, although these are mainly focused on the spatial memory extinction consequences, as an extinction-induced despair, a useful model to study depression processes. A few authors as Porte et al. [26] tried to perform brain mapping analysis of this process, including the hippocampus and the amygadala. In addition, brain structures like the prefrontal cortex, or the mammillary bodies have been widely and independently related with spatial navigation performance [18,27–29] or the memory extinction process (see [3,10] for reviews). Nonetheless, the functional brain networks underlying spatial memory extinction remain unclear.

In this experiment, we evaluated the functional involvement of different brain regions in the extinction of spatial memory, using the water maze to submit rats to a reference memory task followed by a multiple extinction trials task. For this purpose, brain activity was measured using c-Fos immunohistochemistry. This is, one of the first studies in which this technique is applied to measure brain changes during the extinction process of spatial memory.

The cellular *c-fos* proto-oncogene is an immediate early expression gene (IEGs) because its induction is one of the first cellular responses after the application of a variety of stimuli. This induction is rapid and transient, so that upon cellular stimulation their levels return near basal level in several hours [30]. Its encoded product, the c-Fos protein, causes membrane depolarization and voltage-gated calcium influx, resulting in neuronal activity changes [31]. The induction of *c-fos* expression has been related to neuronal activation underlying learning and memory processes [32,33] and it is reported to be useful to study neuronal plasticity required for spatial memory processes [33–37].

2. Materials and methods

2.1. Animals

A total of 20 male Wistar rats (*Rattus norvegicus*) between 260 and 360 g were used. The animals were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain). They were housed under standard conditions (12-h light/dark cycle with lights on from 08:00 to 20:00 h), at constant room temperature of 23 ± 2 °C with ad libitum access to food and water. All experimental procedures carried out with animals were approved by a local veterinary committee from the University of Oviedo vivarium and subsequent handling strictly followed the European Communities Council Directive 2010/63/UE and RD 1201/2005. All efforts were made to minimize the number of animals used and their suffering.

2.2. Apparatus

Animals were trained in a Morris water maze (MWM). The maze was a circular water tank made of black fiberglass, measuring 1.5 m in diameter by 75 cm in height, placed 50 cm above the floor [38]. The pool 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 23 ± 1 °C during the entire training period. The experimental room had numerous visual cues such as posters fixed on the walls, a shelf, covered windows and colored patterns on black panels. The swimming pool was illuminated by two halogen spotlights (500 W) placed on the floor and facing the walls. Each trial was recorded and path of the animals analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

2.3. Behavioral procedure

In order to discard possible motor and sensory deficiencies, animals were tested in a neurological assessment battery after the first handling session. The neurological tests used include the following tests: abduction response of hind limbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response and righting reflex [39].

Before training, the rats were handled daily during 5 days. The spatial memory task was performed between 09:30 and 13:30 h. For a behavioral protocol summary see Fig. 1.

2.3.1. Habituation

During habituation, rats received two sessions spaced 1 h apart. The water maze was divided virtually into four equal quadrants (called A, B, C and D) and a visible escape platform was located in the center of the quadrant D, 2 cm above the surface of the water. In each session the rats were released facing the pool walls from the central part of each quadrant following a pseudorandom sequence, four times each session. Rats were returned to their home cages between sessions. Rats were allowed to swim during 60 s to locate the platform in each trial, or gently guided to it after that period of time. They remained there for 15 s and then were placed in a plastic bucket during 30 s until the next trial.

2.3.2. Reference memory task

During the training phase animals received one session of four trials each on a daily basis, during five consecutive days. They were released from the central border of each of the quadrants in a pseudorandom order to search for a hidden escape platform beneath the water surface. The platform was located in the same quadrant (escape quadrant) during the training days, and rats were required to find it using spatial cues available in the room (reference memory training). Rats were allowed to swim during 60 s to reach the platform or gently guided to it after that time; they spent 15 s on the platform and rested during 30 s in a black plastic bucket within trials.

2.3.3. Retention probe

After the last training trial, rats were submitted to a retention probe test. In this case, the platform was removed from the maze. Rats were released from the opposite quadrant and they were allowed to swim during 60 s. After this period, they were picked up from the pool and placed again in the plastic bucket. In order to

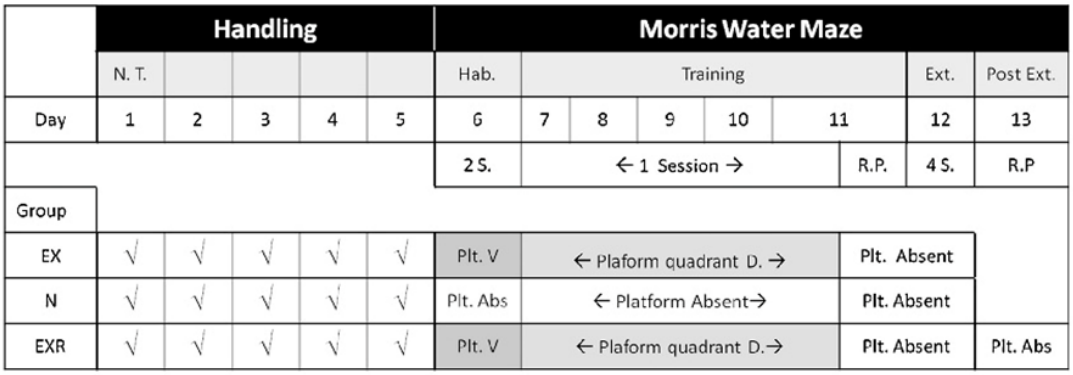

Fig. 1. Timeline and protocol of the experiment. Handling was carried out during 5 min each day. All sessions consisted in four trials of 60 s maximum. *Abbreviations*: neurological test (N.T), habituation (Hab.), extinction (Ext), post-extinction (Post. Ext.); extinction group (EX), naïve group (N), extinction and retention probe group (EXR); session (S), retention probe (R.P.); platform (Plt.), visible (V), in escape quadrant (D), and platform absent (Plt. Abs.). Please see Section 2 for more details.

prevent early extinction of the previously learned task, after completion of the probe all animals received a following trial (30 s) in which the platform was replaced to its original place.

2.3.4. Extinction protocol

Following the protocol by [40] the day after the learning retention probe, the animals (EX, $n = 7$) received four extinction sessions, each one consisted in four single non-reinforced trials of 60 s each, resting 30 s in a plastic bucket between trials. Period within sessions for the same animal was 30 min.

A control naïve group (N, $n = 7$) was included in order to ensure that brain activity changes were induced by the learning condition. This group swam for an equivalent amount of time as compared to the trained group. This time included every phase followed by trained animals (i.e. habituation, reference memory training, retention probe, extinction procedure and post-retention trial) but without any escape platform available during the entire testing period.

2.3.5. Extinction probe

To assess that there was no spontaneous recovery of previously learned behavior an additional group of animals (EXR, $n = 6$) was included into the study. This group followed the same procedure used for the EX group: they were submitted to habituation and reference memory training, after the acquisition they were tested in a retention probe. The following day they were subjected to an extinction procedure carried out under the same conditions as the EX group. Additionally, 24 h after the extinction protocol has finished, extinction of the previously acquired behavior was tested in another retention probe. This retention probe was carried out exactly under the same conditions as the first one.

2.4. Immunohistochemistry

2.4.1. Staining procedure

One hour and a half after the extinction procedure has finished, the animals were decapitated and their brains were removed intact, frozen rapidly in isopentane (Sigma–Aldrich, Germany), and stored at −40 °C. Coronal sections (30 μm) of the brain were cut at −20 °C in a cryostat (Microm HM-505E, Heidelberg, Germany) and then mounted on gelatinized slides. The tissue were post-fixed in buffered 4% paraformaldehyde (0.1 M, pH 7.4) for 30 min and rinsed in phosphate-buffered saline (PBS) (0.01 M, pH 7.4). They were subsequently incubated for 15 min with 3% hydrogen peroxide in PBS to remove endogenous peroxidase activity and washed twice in PBS. After blocking with PBS solution containing 0.3% Triton X-100 (PBS-T) (Sigma, USA) and 1% bovine serum albumin for 30 min, sections were incubated with a rabbit polyclonal anti-c-Fos solution (1:10,000) (Santa Cruz Biotech, sc-52, USA) diluted in PBS-T for 24 h at 4 °C in a humid chamber. Slides were then washed 3 times with PBS, and incubated in a goat anti-rabbit biotinylated IgG secondary antibody (Pierce, USA; diluted 1:200 in incubating solution) for 2 h at room temperature. They were washed 3 times in PBS and were treated with avidin–biotin peroxidase complex (Vectastain ABC Ultrasensitive Elite Kit, Pierce, USA) for 1 h. After 2 rinses in PBS, the reaction was visualized treating the sections for about 3 min in a commercial nickel-cobalt-intensified diaminobenzidine kit (Pierce, USA). The reaction was finalized by washing the sections twice in PBS. Slides were then dehydrated through a series of graded alcohols, cleared with xylene and coverslipped with *Entellan* (Merck, USA) for microscopic observation. All immunocytochemistry procedures included sections that served as controls because the primary antibodies were not added. Slides were coded so that the investigator who performed the entire analysis would have no knowledge of the treatment of the individual subjects.

2.4.2. Cell counting

The total number of c-Fos positive nuclei was quantified in three alternate sections spaced 30 μm apart containing the cingulate (CG), the prelimbic (PL), and the infralimbic (IL) regions of the medial prefrontal cortex; the dorsal hippocampus including CA1d, CA3d and the dentate gyrus (DGd); the ventral hippocampus (in CA1v, CA3v and DGv subfields); the retrosplenial granular (RSG) and agranular (RSA) cortex; the medial (Me), basal (Ba), and central (Ce) amygdala nuclei; lastly, we included also the medial (MM) and lateral (LM) nuclei of the mammillary bodies. Coronal sections of these brain regions were located using the stereotaxic atlas of Paxinos and Watson [41]. Distances in mm of brain regions counted from bregma were: +3.2 for CG, PL and IL, −3.14 for dorsal hippocampus and amygdala complex, −4.30 for ventral hippocampus and retrosplenial cortex, and −4.52 for mammillary bodies.

The profiles of the brain regions in the right hemisphere were first outlined in the slides using a permanent marker and their areas were estimated by drawing the brain regions using specific image analysis software (Jandel Scientific, San Rafael, CA, USA). Quantification was done by sampling each region selected using counting frames superimposed over the region. Size of the counting frame was 0.0576 mm^2 although the number of frames counted varied according to the sampled area size. The percentage of area sampled with respect to the total area of the three sections selected was always higher than 10%. Identification of c-Fos positive nuclei to be counted was based on their visual appearance as homogeneous dark stained elements with well-defined borders using a microscope (Olympus BH-2, Japan) coupled to an analog camera

(Sony XC-77, Japan) and a TV monitor (300× total magnification). Eventually, the mean number of c-Fos positive nuclei in three consecutive sections was calculated for each subject and region and divided by the total sampling frames mean per region in each slide (quantified area).

2.5. Statistical analysis

2.5.1. Behavioral data

Data were analyzed by Sigmastat 3.2 software (Systat Software, Chicago, USA).

2.5.1.1. Acquisition. Group differences in escape latencies during the training phase were analyzed using one-way repeated measures ANOVA, with training day as the repeated-measures factor (four trials per session). During the retention probe test, the mean time in the different quadrants was analyzed using one-way repeated measures ANOVA design. Tukey's HSD post hoc tests were applied when significant ANOVA results were found. If the normality assumption was violated or there were no homogeneous variances an ANOVA on ranks was carried out.

2.5.1.2. Extinction procedure. Two-way repeated measures ANOVA were used to evaluate differences between groups (naïve and EX groups) in mean distance swum and velocity. Differences were considered as statistically significant when $p < 0.05$ throughout the experiment.

2.5.1.3. Extinction probe. Similar to the retention probe test, one-way repeated measure ANOVA was used to evaluate differences in the time spent in each quadrant during this phase.

2.5.2. c-Fos data

For multiple group comparisons factors, two-way ANOVAs were used with group (EX, N) and area or nuclei in each structure as principal factors. In short, the cerebral structures analyzed were the following: the prefrontal cortex (prelimbic (PL) and infralimbic (IL) areas); the dorsal (DGd, CA1d and CA3d areas) and ventral (DGv CA1v and CA3v areas) hippocampus; the retrosplenial cortex (granular (RSG) and subgranular (RSA) regions); the amygdala complex (medial (Me), basolateral (Ba), and central (Ce) nuclei) and the mammillary bodies (medial (MM) and lateral (LM) nuclei). Pairwise contrast tests were applied for post hoc analysis of significant differences using the mixed procedure based on Kenward–Roger's adjusted degrees of freedom (df) solution and fitting the *p*-values in step-up fashion for controlling the familywise error rate (FWE). Schaffer's sequentially rejective step-down Bonferroni, and Hochberg's sequentially rejective step-up Bonferroni procedures were carried out to detect true pairwise differences. c-Fos data were analyzed by SAS PROC MIXED software (SAS Institute).

3. Results

3.1. Spatial learning and extinction

No animals were discarded due to abnormal neurological responses.

3.1.1. Reference memory task and retention probe

As shown in Fig. 2, escape latencies significantly decreased during training days in the EX group ($F_{1,6} = 7.82$, $p < 0.001$). Post hoc Tukey test showed differences in escape latencies between the first and fourth ($p = 0.001$) and between day 1 vs. fifth day ($p < 0.001$) and within the second and fourth day ($p = 0.019$) or day 2 as compared with the last training day ($p = 0.010$). Furthermore, the retention probe showed significant differences between the time spent within the four virtual maze quadrants ($F_{1,6} = 28.58$, $p < 0.001$) and the post hoc Tukey test showed that the D quadrant differed significantly from the others ($p < 0.001$) (Fig. 3).

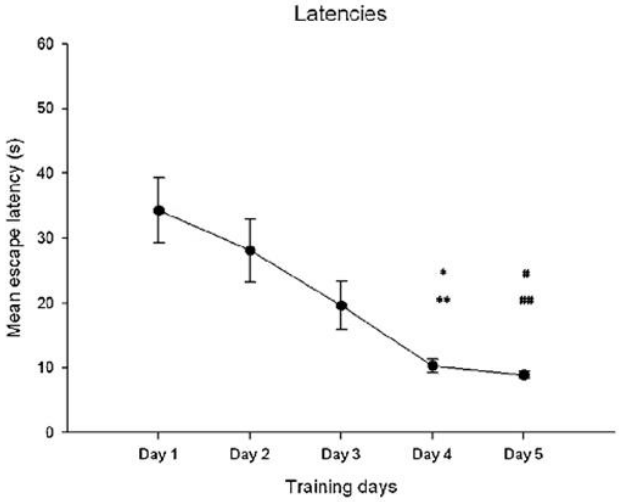

Fig. 2. Mean escape latencies (±S.E.M.) to reach the hidden platform during the daily session over 5 days in EX rats ($p < 0.001$). Post hoc Tukey's test analysis showed differences between the first vs. fourth *($p = 0.001$) and between first vs. fifth #($p < 0.001$) days, similarly to second vs. fourth *($p = 0.0019$) and within second and fifth ##($p < 0.014$) days.

As regards to the extinction-retention group (EXR), the ANOVA on Ranks test was carried out to test latencies within learning days ($p = 0.002$), followed by post hoc Tukey's test that reflects differences between first and fifth days ($p < 0.05$). The retention probe carried out after the last reference memory trial showed differences in the time spent swimming in the four maze quadrants ($F_{5,3} = 26.554$, $p < 0.001$). In particular, significant differences in swimming time between quadrant D and the non-reinforced ones ($p < 0.001$; Tukey's post hoc test) showed that animals achieved the learning criterion.

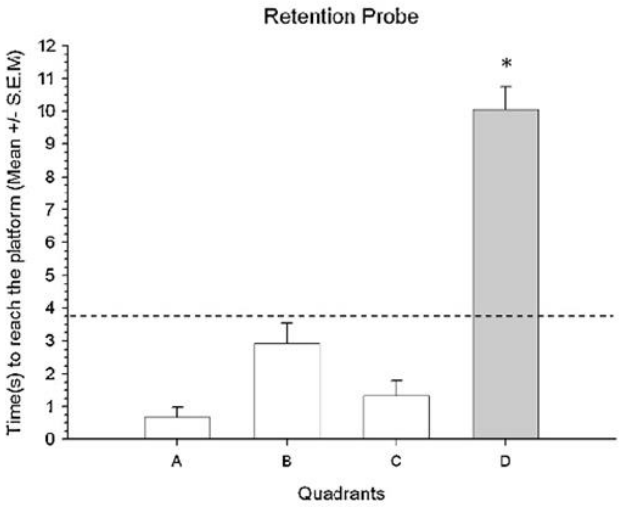

Fig. 3. There were significant differences ($p < 0.001$) in the mean time spent in each of the four virtual quadrants during the first 15 s of the retention probe that followed the learning protocol. All pairwise multiple comparison procedures (Tukey's test) showed differences between the escape quadrant (D) and the rest of them A, B and C *($p < 0.001$). Random probability (25% time in each quadrant) is represented by a broken line.

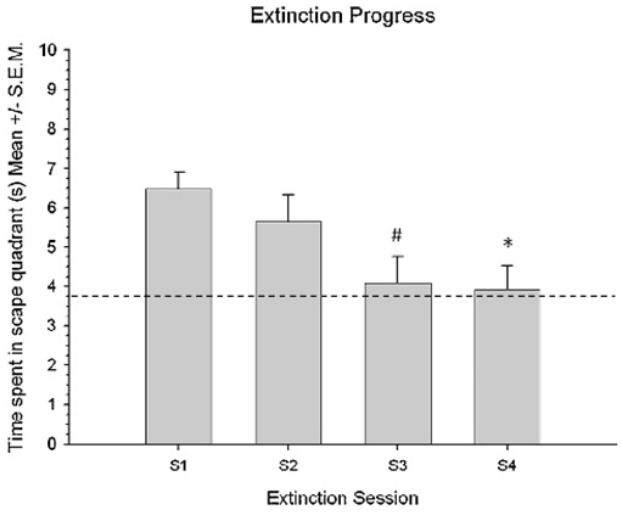

Fig. 4. Time spent during the first 15 s in the former escape quadrant in the extinction protocol, when no platform was available (mean ± S.E.M.). A one way repeated measures ANOVA test resulted in significant differences ($p < 0.02$) between sessions, then, to isolate the groups that differ from the others we used a multiple comparison procedure (Tukey's test) that resulted in differences between sessions 1 and 3 #($p < 0.04$), and between sessions 1 and 4 *($p < 0.03$). Each session consisted in the four trials per session. Random probability (25% time in each quadrant) is represented by a broken line.

3.1.2. Spatial memory extinction

Extinction procedure in the extinction group (EX) resulted in a significant decrease in the mean time spent in the escape quadrant across the four extinction sessions ($F_{1,6} = 4.75$, $p < 0.02$). In particular, significant differences were found between the first session and the third one ($p < 0.04$), and between session 1 and session 4 ($p < 0.03$, Tukey's test) (Fig. 4). Likewise, the EXR group animals showed similar results to EX animals because time spent in D quadrant changed within sessions ($F_{5,3} = 11.136$, $p < 0.001$), and session 1 was different form the following sessions ($p \leq 0.01$).

3.1.3. Extinction probe

No differences were found between quadrants during the retention probe carried out a day after the extinction sessions in the EXR group [ANOVA test; $F_{5,3} = 5.012$ ($p = 0.201$)].

No significant differences were found between the EX and naïve groups in the total distance swum ($p = 0.209$) and mean velocity ($p = 0.724$) during each extinction session.

3.2. c-Fos results

There was a statistically significant group effect in the number of c-Fos positive nuclei when it was analyzed using a two-way ANOVA, with group and areas in each brain region as factors (Fig. 5). Data analysis of the number of c-Fos positive nuclei showed a significant interaction between groups (EX and N) and areas (DG, CA1, CA3) in the dorsal hippocampus ($F_{2,9.4} = 6.88$, $p < 0.015$) and ventral hippocampus ($F_{2,10.5} = 5.59$, $p < 0.03$). Nevertheless, the analysis of pairwise comparisons in the dorsal and ventral hippocampus areas did not show differences between groups in the number of c-Fos positive nuclei in the six areas studied (Fig. 5 and Tables 2 and 3). No significant interaction between factors was found in the rest of areas. However, a statistically significant effect of group were found for the prefrontal cortex ($F_{1,11.9} = 20.16$, $p > 0.001$), the amygdala complex ($F_{1,11.9} = 20.16$, $p < 0.001$) and the mammillary bodies ($F_{1,12} = 28.44$, $p < 0.001$). For all possible contrasts in between-subjects marginal means in each of the areas, the results show after applying Hochberg's sequentially rejective Bonferroni procedure that all comparisons are significant controlling FEW at a level not higher than 0.05. The pairwise contrasts of the groups at areas (see Tables 1, 4 and 5) revealed that EX animals had significantly higher number of c-Fos positive nuclei in the prelimbic and infralimbic areas ($p < 0.05$) and the basolateral, medial and central amygdala nuclei ($p < 0.001$). Moreover, the number of c-Fos positive nuclei was higher in the N group compared to EX group in the lateral mammillary nuclei ($p < 0.001$) (Fig. 5). However, no differences between groups were found in the medial mammillary nucleus.

4. Discussion

Currently, little is known about the neuronal basis underlying the effects of different variables relevant for extinction like trial, temporal or spatial context [10]. Furthermore, previous studies indicate that the brain mechanisms involved in spatial memory acquisition and consolidation may also participate in the process of spatial memory extinction.

The aim of the present research was, therefore, to analyze the number of c-Fos positive cells in adult male rat brains in specific areas typically related to the cognitive processing in a spatial memory extinction process.

4.1. Behavioral procedure

Animals were trained in a spatial reference memory task followed by a spatial extinction protocol of the previously acquired task. The extinction group (EX) acquired the reference memory task and afterwards the formerly learned behavior was successfully extinguished. Spatial memory was revealed by the retention probe carried out after the last training day, in which the animals spent a higher amount of time in the escape quadrant. However, this amount of time decreased through the extinction sessions, reaching values around random probability during the third session of the extinction protocol, indicating the efficacy of the extinction method. Furthermore, extinction showed no spontaneous recovery 24 h after the extinction procedure, as shown in the extinction plus retention probe group (EXR) that did not show preference for the escape quadrant one day after the extinction procedure. Therefore, we might consider that extinction of spatial memory has occurred in our experiment.

According to several studies [4,20,24] the escape platform would act as a reward, being responsible for the reference memory task performance improvement throughout the trials in the MWM. In this way, once the reference memory task is acquired, escape learning extinction can be induced by removing the platform, so that animals are exposed to swimming trials without platform reinforcement. Taking this into account, extinction of spatial memory could be measured by behavioral changes along the extinction sessions, including diminished preference for the escape quadrant [9,21]. However, some authors disagree with the use of this type of protocols using the water maze to produce the extinction of a spatial memory. In this regard, it has been reported spontaneous recovery of the previous extinguished spatial response after a 96 h period delay [6]. This would occur if the number of nonreinforced retrieval trials is insufficient to induce long-lasting extinction. For instance, in a previous study [6] rats were submitted to half of the extinction trials (8 vs. 16 in our case). Moreover, extinction trials were consecutive, instead of using our 30 min intersession delay. Therefore, differences in the experimental settings might explain the apparent discrepancy with our results.

On the other hand, several authors consider that the behavioral protocol used here to induce extinction of spatial memory could in fact induce behavioral despair based on immobility [4,20,24]. Nevertheless, no significant differences were found between the

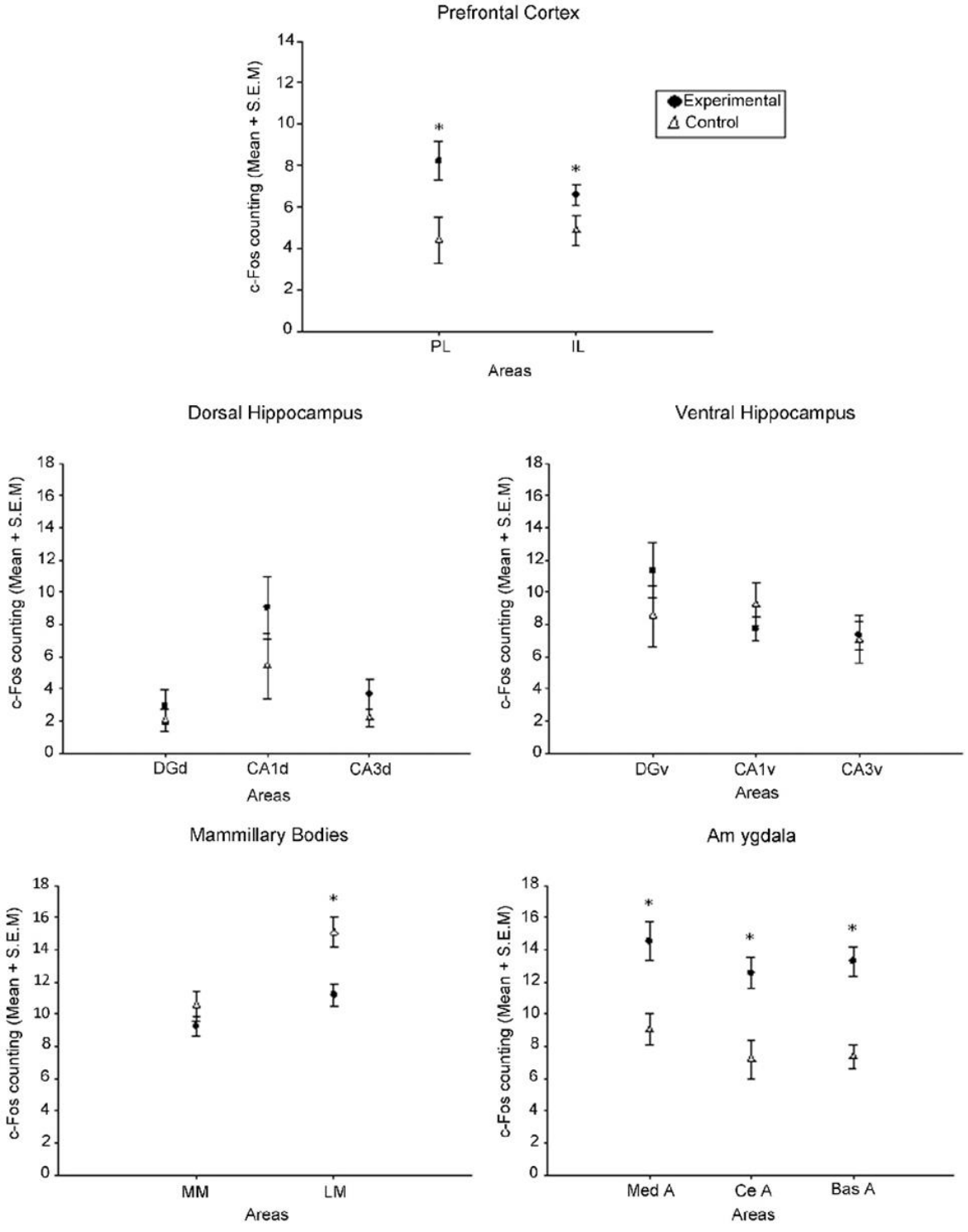

Fig. 5. c-Fos counts expressed as mean (±S.E.M.) per group. An increased number of c-Fos positive nuclei were found in the medial, basal and central nucleus of the amygadala, whereas there were a lower number of c-Fos positive cell-counts in the lateral nuclei of the mammillary bodies *($p < 0.04$). *Abbreviations*: retrosplenial granular (RSG) and agranular (RSA) cortex; cinculate (CG), prelimbic (PL), and infralimbic cortex (IL) of the medial prefrontal cortex; dorsal CA1d, CA3d and dentate gyrus (DGd); and ventral hippocampus (CA1v, CA3v, and DGv) fields; medial (MM) and lateral (LM) nuclei of the mammillary bodies; amygdala medial (Me), basal (Ba), and central (Ce) nuclei.

experimental group (platform available during the reference memory task) and the naïve group (no platform available) regarding the total distance swum and the mean velocity during the extinction protocol. Therefore, we assume that the immobility time of the experimental and pseudo groups was equivalent. Immobility may be possibly caused by other factors different from those attributable to the extinction protocol itself, like fatigue or lack of motivated behavior (also known as behavioral despair) to search for the escape platform after swimming several consecutive extinction trials. In this regard, we could not rule out the possibility that reinforcer

Table 1
Hochberg's adjusted *p* values for the pairwise contrasts in the prefrontal cortex.

Source	DF_1	DF_2	*F* value	*t*-Value	*p*-Value	H-adjusted	Decision
Tests for fixed effects							
Groups	1	10.5	7.79		0.0183		Reject
Areas	2	10.3	8.79		0.0138		Reject
Groups × areas	2	10.3	0.07		0.8037		Retain
Pairwise contrasts of the groups at areas							
EX vs. N at IL		11.0		2.88	0.0149	0.02971	Reject
EX vs. N at PL		10.0		2.43	0.0352	0.03523	Reject

DF_1, numerator degrees of freedom; DF_2, denominator degrees of freedom; *p*-value, probability values; H-adjusted, Hochberg adjustments.

Table 2
Hochberg's adjusted *p* values for the pairwise contrasts in the dorsal hippocampus areas.

Source	DF_1	DF_2	*F* value	*t*-Value	*p*-Value	H-adjusted	Decision
Groups	1	11.3	2.85		0.1189		Retain
Areas	2	9.4	32.45		<0.0001		Reject
Groups × areas	2	9.4	6.88		0.0145		Reject
Pairwise contrasts of the areas							
DGD vs. CA_1 D	11.1			−6.78	<0.0001	0.00009	Reject
CA_1 D vs. CA_3D	12.0			2.74	0.0002	0.00003	Reject
DGD vs. CA_3 D	8.3			−1.97	0.0831	0.08308	Retain
Pairwise contrasts of the groups at areas							
EX vs. N DGD vs. CA_1D	11.1	2.80		0.0171	0.05126		Retain
EX vs. N CA_1 D vs. CA_3D	12.0	1.99		0.0705	0.10568		Retain
EX vs. N DGD vs. CA_3D	8.3	−1.38		0.2037	0.20370		Retain

DF_1, numerator degrees of freedom; DF_2, denominator degrees of freedom; *p*-value, probability values; H-adjusted, Hochberg adjustments.

Table 3
Hochberg's adjusted *p* values for the pairwise contrasts in the ventral hippocampus areas.

Source	DF_1	DF_2	*F* value	*t*-Value	*p*-value	H-adjusted	Decision
Tests for fixed effects							
Groups	1	9.9	0.15		0.7044		Retain
Areas	2	10.5	30.18		<0.0001		Reject
Groups × areas	2	10.5	5.59		0.0222		Reject
Pairwise contrasts of the areas							
DGV vs. CA_3V	11.9			6.79	<0.0001	0.00006	Reject
CA_1 V vs. CA_1V	11.3			2.68	0.0211	0.03164	Reject
DGDV vs. CA_3 V	12.0			2.11	0.0567	0.05673	Retain
Pairwise contrasts of the groups at areas							
EX vs. N DGD vs. CA_1D	11.1		3.28		0.0066	0.01053	Reject
EX vs. N CA_1 D vs. CA_3D	12.0		3.25		0.0070	0.01053	Reject
EX vs. N DGD vs. CA_3D	8.3		−1.95		0.0761	0.07614	Retain

DF_1, numerator degrees of freedom; DF_2, denominator degrees of freedom; *p*-value, probability values; H-adjusted, Hochberg adjustments.

Table 4
Hochberg's adjusted *p* values for the pairwise contrasts in the amygdala nuclei.

Source	DF_1	DF_2	*F* value	*t*-Value	*p*-value	H-adjusted	Decision
Tests for fixed effects							
Groups	1	11.9	20.16		0.0008		Reject
Areas	2	11.4	5.30		0.0258		Reject
Groups × areas	2	10.4	0.14		0.8689		Retain
Pairwise contrasts of the areas							
Me vs. Ba	11.7			3.03	0.0109	0.02788	Reject
Me vs. Ce	11.5			2.74	0.0186	0.02788	Reject
Ce vs. Ba	11.5			−0.69	0.5011	0.50115	Retain
Pairwise contrasts of the groups at areas							
EX vs. N at Ba	11.5			4.94	0.0004	0.00117	Reject
EX vs. N at Me	11.6			3.62	0.0037	0.00511	Reject
EX vs. N at Ce	11.6			3.44	0.0051	0.00511	Reject

DF_1, numerator degrees of freedom; DF_2, denominator degrees of freedom; *p*-value, probability values; H-adjusted, Hochberg adjustments.

Table 5
Hochberg's adjusted p values for the pairwise contrasts in the mammillary bodies nuclei.

Source	DF_1	DF_2	*F* value	*t*-Value	*p*-value	H-adjusted	Decision
Tests for fixed effects							
Groups	1	12.0	28.44		0.0002		Reject
Areas	2	10.1	16.29		0.0023		Reject
Groups × Areas	2	10.1	2.62		0.1361		Retain
Pairwise contrasts of the groups at areas							
EX vs. N at LM	11.4			−4.03	0.0018	0.00368	Reject
EX vs. N at MM	9.9			−1.39	0.1942	0.19421	Retain

DF_1, numerator degrees of freedom; DF_2, denominator degrees of freedom; *p*-value, probability values; H-adjusted, Hochberg adjustments.

(escape platform) withdrawal in the water maze could also induce behavioral despair in both groups. In addition, it could be possible that this kind of extinction would be understood as a new learning process, since behavior after extinction is, at least, partly influenced by a context specific form of inhibitory learning [10,42].

4.2. c-Fos pattern

The results of this investigation suggest that brain regions located in the prefrontal cortex, the amygdala and the mammillary bodies are actively engaged during spatial navigation during the extinction process. Expression of c-Fos protein was increased in PL-IL areas of the prefrontal cortex and the amygdala complex after the extinction of spatial memory. Association of the amygdala with spatial memory extinction is not entirely surprising, given the wealth of evidence involving this brain structure in learning under stress conditions, which might be, for instance, the case for the extinction of spatial memory. Furthermore, previous studies have pointed out the importance of the amygdala in the extinction of spatial memory. In particular, the basolateral amygdala is thought to mediate the initial stage of extinction [43,44] and the expression of extinction via inhibition of central amygdala output neurons [45,46]. In this regard, the activation of basolateral nuclei seems to be necessary for the long-term retention of fear extinction [47]. Likewise, Porte et al. [26] have suggested an active role of the lateral nucleus of the amygdala both during early and late stages of spatial memory extinction processes in the water maze. According to these authors, this structure would act as a modulator of the non-emotional (hippocampus-based) vs. emotional (amygdala-based) aspects of the spatial learning experience in the water maze. Similarly, both behavioral and pharmacological studies in human and rodent models pointed to the amygdala as an involved structure in the modulation of hippocampal dependent-learning under stressful conditions [48,49]. In this regard, pharmacological studies suggest that the amygdala forms part of a circuit involved in spatial behavior by maintaining the association within the hippocampal-dependent place representation and its behavioral significance [50]. Extinction-related changes in neuronal activity in the lateral amygdala [51,52] are modulated by the hippocampus [53,54] and hippocampal inactivation impairs extinction of spatial memory [55]. However, we have found no significant group differences in the dorsal neither in the ventral hippocampus, as previously reported [42,56–58].

Currently, there is some disagreement regarding the specific roles of the aforementioned brain structures on spatial learning. Some authors suggest the existence of a temporal time window for the involvement of different structures as prefrontal cortex or the amygdala itself in the modulation of the hippocampal-dependent learning processes [26,27,59]. However, it should be considered that in this study c-Fos activity is reflecting evoked cellular activation at the end of the extinction process, but not at earlier stages. Accordingly, it is possible that the hippocampus might be playing an important role at early stages during the extinction of the previously acquired spatial memory, but not during the consolidation phase. Moreover, it has been reported that the dorsal hippocampus is important for the initiation of extinction of one-trial avoidance learning [60]. Therefore, a possible explanation for the lack of significant changes could be due to the highly specific time window for hippocampal activation during this process [61]; however, we cannot rule out the possibility of an involvement of the hippocampus during early stages of spatial memory extinction.

As in the case of the amygdala complex, we found also increased numbers of c-Fos inmunoreactive cells in the prelimbic and infralimbic areas of the prefrontal cortex, as related to the extinction of spatial memory. These cortical areas have been associated with later stages of spatial learning [27,62,63]. Although little is known about their implication in spatial memory extinction, both cortical areas have been involved in the extinction of several learning tasks [64–68]. It should be also considered that during the spatial memory extinction, the reinforcing stimulus (platform) is removed, suppressing thereby the previously reinforced behavior. Consequently, the animal changes its behavior, and continues to explore the environment in which it is located. Strategy switching requires that the animals integrate differently the possible configurations among the same stimuli and their responses to try to find the removed platform, and this is impaired by medial prefrontal cortex (mPFC) inactivation [69,70]. We could therefore support that mPFC is a critical structure for the extinction of spatial memory.

On the other hand, spatial orientation requires a constantly updated neural representation of directional heading, which is conveyed by head direction cells [71]. Many behavioral lesion studies in rodents involving mammillary bodies are related with spatial memory, stressing the relevance of this brain region on learning processes [28,72]. Despite the already demonstrated contribution of the mammillary bodies to spatial and basic associative learning, only a few studies have used functional gene mapping to evaluate specific contributions of this structure. This could be partly related to the very low baseline levels of immediate-early gene expression detected in this structure like c-fos or zif268 [73,74]. However, when changes in c-Fos protein levels were assessed, changes in the lateral, but not in the medial mammillary nucleus have been found both after a contextual fear conditioning [75], and after training in spatial working memory tasks [35]. However, we have found a higher number of c-Fos positive nuclei in the naïve group as compared to the experimental group in the lateral mammillary nucleus. It has been previously published that the lesion of the lateral mammillary body could produce mild impairments in spatial memory, but only in cases of rapid new spatial learning [76]. More recently, it has been reported [77] that lateral mammillary nuclei lesions prevent the normal use of visual allocentric cues and geometric cues while leaving animals able to use simple direction cues. Therefore, it would appear that the head-direction signal is not important for spatial navigation in our case, although it could be needed to interact with different spatial coding schemes in downstream brain structures in order to form accurate spatial representations [78,79]. Taking into account that c-Fos activity reflects the last part of this process of extinction in this study, we cannot preclude the possibility of a new inhibitory learning taking place at the time.

According to some authors, the extinguished memory is not erased but inhibited [10,42], so that the observed c-Fos changes may reflect this new learning process that could be occurring at the end of the extinction procedure.

4.3. Conclusions

We applied for the first time c-Fos protein imaging analysis to study spatial memory extinction. These results add further support for a growing literature suggesting that medial prefrontal cortex or the amygdala complex are neural substrates relevant for the extinction of spatial memory. Moreover, it is the first to suggest a possible role of diencephalic structures like the mammillary bodies. However, unlike previous results obtained in conditioning studies, the hippocampus seems not to be involved during the final stage of this extinction process. These finding provide new insights on the anatomical basis underlying the spatial memory extinction, although further studies would be necessary to fully understand the dynamic nature of this process.

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Artículo 6


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Research report

Brain functional network changes following Prelimbic area inactivation in a spatial memory extinction task

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ABSTRACT

Several studies suggest a prefrontal cortex involvement during the acquisition and consolidation of spatial memory, suggesting an active modulating role at late stages of acquisition processes. Recently, we have reported that the prelimbic and infralimbic areas of the prefrontal cortex, among other structures, are also specifically involved in the late phases of spatial memory extinction. This study aimed to evaluate whether the inactivation of the prelimbic area of the prefrontal cortex impaired spatial memory extinction. For this purpose, male Wistar rats were implanted bilaterally with cannulae into the prelimbic region of the prefrontal cortex. Animals were trained during 5 consecutive days in a hidden platform task and tested for reference spatial memory immediately after the last training session. One day after completing the training task, bilateral infusion of the $GABA_A$ receptor agonist Muscimol was performed before the extinction protocol was carried out. Additionally, cytochrome c oxidase histochemistry was applied to map the metabolic brain activity related to the spatial memory extinction under prelimbic cortex inactivation. Results show that animals acquired the reference memory task in the water maze, and the extinction task was successfully completed without significant impairment. However, analysis of the functional brain networks involved by cytochrome oxidase activity interregional correlations showed changes in brain networks between the group treated with Muscimol as compared to the saline-treated group, supporting the involvement of the mammillary bodies at a the late stage in the memory extinction process.

1. Introduction

Besides the explicit need to learn and remember new information and experiences by living organisms, an essential factor for efficient learning and survival is to discard useful information learnt in the past that is no longer relevant. At a certain point, the original memory and associated behavior must be suppressed, a process known as extinction learning [1]. According to several authors, the extinction process in the water maze would follow the same laws that govern instrumental learning [2], and even those which determine conventional classical and operant conditioning, so we might think that similar processes would underlie spatial and nonspatial associative learning [3–5]. It has been suggested that the molecular mechanisms underlying the acquisition or consolidation of extinction memory would be similar to those described for the acquisition or consolidation of the original task [6,7]. In this context, extinction may be considered as a new form of learning, involving memory formation although preserving the original memory trace. Many studies have tried to explain neural basis underlying this stage in terms of conditioning [6,8–10]; however, the neural substrates of a previously acquired spatial memory task remain elusive. The

Abbreviations: AcC, nucleus accumbens core; AcSh, nucleus accumbens shell; AD, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; BaA, basal amygdaloid nucleus; CA1d, Cornu Ammonis 1 subfield of the dorsal hippocampus; CA1v, Cornu Ammonis 1 subfield of the ventral hippocampus; CA3d, Cornu Ammonis 3 subfield of the dorsal hippocampus; CA3v, Cornu Ammonis 3 subfield of the ventral hippocampus; CeA, central amygdaloid nucleus; CFI, comparative fit index; CO, cytochrome oxidase; DGd, dorsal portion of the dentate gyrus; DGv, ventral portion of the dentate gyrus; Ent, entorhinal cortex; IL, infralimbic region of the prefrontal cortex; LaA, lateral amygdaloid nucleus; LM, lateral nucleus of the mammillary bodies; LS, lateral septum; M1, primary motor cortex; MD, medio-dorsal thalamic nucleus; MeA, medial amygdaloid nucleus; MM, medial mammillary nuclei; MS, medial septum; Mu, Muscimol-infused group; NNFI, non-normed fit index; PL, prelimbic region of the prefrontal cortex; PM, premammillary nucleus; PRh, perirhinal cortex; RMSEA, root mean square error of approximation; RSA, Agranular retrosplenial cortex; RSG, granular retrosplenial cortex; Sa, Saline vehicle-infused group; SE, standard error; SRW, standardized regression weights; SuM, supramammillary nucleus; URW, unstandardized regression weights; VTA Post, posterior ventral tegmental area.

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extinction of spatial learning evaluated in the Morris water maze has been studied at a behavioral level [3,4] and used in a despair- and depression-like model [2,11–13]. Recently, we have reported results on late stages of spatial learning memory extinction [14], in which the infralimbic and prelimbic areas of the medial prefrontal cortex, together with the lateral mammillary nucleus and the amygdala, showed changes in c-Fos protein expression following the execution of extinction task.

Currently, there is mounting scientific evidence about the variety and complexity of learning and memory processes that would require a multidisciplinary approach to fully understand their underlying neurobiological mechanisms. However, although significant progress has been made at the molecular and cellular level to understand learning and memory mechanisms, it is believed that only by the description of the functional brain networks involved at memory systems level, it would possible to fully understand the processes supporting memory as a whole. Unfortunately, this might represent the most difficult approach [15].

In this regard, some authors have tried to establish a map of the involved structures in spatial memory extinction, highlighting the role played by the amygdala and the hippocampus [16]. In addition, the mammillary bodies and the medial prefrontal cortex have been largely related with spatial navigation [17–19] and memory extinction processes [20,21]. In particular, we reported a key role of the prefrontal cortex at late stages of spatial learning acquisition [19] that was later found at late phases of the extinction task [14].

Taking the above into account, the objective of the present study is to assess the participation of the prelimbic region of the prefrontal cortex in the brain networks underlying spatial memory extinction and its relationship with previously related brain areas through the extinction of a acquired task evaluated in the Morris Water maze. For this purpose, selective temporal inactivation of the prelimbic region will be performed by intracerebral administration of the GABA-A agonist Muscimol. In order to evaluate possible changes in the brain networks associated with the inactivation of the prelimbic cortex, metabolic brain mapping will be carried out by cytochrome c oxidase (CO) quantitative histochemistry. CO is a mitochondrial enzyme of the respiratory chain and its activity is commonly used as a marker of brain oxidative metabolism.

CO histochemistry will be used in the present study to determine possible changes in local brain metabolism in rats after extinction of a previously learnt spatial reference memory task in the Morris water maze and with bilateral inactivation of the prelimbic area. For this purpose, the oxidative metabolism of different brain areas related to spatial memory and extinction processes will be evaluated using cytochrome oxidase (CO) histochemistry. This technique is useful to measure changes in regional brain activity associated with spatial learning or navigation [19,22,23]. In addition, CO histochemistry has been previously used to assess changes in these functional networks following experimental reversible inactivation [24]. Finally, interregional correlations in CO activity among areas of the medial prefrontal cortex and additional brain regions related with spatial memory extinction will be analyzed to determine functional changes in the neural networks involved in spatial memory extinction after temporal inactivation of the prelimbic area in the prefrontal cortex.

2. Material and methods

2.1. Animals

Seventeen male adult Wistar rats (*Rattus norvegicus*) weighing between 250 and 330 g were used in our experiments. Rats were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain) and they were housed in a temperature- and humidity-controlled room (23 ± 2 °C; 60 ± 10% relative humidity). Lighting was kept on a 12 h light/dark cycle with lights on from 08:00 to 20:00 h. Water and food were available with ad libitum access throughout the experiment. All experimental procedures carried out with animals were approved by a local ethical committee from the University of Oviedo vivarium and in accordance with the European Communities Council Directive 2010/63/UE and the Spanish legislation on care and use of animals for experimentation (R.D. 53/2013; Law 32/2007). All efforts were made to minimize the number of animals used and their suffering.

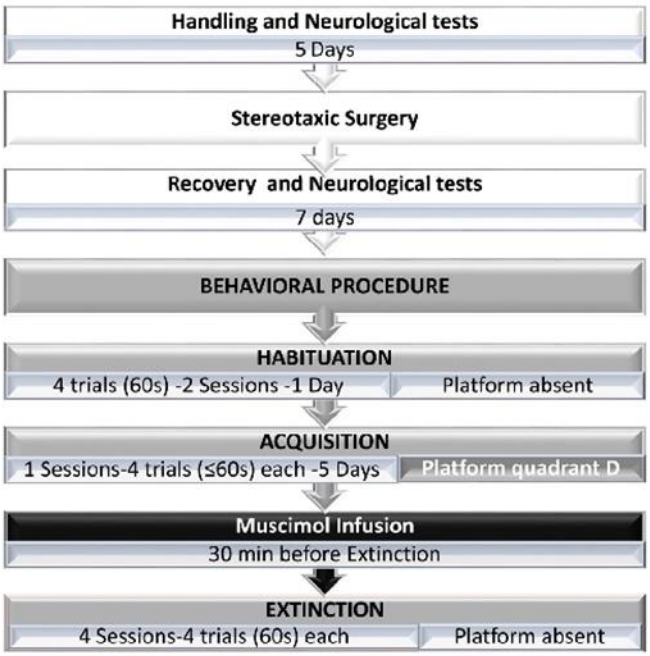

Fig. 1. Time line of the experiment.

2.2. Neurological tests and brain surgery

Before starting the experimental procedure, animals were tested in a neurological assessment battery in order to discard possible motor and sensory deficits. The neurological tests used include: abduction response of hind limbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response and righting reflex [25]. Rats were handled daily for 5 days prior surgery to reduce anxiety related with manipulation procedures. See Fig. 1 for a time-line.

On the surgery day, rats were deeply anesthetized with xylazine (5 mg/kg i.m.) and ketamine (80 mg/kg i.p.) and placed in a David Kopf (Tujunga, CA) or Narishige (Japan) sterotaxic frame. They were stereotaxically implanted with bilateral stainless-steel cannulae (22 G) (Becton Dickinson S.A., Spain) in the prelimbic region of the prefrontal cortex (coordinates from bregma: AP +3.1, L ±0.07, DV −3.0 mm). They were allowed to recover from surgery during 7 days, prior to the beginning of behavioral procedures carried out in the Morris water maze. Cannulae and anchor screws (two per subject) were encased in dental acrylic dental (Glaslonomer Cement, Shofu Inc., UK).

After post-surgical recovery period, animals were tested in the neurological examination battery explained above to discard any possible abnormalities caused by surgical procedures.

2.3. Behavioral procedure

Animals were trained in a Morris water maze. The maze was a circular water tank made of black fiberglass, measuring 1.5 m in diameter by 75 cm in height [26]. The pool was filled with tap water and an escape platform was placed hidden beneath the water surface. The water temperature was kept at 20 ± 1 °C during the whole testing period. The pool was surrounded by numerous visual cues as explained in previous articles [14,27]. Each trial was recorded and later analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

2.3.1. Habituation

During habituation in the Morris water maze, rats received two sessions spaced 1 h apart. The maze was virtually divided into four equal quadrants (A, B, C and D), and the escape platform was set in the center of the quadrant D, 2 cm above the surface of the water during the habituation phase, so that it was visible for the animals. In each session, the rats were released facing the pool walls from the central part of each virtual quadrant following a pseudorandom sequence, four times each session. Rats were allowed to swim up to 60 s to locate the platform in each trial, or guided to it after that. They were remained there for 15 s and then they rest in a black plastic bucket for 15 s until the next trial. Animals were returned to their home cages between sessions.

2.3.2. Reference memory task

Animals were trained during 5 consecutive days in a hidden platform task and tested for retention test immediately after the last acquisition trial. The spatial memory task was performed between 09:30 and 13:00 h.

During the acquisition phase, animals received a daily session of four trials, in which they were released from the central border of each of the quadrants in a pseudorandom order to search for a hidden escape platform beneath the water surface (1.5 cm). The platform was located in the same quadrant (escape quadrant, D) along the acquisition procedure, and rats were required to find it using spatial cues available in the room following training. Rats were allowed to swim for 60 s to reach the platform or gently guided to it after that time; they were 15 s on the platform and then they rested during 30 s in the aforementioned plastic bucket within trials.

2.3.3. Retention probe

After the last training day, rats were submitted to a retention test in a single probe trial. During this probe, the platform was removed from the maze, and rats were released from the contra lateral quadrant. They were allowed to swim for 60 s. After this period, animals were placed again in the bucket. In order to prevent premature extinction of the previously learned task, all animals received an additional trial in which the platform was available again in its original place. In this last trial, all animals were released from the quadrant B.

Even though the retention probe trial lasted 60 s, only the first 30 were included into the analysis. It was observed that the majority of animals tended initially to swim in the correct quadrant, but then quickly started to search in the rest when they failed to locate the absent platform [28,29]. For this reason, the inclusion of the entire period could lead to a misinterpretation of the data.

After finishing the training procedure, they were randomly divided into the two different groups Muscimol (Mu, $n = 9$) and Saline (Sa, $n = 8$).

2.3.4. Extinction protocol

The following day, rats were bilaterally infused in the prelimbic cortex with 0.5 μl of 1 μg/μl Muscimol dissolved in 0.9% saline (Muscimol group) or only with 0.9% saline in case of the Saline group. After 30 min post-infusion, they were submitted to four extinction sessions in the water maze.

Following the extinction protocol by [30] and previously explained in [14], animals had four extinction sessions the day after the learning retention probe. Each session consisted in four single non-reinforced trials in which no platform was available in the maze, of 60 s each, making a total of 16 trials. The resting period and in-between trial lasted 30 s in the aforementioned black plastic bucket and the inter-session interval was 30 min that took place in the home cage.

2.4. Cytochrome oxidase histochemistry

Ninety minutes after finishing the behavioral tasks, all animals were decapitated. Their brains were quickly removed then frozen in isopentane at −70 °C (Sigma-Aldrich, Madrid, Spain) and stored at −40 °C to preserve the brain tissue and enzyme activity. Brains were subsequently cut at 30 μm thick coronal sections using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). These sections were mounted on slides and stored at −40 °C until processing with quantitative CO histochemistry. One of the Saline group animals' brain and some sections from a few subjects of all of the groups could not be used as a result of tissue processing, although the final number of sections available for histochemistry per group was equal to seven or greater than the former in all cases.

A modified version of the method based on the quantitative CO histochemical method developed by Gonzalez-Lima and Jones [31] was used. Staining variability across different baths was controlled by sets of tissue standards. These standards were obtained from Wistar rat brain homogenates of known CO activity determined spectrophotometrically at different thicknesses (10, 30, 50 and 70 μm). Following the previously described protocol by Conejo et al. [24], the standards were included with each bath of slides. Each set of slides were fixed for 5 min with a 0.5% glutaraldehyde solution, rinsed three times in phosphate buffer and preincubated for 5 min in a solution containing 0.05 M Tris buffer, pH 7.6, with 275 mg/l cobalt chloride 10% (w/v) sucrose and 5 ml of dimethylsulfoxide. After the sections had been rinsed in phosphate buffer (pH 7.6, 0.1 M), they were incubated at 37 °C for 1 h in the dark and with continuous stirring in a solution containing 50 mg of 3,3′-diaminobenzidine, 15 mg of cytochrome c (Sigma, St. Louis, MO, USA) and 4 g of sucrose per 100 ml phosphate buffer (pH 7.4; 0.1 M). The reaction was stopped by fixing the tissue in buffered formalin (10%, w/v, sucrose and 4% formaline) for 30 min at room temperature. After being fixed, the slides were dehydrated, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

CO histochemical staining intensity was measured by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, UK) which includes specific image analysis software. Four measurements of relative optical density in three consecutive sections were done per region, i.e. 12 in total. In order to establish comparisons and considering possible staining variations across brain sections from different staining baths, measurements were also taken from CO-stained brain homogenate standards. Regression curves between section thickness and known CO activity, previously measured by spectrophotometric assay in each set of standards, were calculated. Lastly, average relative optical density measured in each brain region was converted into CO activity units (1 unit: 1 μmol of cytochrome c oxidized/min/g tissue wet weight at 23 °C) using

the previously calculated regression curve in each homogenate standard. The averaged measure per region was carried out for each region and animal. Those included prelimbic (PL), and infralimbic cortex (IL) of the medial prefrontal cortex and primary motor cortex (M1), all of them measured at ±3.70 mm from Bregma; granular (RSG) and agranular (RSA) retrosplenial cortices at ±4.52 mm; parietal (PAR) ±3.80 mm, entorhinal (Ent), and perirhinal (PRh) cortices at ±4.52 mm. In addition, the following subcortical regions were also taken: medio dorsal (MD), anterodorsal (AD) and anteroventral (AV) thalamic nuclei, measured at ±1.40 mm; lateral (LS) and medial septum (MS) at ±0.20 mm, nucleus accumbens core (AcC) and shell (AcSh) measured at ±1.00 mm; dorsal fields of hippocampus including CA1, CA3 and dentate gyrus of the dorsal (CA1d, CA3d, DGd fields) at ±3.30 mm; and ventral hippocampus (CA1v, CA3v, DGv fields), taken at ±4.52 mm from Bregma. Medial (MeA), basal (BaA), lateral (LaA) and central (CeA) amygdaloid nuclei were measured at ±3.14 mm from Bregma; medial (MM), lateral (LM) and supramammillary nucleus (SuM) of the mammillary bodies measured at ±4.52 mm, as well as the premammillary nucleus (PM) taken at ±4.16 mm. The selected brains regions anatomically were defined according to Paxinos and Watson [32].

2.5. Data analysis

2.5.1. Behavioral data

The behavioral data were analyzed using SAS 9.4 PROC MIXED (SAS Institute Inc., USA).

For statistical analysis of latency outcomes, a mixed-effects model repeated-measures (MMRM) was applied. This approach provides an appropriate general analytic framework to test the null hypothesis that there were no changes in the mean response over time [33]. The MMRM implemented herein included an unstructured (UN) modeling of time and the within-participant error correlation structure. In the analysis of response profiles, the repeated factor was regarded as qualitative instead of quantitative. Following rejection of an omnibus hypothesis, the next step was to determine to which contrasts among the population means are not equal to zero. To control the family-wise error rate for all possible pairwise comparisons, the Hochberg [34] step-up Bonferroni inequality was applied using the ESTIMATE statement in SAS PROC MIXED and the HOC option in SAS PROC MULTTEST. Dataset was analyzed using MMRM with REML estimation as implemented in SAS Version 9.4.

Repeated-measures one-way ANOVA test was used to test the quadrant preference during the retention memory test carried out immediately after the last learning session. In this case, the Holm–Sidak method was applied as post hoc test.

One-way repeated-measures ANOVA was carried out independently for each experimental group using the escape quadrant across the four extinction sessions as factor.

After the drug or saline infusion, the extinction progression was analyzed separately by the time spent in the escape quadrant per session along the four extinction sessions with two-way repeated-measures ANOVA with group and sessions as factors.

2.5.2. CO activity and brain network analysis

As in the above-mentioned case, the averaged CO activity differences per region between both groups were analyzed using Student's *t*-tests.

In addition, exploratory path analyses were carried out to study metabolic brain networks established both in Muscimol and Saline groups. In order to examine the functional relationships between observed brain regions, exploratory path analyses were conducted. Data were analyzed using SAS 9.4 PROC CALIS (SAS Institute Inc., 2013). The analysis of covariance structure was performed using maximum likelihood estimation. The model χ^2/χ^2 *df* ratio was used as a preliminary measure of overall fit, with conventional values for an acceptable fit being <2. Because the null hypothesis was that the specified model would fit the data (i.e., the predicted and observed covariance matrices would not differ significantly), non-significant χ^2 *P*-values were required as evidence to support the specified model. Comparative indices of fit reflected the improvement in fit obtained when using the hypothesized model instead of the null or baseline model. Therefore, a non-normed fit index (NNFI) > 0.9 and a comparative fit index (CFI) > 0.9 were interpreted as indicating a reasonable fit for the model. We also used the root mean square error of approximation (RMSEA), an index of the error between the model and the observed data, with values <0.05 or <0.08 indicating excellent and modest approximations, respectively. Convergence of the estimated covariance matrix with the sample covariance matrix was assessed by inspection of the matrix of normalized residuals. Parameter estimates for manifest variable equations were calculated and construed as indicating the strength of each individual path within the model. In post hoc assessments of the strength of each path within the model, we considered as significant those standardized parameter estimates in which the absolute *t*-values were >1.96.

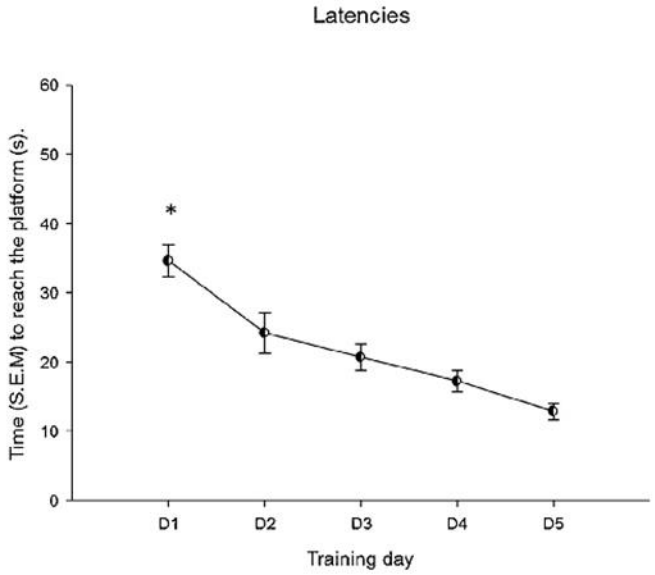

Fig. 2. Mean escape latencies (±S.E.M.) to reach the hidden platform during the daily session over 5 days in both future Muscimol and Saline groups rats. *$p < 0.001$. Hochberg's step-up procedure showed all possible pairwise comparisons were statistically significant at the 5% family-wise significance level, with the exception of the comparisons for the 2–4 days and days 3–4.

3. Results

3.1. Behavioral data

No animals were discarded for motor or sensory deficits previously or due to the surgical procedure.

3.1.1. Latencies and retention memory test

Animals of both groups decreased the spent time to reach the platform along the reference memory training. MMRM analysis results revealed statistically significant differences ($F_{4,13} = 14.35$, $p < 0.0001$) for the training-days factor; Therefore, rat performance changed over time. According to the Hochberg's step-up procedure, all possible pairwise comparisons were statistically significant at the 5% family-wise significance level, with the exception of the comparisons between the 2nd and 4th days and day 3 to day 4, as shown in Fig. 2.

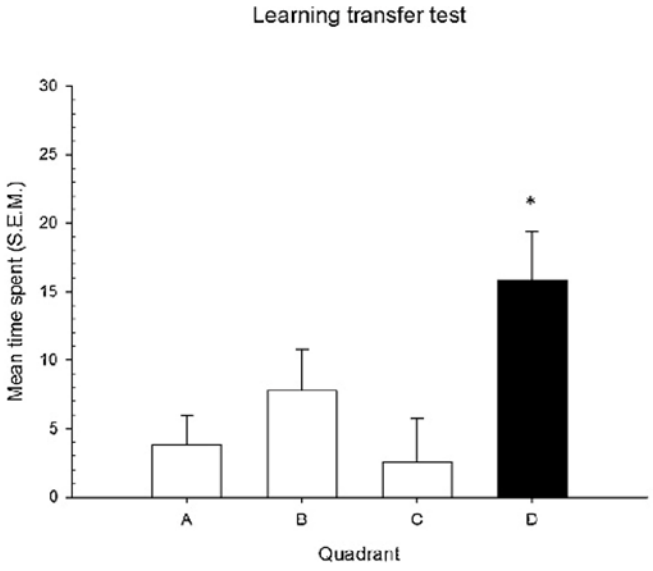

Fig. 3. Mean time spent (±S.E.M.) in each virtual quadrant during the retention memory test performed right after the last acquisition session. There were differences between the escape quadrant (D) and the rest of the quadrants ($p < 0.001$).

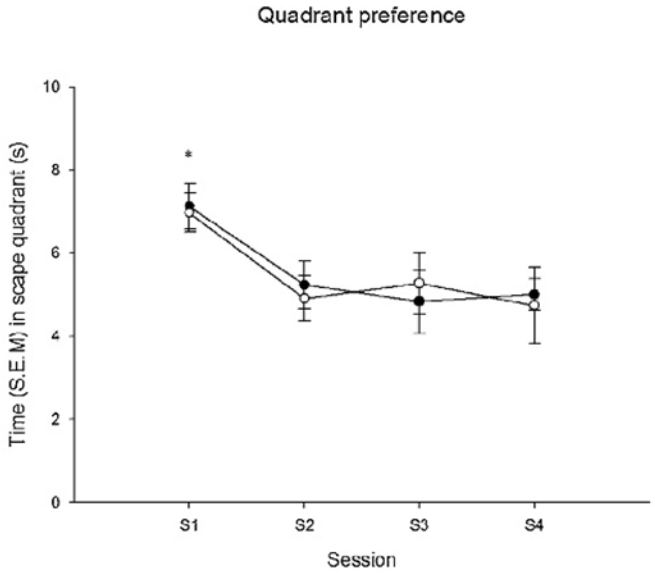

Fig. 4. There were significant differences (*$p = 0.01$) in the mean time spent in the formerly reinforced quadrant along extinction sessions during the first 15 s of each extinction session. Post hoc Holm–Sidak tests showed differences between the first session and the rest of them in both groups. However, no differences were found between groups in the mean time spent in the escape quadrant.

Results showed also differences in the amount of time spent among the four different quadrants that virtually divided the pool along the retention probe carried out the last day during the acquisition reference memory ($F_{3,48} = 50.02$; $p < 0.001$). Animals spent significantly more time swimming in the formerly reinforced quadrant as compared with the others ($p < 0.001$) and in the adjacent quadrant B versus quadrants A and C ($p < 0.01$) (see Fig. 3).

3.1.2. Extinction procedure

Both Muscimol and Saline groups successfully extinguished the formerly acquired hidden platform task, as the two-way ANOVA showed. No interaction between session and quadrant was found ($F_{3,45} = 0.18$; $p = 0.91$) during the extinction procedure, similarly, groups did not differ in the time spent in the escape at the formerly reinforced quadrant along extinction sessions during the first 15 s of each extinction session ($F_{1,15} = 0.02$; $p = 0.86$), however, there was a significant main effect of session ($F_{3,45} = 5.98$; $p < 0.01$). Holm–Sidak test showed that animals spent significant different time during the first session of the extinction procedure as compared to the rest of the sessions ($p < 0.01$) (Fig. 4).

3.2. Functional brain networks of CO activity

There were significant differences between groups in the mean CO activity in the lateral nuclei of the mammillary bodies ($p < 0.05$).

Functional brain networks differed in both groups. The Muscimol-treated group showed a modified network as compared to the Saline group. Specifically, this group had a simple network with connections projecting from the CA3v field of the ventral hippocampus and to the medial nucleus of the amygdala, which also receives connections from the retrosplenial agranular cortex and the lateral mammillary bodies. The medial nucleus of the amygdala complex sends in turn output to the ventral tegmental nucleus, which has unidirectional connections to the prelimbic cortex and the lateral mammillary bodies. The latter brain regions project both to the medial amygdala nucleus and the anterodorsal thalamus that maintains new unidirectional connections with the retrosplenial cortex to finish the loop.

However, the Saline group showed a more intricate network in which we can observe numerous reciprocal connections. Brain regions involved in this network were the ventral hippocampus, the prelimbic cortex, the anterodorsal thalamic nucleus, the medial amygdala nucleus, the retrosplenial agranular cortex and the ventral tegmental area. See Fig. 5 and Tables 1A and 1B for details.

4. Discussion

4.1. Prelimbic inactivation and extinction of spatial memory

It is known that the prefrontal cortex plays an important role in various conditioned learning extinction processes, late stages of acquisition [19] and extinction of spatial memory [14]. In the mentioned study, higher c-Fos expression both in the prelimbic and infralimbic regions of the prefrontal cortex was reported at late stages of the extinction process.

However, the specific role played by the prelimbic cortex during the extinction process or even the brain network associated with this brain region underlying the extinction process remains unclear. Therefore, this study aimed to elucidate the role of the prelimbic cortex on spatial extinction memory, and its relationship with structures typically related to cognitive processes and extinction memory. For this purpose, we temporally inactivated this region during the extinction of a spatial memory task in the water maze and later the neural networks involved were analyzed by measuring the regional brain metabolic activity using CO histochemistry.

The development of a variety of reversible inactivation techniques has derived in a wide range of tools that allow us to study learning and memory processes at different levels, since they were first introduced five decades ago [35]. Those techniques offer a number of advantages over traditional brain lesion methods. In this regard, if a brain region is believed to be involved throughout the whole memory process, involving acquisition, consolidation, retrieval, or extinction stages, brain lesions cannot be used to evaluate the involvement of this region at a particular memory stage. On the other hand, reversible inactivation would be a useful method to be applied within a temporal time window. Moreover, the target brain area that could be inactivated is in general terms much smaller and therefore its location can be more precise [36]. See [35] for a review about this topic. This method could be especially

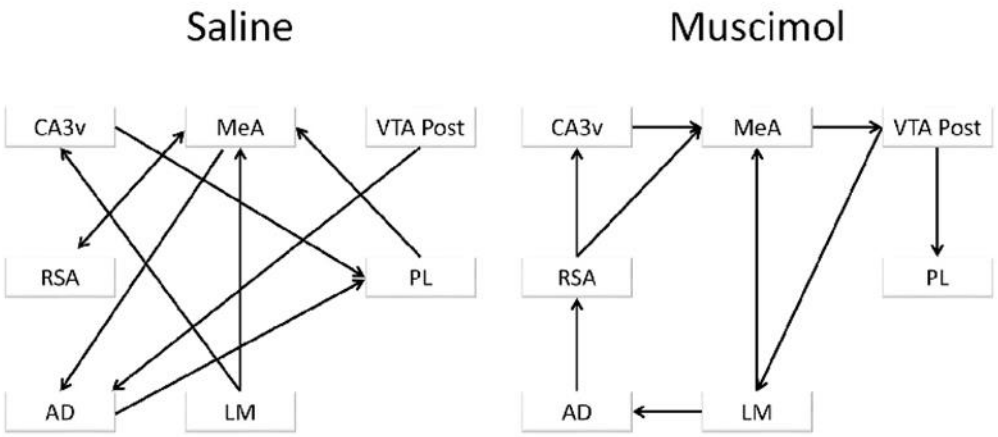

Fig. 5. Path analysis of correlations in regional brain cytochrome oxidase activity. Abbreviations: AD, anterodorsal thalamus; CA3v, Cornu Ammonis 3 of the ventral hippocampus; LM, lateral nucleus of the mammillary bodies; VTA Post, posterior ventral tegmental area; MeA, medial nucleus of the Amygdala; PL, prelimbic area of the prefrontal cortex; RSA, retrosplenial agranular cortex.

useful to explore brain circuits involved in independent but at least temporally overlapping processes [37].

In order to reversibly inactivate a particular brain region, several drugs can be injected such as specific sodium channel blockers like tetrodotoxine, local anesthetics which block the transmission of action potentials in axons and cellular bodies or neurotransmitter agonists/antagonists which interfere with neuronal activity at synaptic level. In these cases, local drug administration by microinjection is required [38]. The latter technique presents several advantages for behavioral studies, because local drug administration is possible through permanently implanted intracerebral cannulae. Furthermore, the microinjection procedure in deep brain areas allows the inactivation of smaller areas as compared to the previously used inactivation techniques, thus minimizing damage to the surrounded tissues due to smaller cannula diameter [39,40].

Our results showed that both groups of animals successfully learned the hidden platform task, as demonstrated by the higher amount of time spent in the target quadrant, as well as the

Table 1A
Standardized and unstandardized parameter estimates, standard errors and *t*-values—path analysis for the saline group.

Variables			URW	SE	*t*-Value	SRW	SE	*t*-Value
CA3v	→	PL	0.8646	0.3316	2.61***	0.7601	0.1979	3.87***
PL	→	MeA	1.4334	0.3674	3.90***	1.2195	0.4379	2.78***
RSA	→	MeA	−2.9849	0.7522	−3.97***	−1.3269	0.4010	−3.31***
AD	→	PL	−0.3190	0.2347	−1.36	−0.4072	0.2438	−1.67*
LM	→	MeA	1.1036	0.3923	2.81***	0.7233	0.2976	2.43**
LM	→	CA3v	0.5710	0.3525	1.62	0.4958	0.2315	2.24**
MeA	→	RSA	0.4698	0.2052	2.29**	1.0567	0.3073	3.43**
MeA	→	AD	0.5675	0.2380	2.38**	0.5226	0.1995	2.62***
VTA Post	→	AD	−1.0319	0.3545	–	−0.6222	0.1607	−3.87***

Note: Variances of the observed variables are not included. URW (unstandardized regression weights), SRW (standardized regression weights, transformations of unstandardized estimates that remove scaling and can be used for informal comparisons of parameters throughout the model), SE (standard error). The structural equation model showed acceptable fit on three measures, chi-square (6.25, df = 17, $p = 0.99$), CFI (0.999), and Pr. Close Fit (0.99).
*Significant at 10%.
**Significant at 5%.
***Significant at 1%.

Table 1B
Muscimol group's standardized and unstandardized parameter estimates, standard errors and *t*-values—path analysis.

Variables			URW	SE	*t*-Value	SRW	SE	*t*-Value
CA3v	→	MeA	3.2272	1.0242	3.15***	4.8828	2.2351	2.18**
PL	→	RSA	1.4668	0.5031	2.92***	2.2789	1.073	2.12**
RSA	→	CA3v	−1.1362	0.5719	−1.99**	−1.1157	0.4698	−2.37**
RSA	→	MeA	−2.1567	0.7582	2.84***	−3.2026	1.6003	−2.01**
AD	→	RSA	−0.6682	0.2429	2.75***	−2.1169	1.0910	1.96**
LM	→	AD	−2.9465	0.5817	5.07***	−1.0125	0.0697	14.53***
LM	→	MeA	−2.9550	0.9613	3.06***	−4.7546	2.4359	1.96**
MeA	→	AD	−1.8210	0.9054	2.01**	−0.3877	0.2310	1.67*
MeA	→	VTA Post	2.5905	0.7970	3.25***	2.2676	0.6436	3.52***
VTA Post	→	LM	1.7838	0.7447	2.40**	1.2634	0.4444	2.84***
VTA Post	→	PL	1.4673	0.7975	1.84*	0.7263	0.3488	2.08**

Note: See note to Table 1A. The model provided acceptable fit on three measures, chi-square (13.34, df = 16, $p = 0.79$), CFI (0.961), and Pr. Close Fit (0.80). AD, anterodorsal thalamus; CA3v, Cornu Ammonis 3 of the ventral hippocampus; LM, lateral nucleus of the mammillary bodies; VTA Post, posterior ventral tegmental area; MeA, medial nucleus of the amygdala; PL, prelimbic area of the prefrontal cortex; RSA, retrosplenial agranular cortex.

significant reduction of latencies to find the platform during the acquisition phase as previously described [14]. Furthermore, after Muscimol infusion, the experimental animals were similarly able to complete the extinction phase like those who received the saline solution. This result was evidenced by a decreased preference for the previously reinforced escape quadrant throughout the extinction sessions.

Our data are consistent with previous findings, which suggest that the escape platform would act as a rewarding stimulus, because it contributes to improve spatial memory performance along sessions in the Morris water maze [2,11,41]. Similarly, after acquisition of the task, extinction could be induced by platform removal, thus forcing the animal to swim in the absence of rewards. By taking the latter into account, the extinction of a spatial memory task could be assessed by evaluating the behavioral changes throughout extinction sessions, like reduced preference for the previously reinforced virtual quadrant [7,13]. Therefore, we might think that extinction of spatial memory has occurred in both groups.

The extinction process is a very well-preserved learning process. According to Delamater and Westbrook [42], extinction may fit into the prevalent learning theory, assuming that extinction results in partial erasure of the original learning together with new inhibitory learning. Since acquisition and extinction of spatial learning in the Morris water maze follow the same laws that determine classical and operant conditioning, we might think that similar processes would underlie spatial and non-spatial learning [4,5]. Although further research is required, it seems that this learning phase could only be fully explained by elucidating the underlying brain networks.

4.2. Brain CO activity changes and reorganization of functional networks

Regarding regional CO brain activity, only the mammillary bodies differed among groups. However, path analysis revealed that the functional networks activated after the extinction of a spatial reference memory task were different in animals that performed the task under prelimbic area temporal inactivation, as compared to animals treated with saline (i.e. control) conditions. The Saline group showed a more intricate pattern among structures, meanwhile these connections changed and they were limited to a few structures after inactivation of the prelimbic cortex. According to the current perspective of brain function, which mostly derives from neuroimaging studies, brain network patterns would depend on the effective or functional connectivity at a local or distal anatomical brain pathway [43–45]. In addition, these authors suggest that functional interactions among brain regions could be different between control and experimental groups, even when no significant changes are shown in their neuronal activity [45]. As a result, particular regions could be differentially involved at different time points throughout a particular task by changing its interactions with other regions, like the well-known case of the hippocampus along spatial learning [24,46]. It would be therefore a similar case for the prefrontal cortex during the extinction of spatial memory. In particular, the medial prefrontal cortex could be involved in the extinction of spatial memory by changing its interactions with other areas throughout the process, which could explain why this region is activated at late stages of spatial memory extinction [14], meanwhile its early inactivation does not appear to affect the extinction task acquisition performance.

As already mentioned, the lateral mammillary nuclei showed differences between both groups. In this regard, lesion studies support the idea of the mammillary bodies being involved in spatial memory [17]. Additionally, lateral mammillary nuclei has been related to rapid new spatial learning, as it lesion produce mild impairments in this task execution [47]. As to the spatial memory extinction phase, the extinction-induced change in c-Fos expression was found in this structure as previously reported [14], so that it could be involved not only in the latter phase of the extinction process, but also at the beginning of this new learning phase.

The medial amygdala shows interactions both with the mammillary bodies as well as with the hippocampal system, including CA3v and retrosplenial cortex. There is a body of evidence supporting the idea that the amygdala complex is involved in spatial memory. Specifically, Porte et al. [16] have shown an active role of the amygdala, together with the hippocampus, both in early and late stages of the extinction of spatial memory. These authors suggest a regulatory role for the amygdala during the extinction process, modulating the hippocampus-based non-emotional aspects and the amygdala-dependent emotional processes that spatial learning in the water maze involves. Its connection with the ventral hippocampus and extended hippocampal areas, like the retrosplenial cortex, seems to be required to acquire a spatial memory task. Specifically, it is well known that the ventral hippocampus, the basolateral amygdala and parahippocampal areas are associated with escape behavior under stress conditions [48], which would agree with our results. However, recent studies with rats reported that the ventral hippocampus modulates memory extinction. In this regard, disconnections between the ventral hippocampus and the amygdala facilitate the recall of fear extinction memory [49], but the inactivation of the ventral hippocampus itself impairs the recall of this extinction memory [50]. Moreover, recent studies showed that low-frequency stimulation of the ventral hippocampus enhances extinction learning [51], so that the ventral hippocampus would be involved not only in the retrieval of the acquired extinction processes, but also in extinction learning. Since the ventral hippocampus did not show any differences in the last stages of this extinction process when examined by brain c-Fos protein [14], it might be that its involvement in this process would be essentially restricted to early acquisition stages.

Similarly, the anterior thalamus has been involved in spatial learning of a location related to multiple distal cues, as was revealed for lesion studies in water maze tasks [52–54]. Furthermore, evidence includes the finding that these nuclei of the thalamus are linked to item-place associations and distal information [55], as in the case of the hidden platform task performed here [49]. Moreover, the anterodorsal thalamus is known to be one of the structures which contain neurons that form part of the head direction system. These cells are believed to represent the animal's perceived directional heading in its environment [56] being this information afterwards processed to form a cognitive map of the environment [57]. The anterodorsal thalamus together with the laterodorsal thalamus receives visual inputs from the retrosplenial cortex, and both regions are reciprocally connected to the postsubiculum [58,59]. This fact would explain why those structures remain connected to perform the extinction spatial task. In this regard, the anterodorsal thalamus remains connected to the lateral mammillary nucleus, which is not surprising if we take into account that this thalamic nucleus receives vestibular and motor information from the lateral mammillary nucleus [60]. In sum, the participation of the anterodorsal thalamus in this process could be associated with its role in the head direction system and spatial navigation in general. On the other hand, a recent study reports the specific role of this structure in memory extinction, as part of a circuit involving prefrontal area and the amygdala in conditioned fear [61]. Although it has not been possible to provide a definite explanation for the participation of the thalamus, it seems that it is required for the extinction of spatial memory task. Future studies are required to evaluate the specific role of the anterior thalamus in spatial memory extinction.

5. Conclusions

The interpretation of our results supports the notion that the prefrontal cortex plays an important role in the extinction of spatial memory. However, the integrity of the prelimbic area seems not to be essential for spatial memory extinction completion. In this way, when this area is temporally inactivated, changes in extinction-associated metabolic brain networks were found, providing the necessary support to successfully complete extinction. Moreover, in agreement with our previous study [14] increased metabolic activity in the mammillary bodies was found in the experimental group, highlighting the role played by this structure in the extinction process. However, we cannot rule out the possibility that additional brain regions could participate in the associated brain network, because the dorsal and ventral hippocampal regions are recruited during the acquisition of this task.

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Artículo 7

CA1 INACTIVATION ALTERS A SPATIAL MEMORY EXTINCTION TASK AND MODIFIES PREFRONTAL CORTEX AND ACUMBENS NUCLEUS METABOLIC ACTIVITY

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ABSTRACT

Functional inactivation enables the study of the dorsal hipocampal involvement in different phases of spatial memory in a lab context. In this study, we applied the GABA-A Muscimol antagonist to temporally inactivate the CA1 field of the dorsal hippocampus to evaluate the role of this structure and its functionally connected structures in a spatial memory extinction task in the Morris Water Maze. Results showed that bilateral inactivation of the dorsal hippocampus resulted in an impairment of the retrieval and posterior extinction task. Differently to the saline-infused group, in which animals steadily reduced the amount of time spent in the previously reinforced quadrant, preference for the scape quadrant for the hippocampus inactivated groups did not change along extinction sessions. Analysis of cytochrome oxidase activity disclosed regional differences between groups. In comparison to the saline group, animals with CA1 inactivation showed decreased CO activity in the prelimbic, infralimbic and cingulate areas of the prefrontal cortex, as well as the perirhinal cortex. As to the subcortical regions, the Core nucleus of the accumbens presented also a lower CO activity level in the experimental group. This study demonstrated that the integrity of the CA1 field of the dorsal hippocampus is essential for the successful completion of spatial reference memory task in the Morris water maze.

KEYWORDS: Extinction; Spatial Memory; Muscimol; Ca1 dorsal hippocampus, Accumbens, Prefrontal cortex; Cytochrome Oxidase.

ABBREVIATIONS

AcC: Nucleus accumbens Core

AcSh: Nucleus accumbens Shell

AD: Anterodorsal thalamic nucleus

AV: Anteroventral thalamic nucleus

BaA: Basal amygdaloid nucleus

CA1d: Cornu Ammonis 1 subfield of the dorsal hippocampus

CA1v: CornusAmmonis 1 subfield of the ventral hippocampus

CA3d: Cornu Ammonis 3 subfield of the dorsal hippocampus

CA3v: Cornu Ammonis 3 subfield of the ventral hippocampus

CeA: Central amygdaloid nucleus

CO: Cytochrome oxidase

DGd: Dorsal portion of the Dentate Gyrus

DGv: Ventral portion of the Dentate Gyrus

Ent: Entorhinal cortex

IL: Infralimbic region of the prefrontal cortex

LaA: Lateral amygdaloid nucleus

LM: lateral nucleus of the mammillary bodies

LS: Lateral septum

M1: Primary motor cortex

MD: Medio-dorsal thalamic nucleus

MeA: Medial amygdaloid nucleus

MM: Medial mammillary nuclei

MS: Medial septum

MuH: Muscimol infused group

PL: prelimbic region of the prefrontal cortex

PM: Premammillary nucleus

PRh: Perirhinal cortex

RSA: Agranular retrosplenial cortex

RSG: Granular retrosplenial cortex

SaH: Saline vehicle infused group

SE: Standard Error

SuM: Supramammillary nucleus

VTA Post: posterior ventral tegmental area

INTRODUCTION

One of the essential factors for animals lives is the ability to learn and remember new information and experiences, however, equally important for learning and survival is being able to discard useful information learnt in the past that is no longer relevant. When the learned behavior is no longer adaptive, over trials of extinctions, the organism learn that the formed response is no longer effective in conducting to reinforcement and will gradually desist to emit this behavior(Bouton et al., 2006, Archbold et al., 2010). At a certain moment, the original memory and associated behavior must be suppressed, a process known as extinction learning (Andre et al., 2014). According to several authors, the extinction process in the water maze would follow the same laws that govern instrumental learning (Huston et al., 2009), and even those which determine conventional classical and operant conditioning, so we might think that similar processes would underlie spatial and nonspatial associative learning (Sanchez-Moreno et al., 1999, Prados et al., 2003, Prados et al., 2008). It has been suggested that the molecular mechanisms underlying the acquisition or consolidation of extinction memory would be similar to those described for the acquisition or consolidation of the original task (Lattal et al., 2003, Szapiro et al., 2003). In this context, extinction may be considered as a new form of learning, involving memory formation although preserving the original memory trace. The extinction of spatial learning evaluated in the Morris water maze has been studied at a behavioral level (Prados et al., 2003, Prados et al., 2008), and to recreate pathological conditions, being used as a despair- and depression-like model (Topic et al., 2005, Schulz et al., 2007, Huston et al., 2009, Huston et al., 2013). Recently, we have reported results on late stages of spatial learning memory extinction (Mendez-Couz et al., 2014), in which, contrary to the prefrontal cortex areas, which showed an late activation, the dorsal hippocampus seems not to be activated, as changes were not observed in a c-Fos protein expression following the execution of extinction task. However, when the prelimbic area of the prefrontal cortex were inactivated before executing a spatial memory extinction task, rats were able to complete it, showing different patterns of associated brain cytochrome oxidase activation networks, in which the dorsal hippocampus seemed to play a key role (Mendez-Couz et al., 2015b).

Currently, there is evidence about the variety and complexity of learning and memory processes that would require a multidisciplinary approach to fully understand their underlying neurobiological mechanisms. However, although much progress has been made at the molecular and cellular level to understand learning and memory mechanisms, it is believed that only by the description of the

functional brain networks involved at memory systems level, it would possible to fully understand the processes supporting memory as a whole. Unfortunately, this might represent the most difficult approach (Kandel and Pittenger, 1999)

In this context, some authors have tried to establish a map of the involved structures in spatial memory extinction, highlighting the role played by the amygdala and the hippocampus (Porte et al., 2011). In addition, the mammillary bodies and the medial prefrontal cortex have been largely related with spatial navigation (Mendez-Lopez et al., 2009, Conejo et al., 2010, Vann, 2010) and memory extinction processes (Delamater, 2004, Bouton et al., 2006). In particular, we reported a key role of the hippocampus at the beginning of spatial learning acquisition (Conejo et al., 2010) that was confirmed necessary to properly retrieve the spatial task one to several weeks after (Conejo et al., 2013, Mendez-Couz et al., 2015a). Additionally, when the prelimbic area of the prefrontal cortex was inactivation, execution of the task was not impaired, but metabolic brain networks changed, showing an important role of the dorsal hippocampus to support spatial extinction learning.

The objective of the present study is, therefore, to elucidate the participation of the CA1 of the dorsal hippocampus in the brain networks underlying spatial memory extinction, and its relationship with previously related brain areas through the extinction of a previously acquired task evaluated in the Morris Water maze. For this purpose, selective temporal inactivation of the dorsal CA1 will be performed by intracerebral administration of the GABA-A agonist muscimol. In order to evaluate possible changes in the brain structures associated with the inactivation of the dorsal hippocampus, metabolic brain mapping will be carried out by cytochrome c oxidase (CO) quantitative histochemistry. CO is a mitochondrial enzyme of the respiratory chain, and its activity is commonly used as a marker of brain oxidative metabolism.

CO histochemistry will be used in the present study to determine possible changes in local brain metabolism in rats after extinction a previously learnt spatial reference memory task in the Morris water maze, and with bilateral inactivation of the dorsal hippocampus. For this purpose, the oxidative metabolism of different brain areas related to spatial memory and extinction processes will be evaluated using cytochrome oxidase (CO) histochemistry. This technique has being proved useful to measure changes in regional brain activity associated with spatial learning or navigation (Villarreal et al., 2002, Hu et al., 2006, Maruani et al., 2008, Conejo et al., 2010, Fidalgo et al., 2012, Arias et al., 2014, Fidalgo et al., 2014). In addition, CO histochemistry has been previously

used to assess changes in this functional activity following experimental reversible inactivation (Conejo et al., 2013, Mendez-Couz et al., 2015b).

1. EXPERIMENTAL PROCEDURES

1.1. ANIMALS

Sixteen male adult Wistar rats (*Rattus norvegicus)* weighing between 250-330g were used in our experiments. Rats were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain) and they were housed in a temperature- and humidity- controlled room (23±2ºC; 60±10 % relative humidity). Lighting was kept on a 12 h light/dark cycle with lights on from 08:00–20:00 h. Water and food was available with *ad libitum* access throughout the experiment. All experimental procedures carried out with animals were approved by a local ethical committee from the University of Oviedo vivarium and in accordance with the European Communities Council Directive 2010/63/UE and the Spanish legislation on care and use of animals for experimentation (R.D. 53/2013; Law 32/2007). All efforts were made to minimize the number of animals used and their suffering.

1.2. NEUROLOGICAL TESTS AND BRAIN SURGERY

Before starting the experimental procedure, animals were tested in a neurological assessment battery in order to discard possible motor and sensory deficits. The neurological tests used include: abduction response of hind limbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response and righting reflex (Bures et al., 1976). Rats were handled daily during 5 days prior surgery to reduce anxiety related with manipulation procedures. See Figure 1 for a time-line.

The surgery day, rats were deeply anaesthetised with xylazine (5mg/kg i.m.) and ketamine (80 mg/kg i.p.) and placed in a David Kopf (Tujunga, CA) or Narishige (Japan) sterotaxic frame. They were stereotaxically implanted with bilateral stainless-still cannulae (22G) (Becton Dickinson S.A., Spain) in the prelimbic region of the CA1 of the dorsal hippocampus (coordinates from bregma: AP -3.6, L ±2.6, DV -2.1 mm).They were allowed to recover from surgery during 7 days, prior to the

beginning of behavioral procedures carried out in the Morris water maze. Cannulae and anchor screws (2 per subject) were encased in dental acrylic dental (Glaslonomer Cement, Shofu Inc., UK).

After post-surgical recovery period, animals were tested in the neurological examination battery explained above to discard any possible abnormalites caused by surgical procedures.

1.3. BEHAVIORAL PROCEDURE

Animals were trained in a Morris water maze. The maze was a circular water tank made of black fiberglass, measuring 1.5 m in diameter by 75 cm in height (Morris, 1984) . The pool was filled with tap water and an escape platform was placed hidden beneath the water surface. The water temperature was kept at 20±1 °C during the whole testing period. The pool was surrounded by numerous visual cues as explained in previous articles (Méndez-Couz et al., Mendez-Couz et al., 2014). Each trial was recorded and later analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

1.3.1. Habituation

During habituation in the Morris water maze, rats received two sessions spaced 1 h apart. The maze was virtually divided into four equal quadrants (A, B, C, and D) and the escape platform was set in the center of the quadrant D, 2 cm above the surface of the water during the habituation phase, so that it was visible for the animals. In each session the rats were released facing the pool walls from the central part of each virtual quadrant following a pseudorandom sequence, four times each session. Rats were allowed to swim up to 60 s to locate the platform in each trial, or guided to it after that. They were remained there for 15 s and then they rest in a black plastic bucket during 15 s until the next trial. Animals were returned to their home cages between sessions.

1.3.2. Reference memory task

Animals were trained during five consecutive days in a hidden platform task and tested for retention test immediately after the last acquisition trial. The spatial memory task was performed between 09:30 and 13:00 h.

During the acquisition phase, animals received a daily session of four trials, in which they were released from the central border of each of the quadrants in a pseudorandom order to search for a

hidden escape platform beneath the water surface (1.5 cm). The platform was located in the same quadrant (escape quadrant, D) along the acquisition procedure, and rats were required to find it using spatial cues available in the room following training. Rats were allowed to swim during 60 s to reach the platform or gently guided to it after that time; they were 15 s on the platform and then they rested during 30 s in the aforementioned plastic bucket within trials.

1.3.3.Retention probe

After the last training day, rats were submitted to a retention test in a single probe trial. During this probe, the platform was removed from the maze, and rats were released from the contra lateral quadrant. They were allowed to swim during 60s. After this period, animals were placed again in the bucket. In order to prevent premature extinction of the previously learned task, all animals received an additional trial in which the platform was available again in its original place. In this last trial, all animals were released from the quadrant B.

Even though the retention probe trial lasted 60 seconds, only the first 30 were included into the analysis. It was observed that the majority of animals tended initially to swim in the correct quadrant, but then quickly started to search in the rest when they failed to locate the absent platform (Spooner et al., 1994, Conejo et al., 2007). For this reason, the inclusion of the entire period could lead to a misinterpretation of the data.

After finishing the training procedure they were randomly divided into the two different groups Muscimol (MuH, n=8) and Saline (SaH, n=8).

1.3.4.Extinction protocol

The following day, rats were bilaterally infused in the dorsal hippocampus with 0.5 µl of 1µg/µl muscimol dissolved in 0.9% saline (Muscimol group) or only with 0.9% saline in case of the Saline group. After 30 min post-infusion they were submitted to 4 extinction sessions in the water maze.

Following the extinction protocol by (Rossato et al., 2006) and previously explained in (Mendez-Couz et al., 2014) animals had four extinction sessions the day after the learning retention probe.

Each session consisted in four single non-reinforced trials in which no platform was available in the maze, of 60 s each, making a total of 16 trials. The resting period an in-between trial lasted 30 s in the aforementioned black plastic bucket and the inter-session interval was 30 min that took place in the home cage.

1.4. CYTOCHROME OXIDASE HISTOCHEMISTRY

Ninety minutes after finishing the behavioral tasks, all animals were decapitated. Their brains were quickly removed then frozen in isopentane at −70 °C (Sigma–Aldrich, Madrid, Spain) and stored at −40 °C to preserve the brain tissue and enzyme activity. Brains were subsequently cut at 30 μm-thick coronal sections using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). These sections were mounted on slides and stored at −40 °C until processing with quantitative CO histochemistry. One of the Saline group animals' brain and some sections from a few subjects of all of the groups could not be used as a result of tissue processing, although the final number of sections available for histochemistry per group was equal to seven or greater than the former in all cases.

A modified version of the method based on the quantitative CO histochemical method developed by Gonzalez-Lima and Jones (Gonzalez-Lima and Jones, 1994), was used. Staining variability across different baths was controlled by sets of tissue standards. These standards were obtained from Wistar rat brain homogenates of known CO activity determined spectrophotometrically at different thicknesses (10, 30, 50 and 70 μm). Following the previously described protocol by Conejo et al. (2013), the standards were included with each bath of slides. Each set of slides were fixed for 5 min with a 0.5% glutaraldehyde solution, rinsed three times in phosphate buffer and preincubated 5 min in a solution containing 0.05 M Tris buffer pH 7.6 with 275 mg/l cobalt chloride 10% (w/v) sucrose and 5 ml dimethylsulfoxide. After the sections had been rinsed in phosphate buffer (pH 7.6; 0.1 M) they were incubated at 37 °C for 1 h in the dark and with continuous stirring in a solution containing 50 mg 3,3′-diaminobenzidine, 15 mg cytochrome c (Sigma, St. Louis, MO, USA) and 4 g sucrose per 100 ml phosphate buffer (pH 7.4; 0.1 M). The reaction was stopped by fixing the tissue in buffered formalin (10% w/v sucrose and 4% formaline) for 30 min at room temperature. After being fixed the slides were dehydrated, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

CO histochemical staining intensity was measured by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) which includes specific image analysis software. Four measurements of relative optical density in three consecutive sections were done per region, i.e. twelve in total. In order to establish comparisons and consider possible staining variations across brain sections from different staining baths, measurements were also taken from CO-stained brain homogenate standards. Regression curves between section thickness and known CO activity, previously measured by spectrophotometric assay in each set of standards, were calculated. Lastly, average relative optical density measured in each brain region was converted into CO activity units (1 unit: 1 μmol of cytochrome c oxidized/min/g tissue wet weight at 23 °C) using the previously calculated regression curve in each homogenate standard. The averaged measure per region was carried out for each region and animal. Those included prelimbic (PL), and infralimbic cortex (IL) of the medial prefrontal cortex and primary motor cortex (M1), all of them measured at $^{+}/_{-}$ 3.70 mm from Bregma; granular (RSG) and agranular (RSA) retrosplenial cortices at $^{+}/_{-}$ -4.52 mm; parietal (PAR) $^{+}/_{-}$ -3.80 mm, entorhinal (Ent), and perirhinal (PRh) cortices at $^{+}/_{-}$ -4.52 mm. In addition, the following subcortical regions were also taken: medio dorsal (MD) anterodorsal (AD) and anteroventral (AV) thalamic nuclei, measured at $^{+}/_{-}$ -1.40 mm; lateral (LS) and medial septum (MS) at $^{+}/_{-}$ 0.20 mm, nucleus accumbens core (AcC) and shell (AcSh) measured at $^{+}/_{-}$ 1.00 mm; dorsal fields of hippocampus including CA1, CA3 and dentate gyrus of the dorsal (CA1d, CA3d, DGd fields) at $^{+}/_{-}$ -3.30 mm; and ventral hippocampus (CA1v, CA3v, DGv fields), taken at $^{+}/_{-}$ -4.52 mm from Bregma. Medial (MeA), basal (BaA), lateral (LaA) and central (CeA) amygdaloid nuclei were measured at $^{+}/_{-}$ -3.14 mm from Bregma; medial (MM), lateral (LM) and supramammillary nucleus (SuM) of the mammillary bodies measured at $^{+}/_{-}$ -4.52 mm, as well as the premammillary nucleus (PM) taken at $^{+}/_{-}$ -4.16 mm. The selected brains regions anatomically were defined according to Paxinos and Watson (Paxinos and Watson, 2004).

1.5. DATA ANALYSIS

1.5.1. Behavioral data

The behavioral data were analyzed using SAS 9.4 PROC MIXED (SAS Institute Inc, USA).

Repeated measures one-way ANOVA test was used to test the latencies across adquisition sessions. The Holm-Sidak method was applied as post-hoc test.

Also, one-way ANOVA test was used to test the quadrant preference during the retention memory test carried out immediately after the last learning session. In this case, the Holm-Sidak method was applied as post-hoc test.

One way repeated Measures ANOVA was carried out independently for each experimental group using the escape quadrant across the four extinction sessions as factor.

After the drug or saline infusion the extinction progression was analyzed separately by the time spent in the escape quadrant per session along the four extinction sessions with two-way repeated measures ANOVA with group and sessions as factors.

1.5.2. CO activity

The averaged CO activity differences per region between both groups were analyzed using Student's *t* tests.

2. RESULTS

2.1. BEHAVIORAL DATA

No animals were discarded for motor or sensory deficits previous or due to the surgical procedure.

2.1.1. Latencies and Retention memory test

Animals of both groups decreased the spent time to reach the platform along the reference memory training. MMRM analysis results revealed statistically significant differences ($p<0.001$) for the training-days factor; Therefore, rat performance changed over time. According to the Hochberg's step-up procedure, all possible pairwise comparisons were statistically significant at the 5% family-wise significance level, with the exception of the comparisons between the 2^{nd}-4^{th} days and day 3 to day 4.

Results showed also differences in the amount of time spent among the four different quadrants that virtually divided the pool along the retention probe carried out the last day during the acquisition reference memory ($F_{3,45}=23.78$; $p<0.001$). Animals spent significantly more time swimming in the formerly reinforced quadrant as compared with the others ($p<0.001$). (See Figure 1)

2.1.2. Extinction procedure

Only the saline group successfully extinguished the formerly acquired hidden platform task, as the two-way ANOVA showed. Interaction between session and quadrant was found ($F_{3,33}$=5.49; p=0.04) during the extinction procedure, so main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor. Holm-Sidak test showed that SaH animals spent significant different time during the first session of the extinction procedure as compared to the rest of the sessions (p<0.01) and between session 2 as compared with the last session. (Figure 2)

3. DISCUSION

Currently, little is known about the neuronal basis underlying the extinction of a previously acquired spatial memory. Precious studies indicate that the brain mechanisms involved in spatial memory acquisition and consolidation may also be involved in the progress of spatial memory extinction. In this context, the extended hippocampal system and particularly, the dorsal hippocampus, is believed to play a key role in the acquisition of a new spatial memory task. Is we take into account that the extinction learning may be understood as a new learning, involving both inhibitory learning as well as new learning to solve the changed circumstances, this structure may be related to the acquisition of a new spatial extinction learning task.

The aim of the present research was, therefore, to analyze the effect of a temporal CA1 of the dorsal hippocampus bilateral inactivation in the extinction of a previously acquired reference spatial memory in the Morris water maze. Additionally, brain structures metabolic activity was analyze to asses brain activity changes related to this inactivation.

CONCLUSIONS

Inactivation of dorsal hippocampus alters spatial memory extinction task, although we cannot rule out the possibility of the necessary retrieval process disruption interference. Additionally, associated brain metabolic activity changes were observed both in cortical regions, specifically prelimbic, infralimbic and cingulate areas of the prefrontal cortex, the perirhinal cortex, and limbic structures as the accumbens nucleus. The present study adds to a growing literature supporting the

dorsal hippocampus CA1 role on spatial memory processes, and specifically highlights its importance at the extinction phase. Nevertheless, further pharmacological inactivation studies need to be accomplished to discard possible retrieval impairments accounting for the spatial extinction memory disruption observed.

ACKNOWLEDGEMENTS

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FIGURES

Figure 1: Mean time spent (±S.E.M.) in each virtual quadrant during the retention memory test performed right after the last acquisition session. There were differences between the scape quadrant (D) and the rest of the quadrants ($p<0.001$).

Figure 2: There were significant differences * ($p<0.01$) in the mean time spent in the formerly reinforced quadrant along extinction sessions during the first 30s of each extinction session. Post hoc Holm-Sidak tests showed differences between the first session and the rest of them in the saline group, ($p<0.01$) as well as between session 2 and 4 $^{+}$ ($p<0.01$) and among the third and last session ($p<0.05$). However, no differences were found in the Muscimol group in between sessions.

Figure 3: Mean CO values in regional brain cytochrome oxidase activity. Abbreviations: 3A) PL: prelimbic area, IL: infralimbic area and Cing: Cingulate area of the prefrontal cortex, Perh: Perirhinal cortex; Ent: Entorhinal cortex. 3B) DGd : Dentate Gyrus, CA1d: Cornu Ammonis 1 and CA3d: Cornu Ammonis 3 of the dorsal hippocampus, AcC: Accumbens Core nucleus; AcSh: Accumbens Shell part.

Figure 1

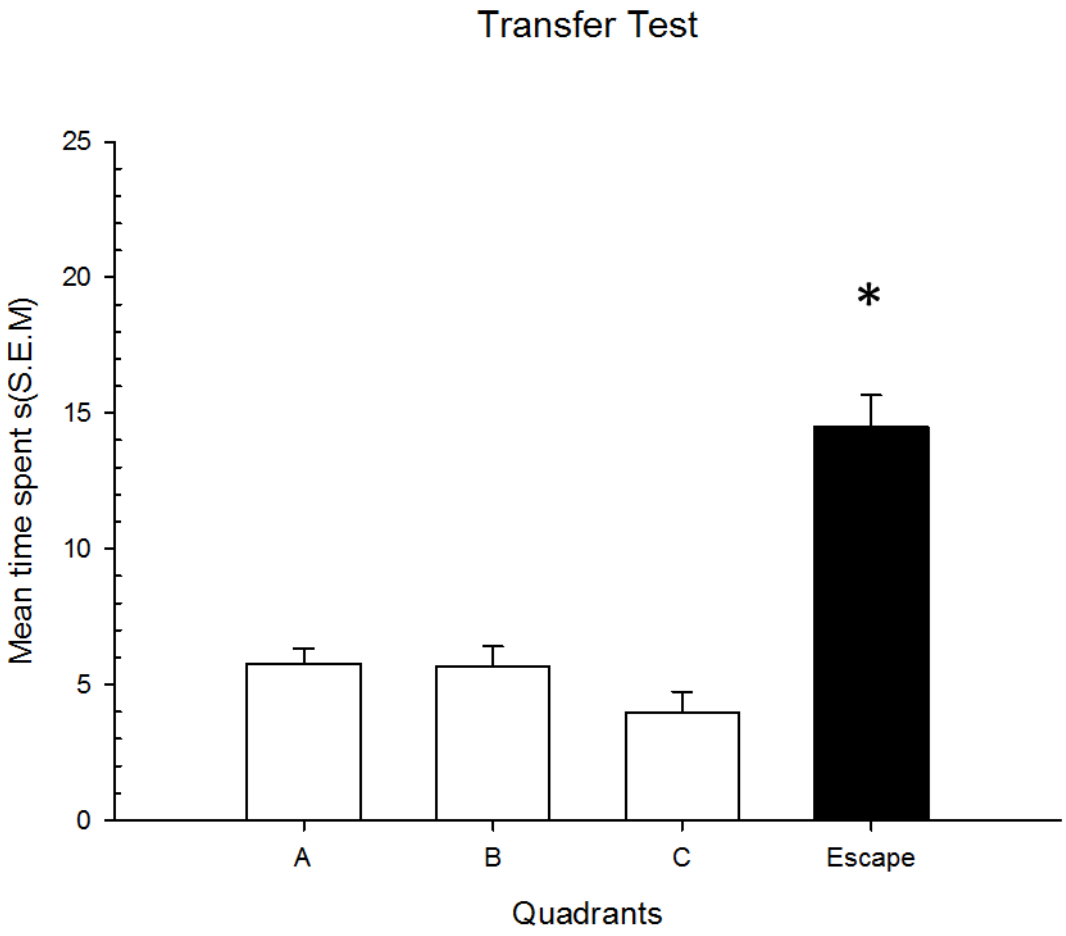
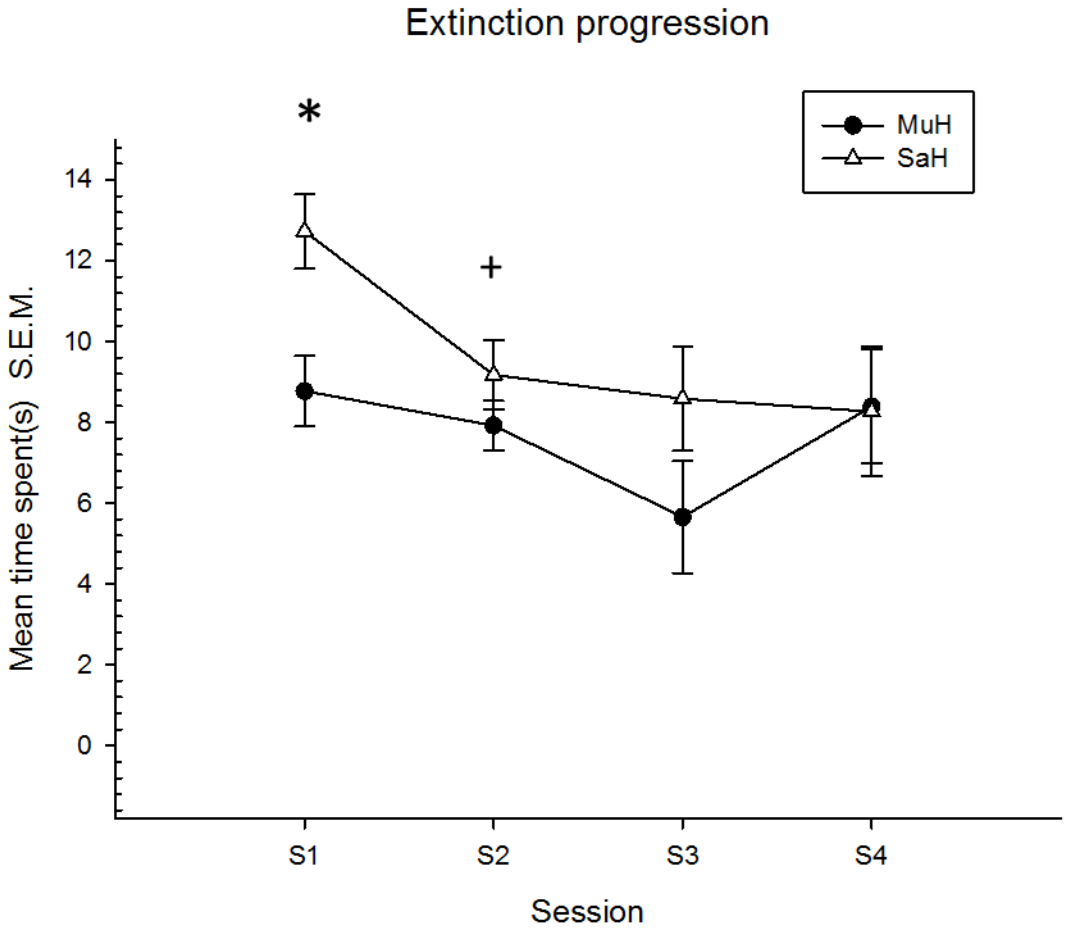
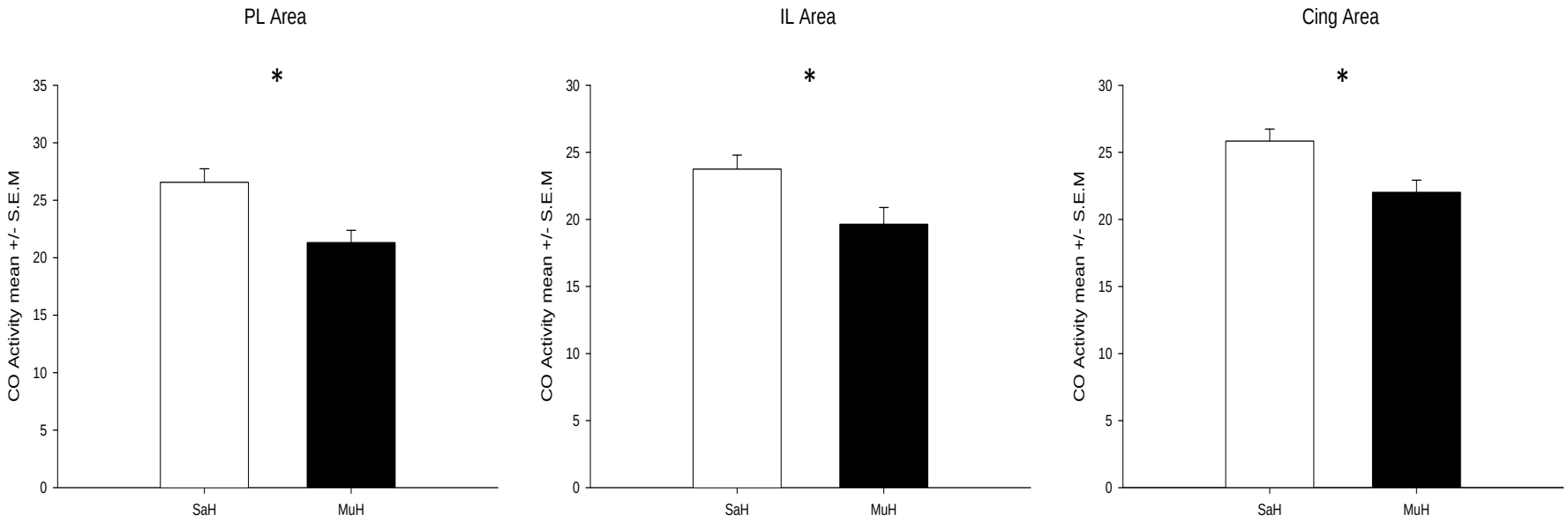
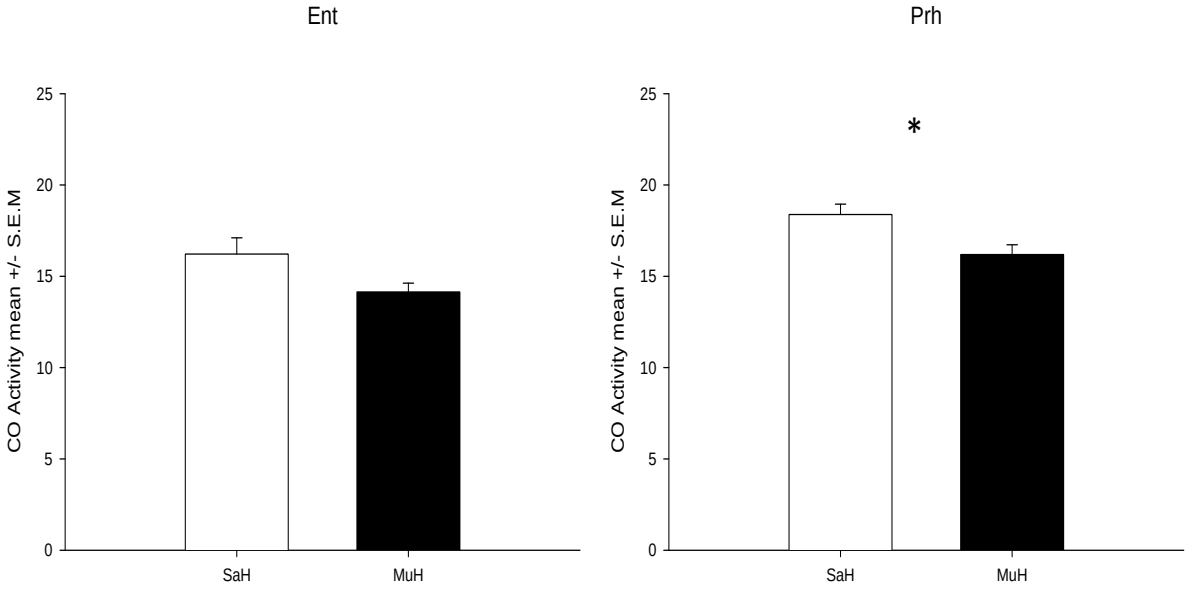
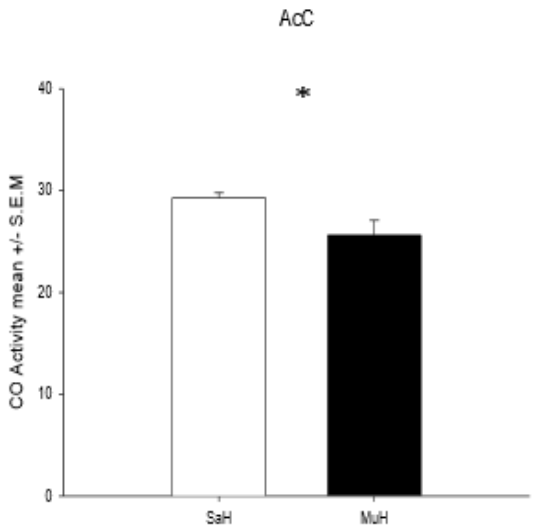

Transfer Test
Mean time spent s(S.E.M)
25
20
15
10
5
0
*
A
B
C
Escape
Quadrants

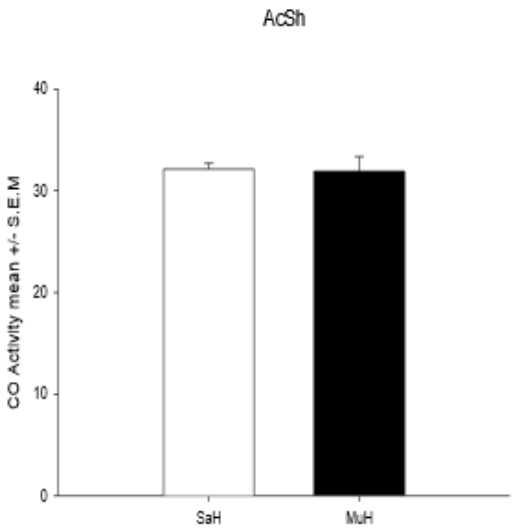
Figure 2

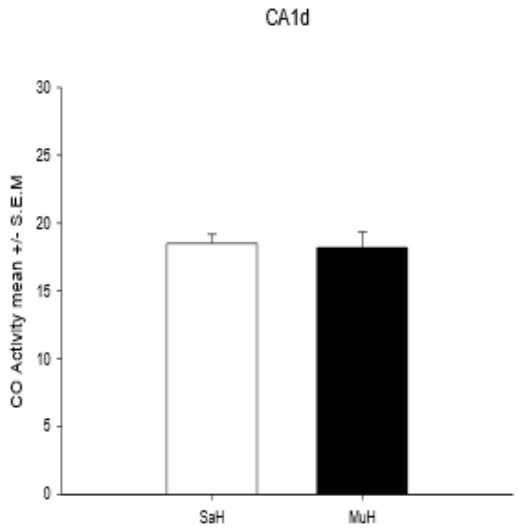

Extinction progression
MuH
SaH
*
+
Mean time spent(s) S.E.M.
0
2
4
6
8
10
12
14
S1
S2
S3
S4
Session

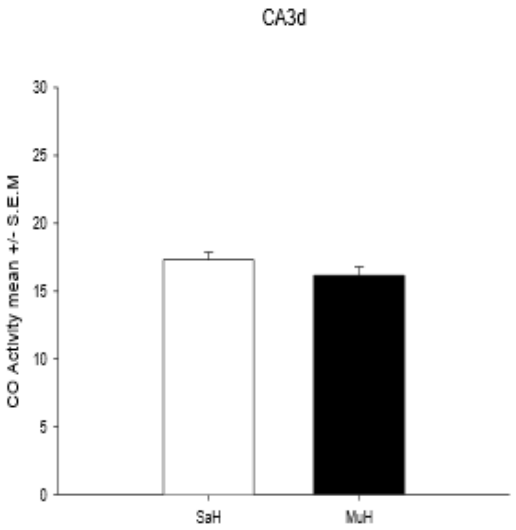

PL Area
IL Area
Cing Area
CO Activity mean +/- S.E.M
SaH
MuH
*

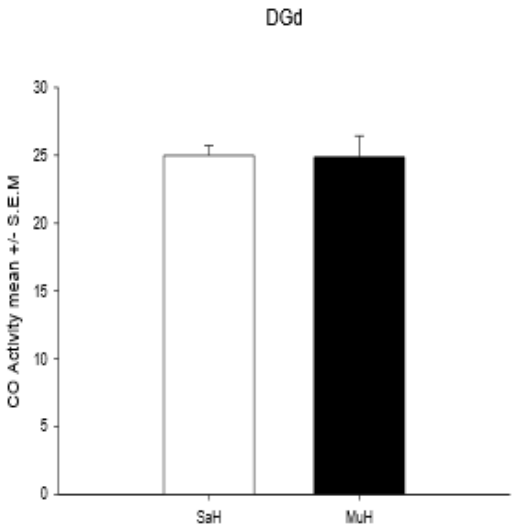

Ent
Prh
CO Activity mean +/- S.E.M
SaH
MuH
*


AcC
*
CO Activity mean +/- S.E.M
40
30
20
10
0
SaH
MuH


AcSh
CO Activity mean +/- S.E.M
40
30
20
10
0
SaH
MuH


CA1d
CO Activity mean +/- S.E.M
30
25
20
15
10
5
0
SaH
MuH


CA3d
CO Activity mean +/- S.E.M
30
25
20
15
10
5
0
SaH
MuH


DGd
CO Activity mean +/- S.E.M
30
25
20
15
10
5
0
SaH
MuH


196

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Artículo 8


Sausalito
Yellow Bluff
Pt. Diablo
GOLDEN GATE
Fort Pt.
Presidio
Black Pt.
South Bay
SAN FRANCI

MODULATION OF SPATIAL MEMORY INDUCED BY CHANGES IN NPY SYSTEM AT REGIONAL RECEBRAL LEVELS.

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KEY WORDS: NPY Y2R antagonist, hippocampus, prefrontal cortex, Morris ater maze, NPY Y1R, Citochrome Oxidase,

ABSTRACT

1. INTRODUCTION

Neuropeptide Y (NPY) was isolated and characterized three decades ago (Tatemoto, 1982). This neuropeptide is widely distributed in the central nervous system (CNS) and it has been associated with many functions including food intake (Stanley and Leibowitz, 1984), cognitive processes (Thorsell et al., 2000), regulation of stress and anxiety responses (see Heilig (2004) for review), mood and neuroprotection (Silva et al., 2005, Malva et al., 2012), and drug addiction (Goncalves et al., 2015)

However, the role of NPY as memory modulator is still controversial. Previous research showed that NPY administration to rodent CNS enhances memory, as shown by passive avoidance tests in mice (Flood et al., 1987). Additionally, some studies report that the NPY system participates in cognitive processes either improving or impairing short- and long-term memory, being these effects both dose- and brain region-specific (Thomas and Ahlers, 1991).

NPY receptors have been described to date (Y_1 to Y_6) in rodents. The NPY exerts complex effects in the CNS mainly through Y_1, Y_2, and Y_5 receptors (Xapelli et al., 2006). Specifically, Y_1 mRNA can be found in hippocampus, amygdala, thalamus and cerebral cortex. Meanwhile, Y_2 receptor is present at high levels in brain areas considered essential for memory processes, such as the hippocampus, the amygdala, and the hypothalamus (Parker and Herzog, 1999).

Accordingly, a wide body of evidence supports a central role of the hippocampus or diencephalic nuclei in spatial memory processes, both in humans and rodents (Aggleton and Brown, 1999, Warburton et al., 2001, Conejo et al., 2007, Fidalgo et al., 2012, Conejo et al., 2013, Dumont et al., 2014, Mendez-Couz et al., 2014). Moreover, recent studies have demonstrated that dynamic time-dependent brain networks underlie the acquisition and consolidation of spatial memory, mainly including diencephalic structures as well as the well-known prefrontal-hippocampal circuits (Conejo et al., 2007, Wang and Cai, 2008, Conejo et al., 2010, Miyoshi et al., 2012, Andre et al., 2014, Fidalgo et al., 2014, Mendez-Couz et al., 2015a, Mendez-Couz et al., 2015b) .

Regarding specific effects of the NPY system on learning and memory, the scarce existing literature suggests the involvement of NPY in neuronal activity, cognitive processes and spatial memory

(Thorsell et al., 2000, dos Santos et al., 2013, Botterill et al., 2014). Showing neuroprotective effects against methamphetamine-induced changes in the hippocampus (Goncalves et al., 2012), hippocampal NPY and Y_2R plasticity related to conditioned fear impairment in epileptic models (Botterill et al., 2014) or neuroprotective effects mediated by Y_2R against oxidative stress that prevents depressive behaviour and spatial deficits in mice (dos Santos et al., 2013).

However, the specific role of NPY on spatial memory, including its temporal and brain region-specific involvement on this process remains unknown. Therefore, in the current study we evaluated the effects of serial intracerebral administrations of NPY Y_2 receptor antagonist BIIE0246 on the dorsal CA1 hippocampus in rats, and its effects on anxiety, spontaneous horizontal activity and spatial memory tasks. Moreover, molecular analysis was carried out to elucidate brain regional changes in NPY, Y_1R and Y_2R expression after completion of the behavioural tasks, as well possible task-related changes in regional brain metabolic activity. Overall, our findings demonstrate the NPY system involvement on spatial memory performance, as shown by changes in NPY levels and Y_1 and Y_2 receptor expression in the hippocampus and the prefrontal cortex and regional brain metabolism following Y_2R antagonist infusion in the dorsal hippocampus.

2. MATERIAL AND METHODS

2.1. ANIMALS

Male adult Wistar rats (*Rattus norvegicus)* weighing between 250-330g were used (43 total). Animals were obtained from the University of Seville central animal facilities (Seville, Spain) and housed in a temperature controlled-room (23±2ºC). Lighting was kept on a 12-h light/dark cycle with lights on from 08:00–20:00 h. Water and food were available *ad libitum* throughout the experimental period. All experimental procedures carried out with animals were approved by a local Animal Ethics Committee of the University of Oviedo and following the European Communities Council Directive 2010/63/UE and the Spanish legislation on care and use of animals for experimentation (RD 53/2013). All efforts were made to minimize the number of animals used and their suffering.

2.2. BEHAVIOURAL PROCEDURE

Animals were first tested in a neurological assessment battery in order to discard possible motor and sensory deficits. The neurological tests used allowed us to evaluate the following reflexes: abduction response of hind limbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response and righting reflex (Bures et al., 1976). Rats were handled daily during 5 day prior surgery, to reduce anxiety-like behavior related with experimental manipulation. See timeline of the experiment in figure 1.

2.3. SURGERY

Rats were deeply anaeastesized with xylazine (5 mg/kg, i.m.) and ketamine (80-100 mg/kg, i.p.) and placed in a David Kopf (Tujunga, CA) or Narishige (Japan) stereotaxic frame. Stainless steel cannulae (inner diameter 22G) (Becton Dickinson S.A., Spain) were stereotaxically implanted bilaterally in the CA1 region of the dorsal hippocampus (coordinates from bregma: AP -3.6, L ±2.6, DV -2.1 mm). Cannulae were fixed to the skull using dental acrylic cement (Glaslonomer Cement, Shofu Inc., UK) and anchor screws. Animals were allowed to recover after surgery during 5 days.

After this resting period, animals underwent the same neurological assessment battery explained above to discard any possible abnormality caused by the stereotaxic procedure.

2.4. BEHAVIOURAL TESTS

Rats received a bilateral injection through the permanent set cannula 30 min before each test, and were divided in two groups as follows: experimental group received 1 nmol/µl BIIE0246 in 0.9% saline serum (1 µl / hemisphere); and the control group was administered with the vehicle (0.9% saline serum). Liquid was infused at 0.5 µl/ min and an additional minute was given before removing the microcannula to prevent fluid to back up into it.

2.4.1. SPONTANEOUS HORIZONTAL ACTIVITY

We started by evaluating the rat spontaneous horizontal activity in actimeters. Each one consisted in a closed acrylic transparent cage that incorporated a recording camera on the top (Noldus PhenoTyper, The Netherlands). During each session, automatic recording of distance travelled was obtained using a video-tracking analysis software (EthoVision XT, Noldus, Wageningen, The Netherlands). Rats were transported to the new room in individual cages. Once there, they were placed into the actimeters, and allowed to freely explore the new environment during a habituation phase (5 min). The horizontal activity was measured during 30 min and analyzed every five minutes. The cages were cleaned between rats with 70% ethanol to remove any possible odor cue.

2.4.2. ELEVATED ZERO MAZE

Two days after the activity test rats were submitted to an elevated zero-maze test. As above mentioned, animals were infused in batches with the drug or vehicle and carried separately to the procedure room. The zero-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 also divided into four sections of equal lengths, two open sections and two closed sections with black acrylic walls 35 cm in height. Rats were taken from the animal facilities in individual cages and left for 5 min before starting the procedure as an habituation to the new environment. After this period they were placed in the center of the open arm under the same lighting conditions as before and their movements were recorded for 5 min. After finishing each session the maze was cleaned with 70% ethanol to remove any possible odor cues. Variables measured included total distance run and time in open arms. Rats movements were recorded with a camera connected to a computer running a video-tracking software (EthoVision XT; Noldus Information Technology, The Netherlands).

2.4.3.MORRIS WATER MAZE

Two days after finishing the Zero maze test, rats started the reference spatial memory task in the Morris water maze. The maze was a circular water tank made of black fiberglass, measuring 1.5 m in diameter by 75 cm in height, (Morris, 1984) . The pool was filled with tap water and an escape platform was placed hidden beneath the water surface. The water temperature was kept at 20±1 °C during the entire training period. The pool was surrounded by numerous visual cues such as coloured boxes, patterns and an air balloon fixed in three black panels surrounding the pool. Additionally, the room was illuminated by two halogen spotlights facing the walls. Each trial was recorded and later analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands). Variables measured included the mean time spent to reach the platform (latencies) and time spent in each of the four virtual quadrants in which the pool was divided (A, B ,C and D).

Habituation phase

During the first stage, rats received one habituation session of four trials, in which the rats were released facing the pool walls from the central part of each quadrant following a pseudo-random sequence. An escape platform was set in the center of the water maze, 2 cm above the surface of the water, so that it was visible for the animals. Rats were allowed to swim up to 60 s to locate the platform in each trial, or guided to it after that. The animals were left in the platform for 15 s, followed by a rest period of 5 s in a black plastic bucket until the next trial.

Training period

Rats were trained during two consecutive days in a hidden platform task and tested for retention test immediately after the last acquisition trial each day.

During the first acquisition day, just after the habituation session, rats received 4 training sessions of four trials each, in which they were released from the central border of each of the quadrants in a pseudorandom order to search for a hidden escape platform beneath the water surface (1.5 cm). The platform was kept in the same quadrant (escape quadrant, C) along the acquisition procedure and rats were required to find it using spatial cues available in the room following training. Rats were allowed to swim during 60 s to reach the platform or gently guided to it after that time; they were 15

s on the platform and then they rested during 5 s in the aforementioned plastic bucket within trials. Once the acquisition sessions were finished animals underwent a retention probe test.

This training procedure was repeated on the following day until reaching the learning criterion of 20 s latency to reach the platform. For that purpose animals underwent three sessions of four trials each in the second day, also followed by a retention probe.

Retention probe

After the last trial in each day, rats were submitted to a retention test in a single probe trial. During this probe, the platform was removed from the maze, and rats were released from the contra lateral quadrant. They were allowed to swim during 60 s. After this period, animals were placed again in the bucket. In order to prevent extinction of the previously learned task, all animals received an additional trial in which the platform was available again in its original place. In this last trial, all animals were released from the quadrant B.

Test

Rats received the last drug or saline infusion in the same conditions as above mentioned at 24 h after finishing MWM training. Then, 30 min after the infusion they followed the same MWM protocol but in this case only one session of four trials were given to the animals. A last retention probe was given to the animals in the same condition as mentioned before.

2.5. MOLECULAR ANALYSIS

2.5.1. WESTERN BLOT

Some rats were sacrificed by decapitation immediately after the last MWM procedure, brains were removed and rapidly frozen in isopentane at −70 °C (Sigma–Aldrich, Madrid, Spain) and stored at −80 °C. Brains were then defrosted and the hippocampus, striata, prefrontal cortex, and cortices were collected. Afterwards, brain regions were homogenized in a lysis buffer supplemented with a protease inhibitor cocktail. Protein concentrations were determined using the BCA assay kit (Pierce,

Rockford, IL, USA), and protein samples (25 to 60 μg) were separated onto sodium dodecyl sulfate–polyacrylamidegel electrophoresis (SDS-PAGE), before being transferred onto polyvinylidene difluoride membrane (PVDF; Millipore, Madrid, Spain). After blocking, membranes were incubated overnight at 4°C, with the following primary antibodies: NPY Y_1 receptor (1:1000, AbD Serotec, Oxfordshire, UK), NPY Y_2 receptor (1:200, Alomone Labs, Jerusalem, Israel). Membranes were then incubated with secondary antibodies, at room temperature (RT) during 1 h, and immunoreactive bands were visualized by enhanced chemofluorescent detection (ECF kit, Amersham), and visualized on the Typhoon 9000 system (GE Healthcare Europe GmbH).

The blots were reprobed with an antibody against β-Actin, which was used as loading control (1:2000; Sigma-Aldrich, St Louis, MO, USA). The immunoblots were analyzed with ImageJ Software (NIH, Bethesda, MD, USA) to measure the optical density of the bands.

An unpaired Student's *t* test was used to compare saline and BII0246 treated rats for each receptor (Y_1 or Y_2) in each region. A non-parametric Mann–Whitney U Statistic was used to analyze these values when normality or equal variances failed. A p value ≤ 0.05 was considered as statistically significant. Data were analyzed using *SigmaStat* 3.5 (Systat Software, Chicago, USA).

2.5.2.NPY INMUNOHISTOCHEMISTRY

Animals were anesthetized with sodium pentobarbital (Sigma-Aldrich) immediately after the last MWM procedure, and intracardially perfused with phosphate-buffered saline (PBS) (10 mL) followed by 4% paraformaldehyde (20 mL). Brains were removed and post-fixed in the same solution, followed by immersion in 20% sucrose. Afterward, coronal sections were cut (30 μm) along its anterior–posterior axis and were mounted directly onto gelatine-coated glass slides until further use. Double-labeling immunofluorescence was performed for NPY and neuron-specific class III beta-tubulin (Tuj-1). Slices were blocked with 10% fetal bovine serum (FBS)/0.5% Triton X-100 in PBS, incubated with polyclonal anti-NPY [1:200; Invitrogen) for 90 min at RT, stained with Hoechst 33342 (Sigma-Aldrich) and mounted in Dako fluorescence medium (Dako North America, Carpinteria, USA). The fluorescent images were recorded using a LSM 710 Meta Confocal microscope (Carl Zeiss, Oberkochen, Germany).

NPY mean absorbance quantification for each experimental group was assessed with ImageJ software. Specifically, 5 equal square samples per slice, obtained from at least two independent slices, were used to evaluate immunoreactivity.

Again, unpaired Student's *t* tests were performed to compare saline and BII0246 treated rats for NPY in each region. Non-parametric Mann–Whitney U tests were used to analyze these values when normality or equal variances failed. A p value less than 0.05 was considered statistically significant. Data has been analyzed with Sigma-Stat 3.5 (Systat Software, Chicago, USA.

2.5.3. **CYTOCHROME OXIDASE HYSTOCHEMISTRY**

Another set of animals were sacrificed 90 min after finishing the behavioral tasks, brains were quickly removed then frozen in isopentane at −70 °C (Sigma–Aldrich, Madrid, Spain) and stored at −40 °C to preserve the brain tissue and enzyme activity. Brains were subsequently cut at 30 μm-thick coronal sections using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). These sections were mounted on slides and stored at −40 °C until processing with quantitative CO histochemistry.

A modified version of the method based on the quantitative CO histochemical method developed by Gonzalez-Lima and Jones (Gonzalez-Lima and Jones, 1994), was used. Staining variability across different baths was controlled by sets of tissue standards. These standards were obtained from Wistar rat brain homogenates of known CO activity, that were determined spectrophotometrically at different thicknesses (10, 30, 50 and 70 μm). Following the previously described protocol by Conejo et al. (2013), the standards were included with each bath of slides. Each set of slides were fixed for 5 min with a 0.5% glutaraldehyde solution, rinsed three times in phosphate buffer and preincubated 5 min in a solution containing 0.05 M Tris buffer pH 7.6 with 275 mg/l cobalt chloride 10% (w/v) sucrose and 5 mL dimethylsulfoxide. After the sections had been rinsed in phosphate buffer (pH 7.6; 0.1 M) they were incubated at 37 °C for 1 h in the dark and with continuous stirring in a solution containing 50 mg 3,3′-diaminobenzidine, 15 mg cytochrome c (Sigma, St. Louis, MO, USA) and 4 g sucrose per 100 ml phosphate buffer (pH 7.4; 0.1 M). The reaction was stopped by fixing the tissue in buffered formalin (10% w/v sucrose and 4% formaline) for 30 min at RT. After being fixed the slides were dehydrated, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

CO histochemical staining intensity was measured by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) which

includes specific image analysis software. Four measurements in three following sections of relative optical density were taken per region, that is to say, twelve in total. To establish comparisons and consider possible staining variations across brain sections from different staining baths, measurements were also taken from CO-stained brain homogenate standards. Regression curves between section thickness and known CO activity, previously measured by spectrophotometric assay in each set of standards, were calculated. Lastly, average relative optical density measured in each brain region was converted into CO activity units (1 unit: 1 μmol of cytochrome c oxidized/min/g tissue wet weight at 23 °C) using the previously calculated regression curve in each homogenate standard. The averaged measure per region was carried out for each region and animal. Those included prelimbic (PL), and infralimbic cortex (IL) of the medial prefrontal cortex and primary motor cortex (M1), all of them meassured $^{+}/_{-}$ 3.70 mm from Bregma; granular (RSG) and agranular (RSA) retrosplenial cortices at $^{+}/_{-}$ -4.52 mm; parietal (PAR) $^{+}/_{-}$ -3.80 mm, entorhinal (Ent), and perirhinal (PRh) cortices at $^{+}/_{-}$ -4.52 mm. In addition, the following subcortical regions were also taken: mediodorsal (MD) anterodorsal (AD) and anteroventral (AV) thalamic nuclei, meassured at $^{+}/_{-}$ -1.40 mm; lateral (LS) and medial septum (MS) at $^{+}/_{-}$ 0.20 mm, nucleus accumbens core (AcC) and shell (AcSh), measured at $^{+}/_{-}$ 1.00 mm; dorsal fields of hippocampus including CA1, CA3 and dentate gyrus of the dorsal (CA1d, CA3d, DGd fields) at $^{+}/_{-}$ -3.30 mm; and ventral hippocampus (CA1v, CA3v, DGv fields), taken at $^{+}/_{-}$ -4.52 mm from Bregma. Medial (MeA), basal (BaA), lateral (LaA) and central (CeA) amygdaloid nucleus all of them measured at $^{+}/_{-}$ -3.14 mm from Bregma; medial (MM), lateral (LM) and supramammillary nucleus (SuM) of the mammillary bodies measured at $^{+}/_{-}$ -4.52 mm, as well as the premammillary nucleus (PM) taken at $^{+}/_{-}$ -4.16 mm. The selected brains regions anatomically were defined according to Paxinos and Watson (Paxinos and Watson, 2004).

3. RESULTS

3.1. BEHAVIOURAL TESTS

No animals were discarded due to their neurological reflexes responses after the surgical procedure.

3.1.1. ESPONTANEOUS HORIZONTAL ACTIVITY.

No differences between groups were found in the spontaneous locomotor activity when measured as the total distance moved, as shown in figure 3

3.1.2. ELEVATED ZERO MAZE

No differences were found in the total distance moved and time spent in open sectors between groups as shown in figure 4.

3.1.3. MORRIS WATER MAZE

Results showed that animals of both groups learned the spatial memory task, as shown by the decreased latencies necessary to reach the platform along training sessions. The two ways repeated measures ANOVA showed no differences between groups along the training sessions, and no interactions between the group and the session presented, however, differences in session were found $F_{(6,239)} = 23.08$; ($p< 0.01$). The Holm-Sidak multiple comparison procedure showed differences between Session 1 and Sessions 3 to 7, ($p<0.01$), as well as between Sessions 2 and 6, and sessions 2 and 7 ($p<0.01$), also between sessions 3 and 6, and between 3 and 7 ($p<0.01$); similarly, differences were found between session 4 and 6, and between 4 and 7,($p<0.01$) , finally, session 5 was significantly different of session 6, and also of session 7, ($p<0.01$) . See figure 5.

Mean time spent in the previously reinforced quadrant in the retention probes carried out after the first and second day of training showed that both groups learned the task by the end of the second day (session 7), so that both in the saline and the experimental group differences were found between the reinforced quadrant as compared to rest of the quadrants ($p<0.01$). See figure 6.

Once the rats were administrated the saline vehicle or the drug for the third time results showed that there was not a statistically significant interaction between Group and Session, but there were significant differences between groups $F_{(1,40)}=6.66$ ($p<0.05$), so that the saline group presented

bigger latencies to reach the platform as compared to the treated group. There was also a significant difference was found between sessions $F_{(3,120)}$= 8.34; (p<0.01). The Holm-Sidak multiple comparisons procedure showed differences between the first trial as compared to the rest of them. (p<0.01). figure 5.

As also shown in figure 6, mean time in the reinforced quadrant in the test day showed that saline group spent more time in the reinforced quadrant as compared to A and D, and the experimental group showed differences between time spent in the reinforced quadrant and the rest of them (p<0.05*).*

3.2. MOLECULAR TESTS

3.2.1. WESTERN BLOT QUANTIFICATION

Differences were found between groups in the receptor Y_2 expression in the hippocampus, finding and increased value in the treated group as compared with the saline one (p<0.05), as well as a decrease in the prefrontal cortex (p<0.05).

No differences were found in the Y1 expression in the hippocampus, striatum or rest of the cortices, but an increased expression was found in the prefrontal cortex (p<0.05). See figure 7.

3.2.2. NPY IMMUNOHISTOCHEMISCTRY QUANTIFICATION

NPY Quantification showed differences between groups in dorsal CA1, t_{18} = -11.21 (p<0.01), dorsal CA3 t_{18} = 5,08 (p<0.01), dorsal DG t_{18} = 3,42 (p<0.05), t_{28} = -8.90 (p<0.01) and Striatum (Mann-Whitney) (p<0.01), finding a decreased value in all of them respect control group, however, an increase NPY expression was found in the prefrontal cortex (p<0.05). See figure 8A for quantification graphic, and 8B-8C for inmunohistochemistry representative images.

3.2.3. CO HISTOCHEMISTRY CUANTIFICATION

T-test showed differences between groups in the Infralimbic area of the prefrontal cortex t_{12}= 2.2, (p<0.01), hippocampus DG dorsal part t_{12}= -2.27, (p<0.05), and ventral CA1 t_{12}= 2.2, (p<0.05), as well as in anterodorsal Thalamic Nucleus t_{12}= -2.27, (p<0.05), medial Mammillary Nuclei t_{10}= 3.6, (p<0.01) and finally in the accumbens Nucleus (core), t_{12}= 2.18, (p≤0.05). See figure 2 and table 1.

4. DISCUSSION

The findings reported in the present study provide the first demonstration, to the best of our knowledge, of the association between brain regional Y_2R expression and mnemonic processes like spatial orientation. Additionally, we showed changes in regional expression of NPY, Y_1R and Y_2R associated with spatial memory-related structures like those included in the extended hippocampal system following Y_2R blockade in the CA1 dorsal hippocampus. Our results fill a gap in our understanding of the role of the NPY system in learning and memory processes at brain regional level.

This conclusion is drawn from experiments in which adult male rats were intracerebral infused with BIIE0246 Y2R antagonist in the CA1 of the dorsal hippocampus. Following Y2R antagonist administration, we observed a performance improvement in the reference spatial memory task in the Morris Water maze, but it failed to affect open-arm avoidance in the elevated zero-maze or spontaneous horizontal activity. Furthermore, when analyzing molecular brain regional changes, differences were found not only in levels of Y_2R expression but also in Y_1R and neuropeptide Y levels in areas related to spatial memory as the prefrontal cortex or dorsal hippocampal fields. These results were also confirmed by the analysis of metabolic brain activity using cytochrome oxidase histochemistry that showed changes not only in hippocampal and prefrontal cortex circuits, but also other well-known spatial orientation related structures as thalamus or mammillary bodies. Together, our findings support the view that the NPY system is involved in spatial memory functions, highlighting the role of brain Y_2R and Y_1R distribution in the process.

It is known that hippocampal or fimbria-fornix lesions could induce hyperactivity in rats. This effect is believed to be caused by the loss of glutamatergic hippocampal inputs to the nucleus accumbens, and associated with changes in dopamine receptors in the latter region (Kimble, 1963, Whishaw and Mittleman, 1991, Bannerman et al., 2001, Bannerman et al., 2004, Douglas and Isaacson, 2014). However, no evidence of hyperactivity was found in the experimental rats after Y2R antagonist infusion in the dorsal CA1, as demonstrated by the lack of differences in the spontaneous horizontal activity measured by the actimeters or the total distance moved during the elevated zero-maze test.

The NPY system is known to be related to anxiety and mood regulation (Thorsell et al., 2000, Thorsell and Heilig, 2002, Heilig et al., 2004, Holzer et al., 2012, dos Santos et al., 2013), however,

no differences were found between experimental and control animals in the amount of time spent in the open arm exploration in an elevated zero-maze, the selected variable related to anxiety reduction in this test. Although anxiolytic effects have been described in this test or the elevated plus-maze, it has been reported that the anxiolytic effects are not only dose-dependent but also region-specific or related with the administration method. Moreover, different receptors as Y_1R, Y_2R or Y_5R might contribute to the anxiolytic effects of NPY (Trent and Menard, 2011). In fact, Zambello et al. (2011) found that anxiolytic-like or antidepressant-like effect could not be confirmed in Y_2R knockout mice.

The last behavioural test used was a spatial orientation task in the MWM paradigm. Our results show that both groups of animals acquired the spatial memory test, as showed by the decreasing latencies to reach the platform along training days and good performance on the retention probe carried out after each session, spending a higher amount of time in the previously reinforced virtual quadrant as compared to the rest of them. However, after the drug infusion, on the third day, the experimental group had better performance as shown by the required time to reach the platform as compared to the control group. Additionally, the experimental group spent significantly more time in the escape quadrant as compared to the rest of quadrants. Briefly, both groups acquired the task as expected, but the experimental group performed better in the reference memory task. These results are in line with Redrobe et al. (2004) data showing impaired performance in MWM test in NPY receptor knockout mice. Nevertheless, dos Santos et al. (2013) claimed that in a model of Alzheimer disease, the ability of NPY to prevent spatial memory deficits is mainly due to a protective effect of this peptide against the AB peptide accumulation toxicity rather than a promnesic effect by itself. This view would be also consistent to the idea of the Y2R as one of the main targets underlying the neuroprotective effect of NPY. Accordingly, previous works showed that the administration of the NPY Y2 receptors antagonist eliminates the neuroprotection effects of NPY (Silva et al., 2003, Silva et al., 2005, Smialowska et al., 2009, dos Santos et al., 2013). Further studies should address the specific mechanisms of impaired performance in spatial orientation found in our study. However, we cannot preclude the possibility of the spatial memory related specific site of infusion of the drug or its associated areas expression changes in both the neuropeptide and its receptors in the behavior.

In this regard, Western blot analysis showed increased expression of Y_2R in the hippocampus, meanwhile decreased expression was observed in the prefrontal cortex, as compared to the control group. As regards to the Y_1R, only the prefrontal cortex showed differences in receptor expression, being higher in the experimental group than in the control group. According to our findings, it

seems that following injection of an Y2R antagonist as BIIE0246, the hippocampus shows up-regulation of this receptor. This might be a way of counteracting the temporary loss in receptor function. Accordingly, it is known that this receptor underlies several functions like neuronal excitability (Silva et al., 2005) and cognitive processes (Botterill et al., 2014) that might explain its function on spatial orientation. Thus, increased levels of Y2R expression in the hippocampus may account for impaired spatial memory performance, although further molecular studies would be necessary to understand this process at a protein level. Accordingly, the increased Y_1R expression found in the PFC is not entirely surprising; taking into account that Y_2R expression was lower so that increased expression of the rest of receptors might be an attempt to counteract its function. Additionally, Y_1 mRNA was already reported to be in high concentration in this region (Parker and Herzog, 1999). The lack of changes in Y1 receptor expression found in the hippocampus could be related with the different functions associated with these receptors. Particularly, the Y_1R plays an important role in anxiety and depression (Holzer et al., 2012) but no anxiogenic or anxiolytic effects were observed in this studty. As mentioned above regarding Y_2R, the molecular regulation of Y1R both in the hippocampus and the PFC would suggest a possible involvement of this receptor in the evaluated learning and memory tasks.

On the other hand, immunohistochemical quantification of NPY protein levels showed widespread decreased NPY expression in the experimental group in the hippocampus, including CA1, CA3 fields and DG. In contrast, the prefrontal cortex showed significant higher expression of NPY in the experimental group as compared to their matched controls.

Administration of Y2R antagonist in the CA1 dorsal hippocampus caused changes in the NPY system, affecting not only the hippocampal areas as expected, but also closely associated areas, like the prefrontal cortex. The hippocampal-prefrontal cortex circuit has been widely related with to the acquisition and consolidation of spatial memory, and specifically, the initially hippocampal-dependent memories that are stored at a later stage within hippocampal-cortical networks and eventually in the neocortex (Squire and Alvarez, 1995, Frankland and Bontempi, 2005, Smith and Squire, 2009, Leon et al., 2010).

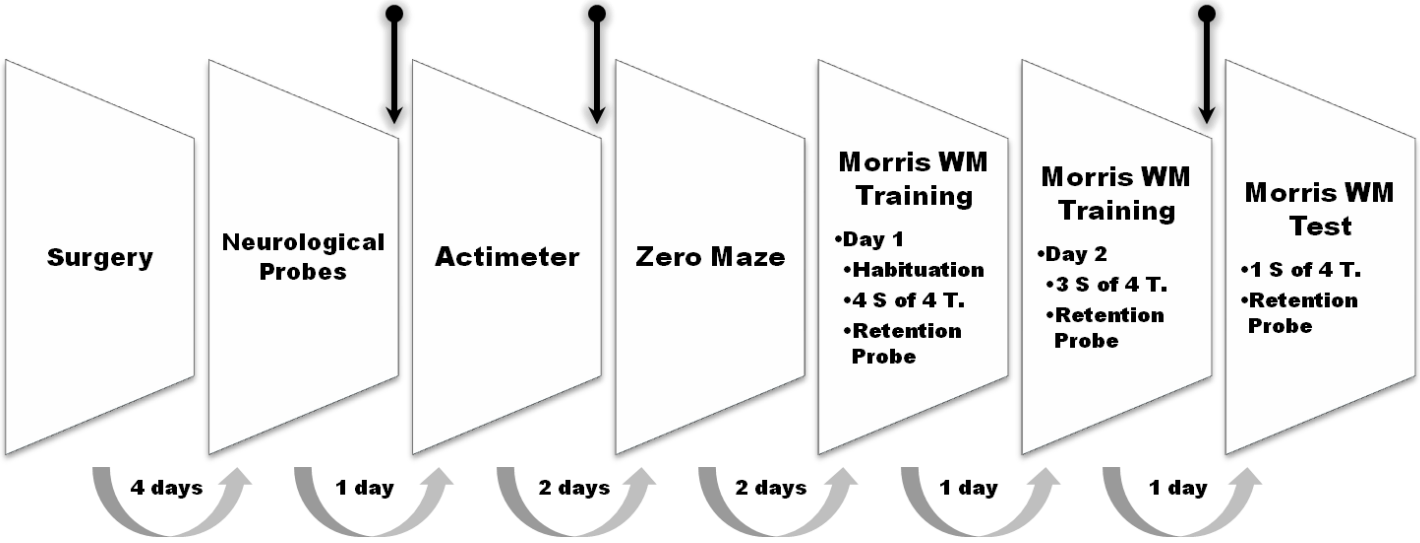
On the other hand, analysis of brain metabolism with CO histochemistry showed drug-induced changes in the aforementioned brain regions. As expected, the prelimbic area of the prefrontal cortex showed lower CO activity meanwhile the DG of the dorsal hippocampus presented an increased CO metabolism. In addition to the classical theory of memory consolidation which supports the idea of a hippocampal-prefrontal circuits supporting spatial memory acquisition, since

changes were observed at different spatial memory stages, like acquisition (Conejo et al., 2007, Conejo et al., 2010, Fidalgo et al., 2012), retrieval (Conejo et al., 2013, Mendez-Couz et al., 2015a) or extinction of spatial memory (Mendez-Couz et al., 2014). Moreover, the CA1 subfield of the ventral hippocampus showed lower activity as compared to the saline group. The ventral portion of the hippocampus has been related to emotional and bodily states (Fanselow and Dong, 2010), contrasting with the spatial visuo-spatial and cognitive functions attributed to the dorsal part (Morris et al., 1982, Moser and Moser, 1998). Therefore, it could be feasible that the differences in CO activation found in this area could be due to drug-infusion effect on anxiety or stress responses. However, no differences in mean CO activity were found brain regions typically associated with stress and anxiety like the perirhinal cortex or the amygdala (Villarreal et al., 2002). These results are consistent with the lack of differences found in the EZM test, so that the effects in the performance of the spatial memory water maze task performance seem not to be related to anxiolytic or anxiogenic drug effects.

Lastly, limbic regions anatomically related with the hippocampal formation like the anterior thalamic nuclei and the mammillary bodies showed group differences in CO activity. Both diencephalic regions are critically involved in spatial orientation because they are included in the so-called head direction system that is essential for spatial navigation (Taube, 1995, Stackman and Taube, 1998, Aggleton and Brown, 1999, Warburton and Aggleton, 1999, Wilton et al., 2001, van Groen et al., 2002, Vann et al., 2003, Mendez et al., 2008, Da Cunha et al., 2009, Lopez et al., 2009, Mendez-Lopez et al., 2009, Vann, 2010, 2011, Loureiro et al., 2012, Mendez-Couz et al., 2014, Mendez-Couz et al., 2015a)

In conclusion, expression of NPY and its receptors Y1R and Y2R were differentially modified by the infusion of an Y2R specific antagonist into the CA1 field of the dorsal hippocampus, being those changes region-specific. Additionally, differences in cognitive process as a MWM task performance and the modified pattern of brain metabolism were found in brain regions usually related with spatial memory like the prefrontal cortex, the dorsal and ventral hippocampus, the thalamus or the mammillary bodies. Therefore, our results support the hypothesis that the NPY system, and specifically Y1R and Y2R play an important role in spatial memory modulating learning and memory processes. Further studies using pharmacological inactivation of the mentioned regions, like PFC areas, and biochemical studies should be of great help for the elucidation of the key role that the NPY system is playing in spatial memory.

Figure 1


Surgery
Neurological Probes
Actimeter
Zero Maze
Morris WM Training
•Day 1
•Habituation
•4 S of 4 T.
•Retention Probe
Morris WM Training
•Day 2
•3 S of 4 T.
•Retention Probe
Morris WM Test
•1 S of 4 T.
•Retention Probe
4 days
1 day
2 days
2 days
1 day
1 day

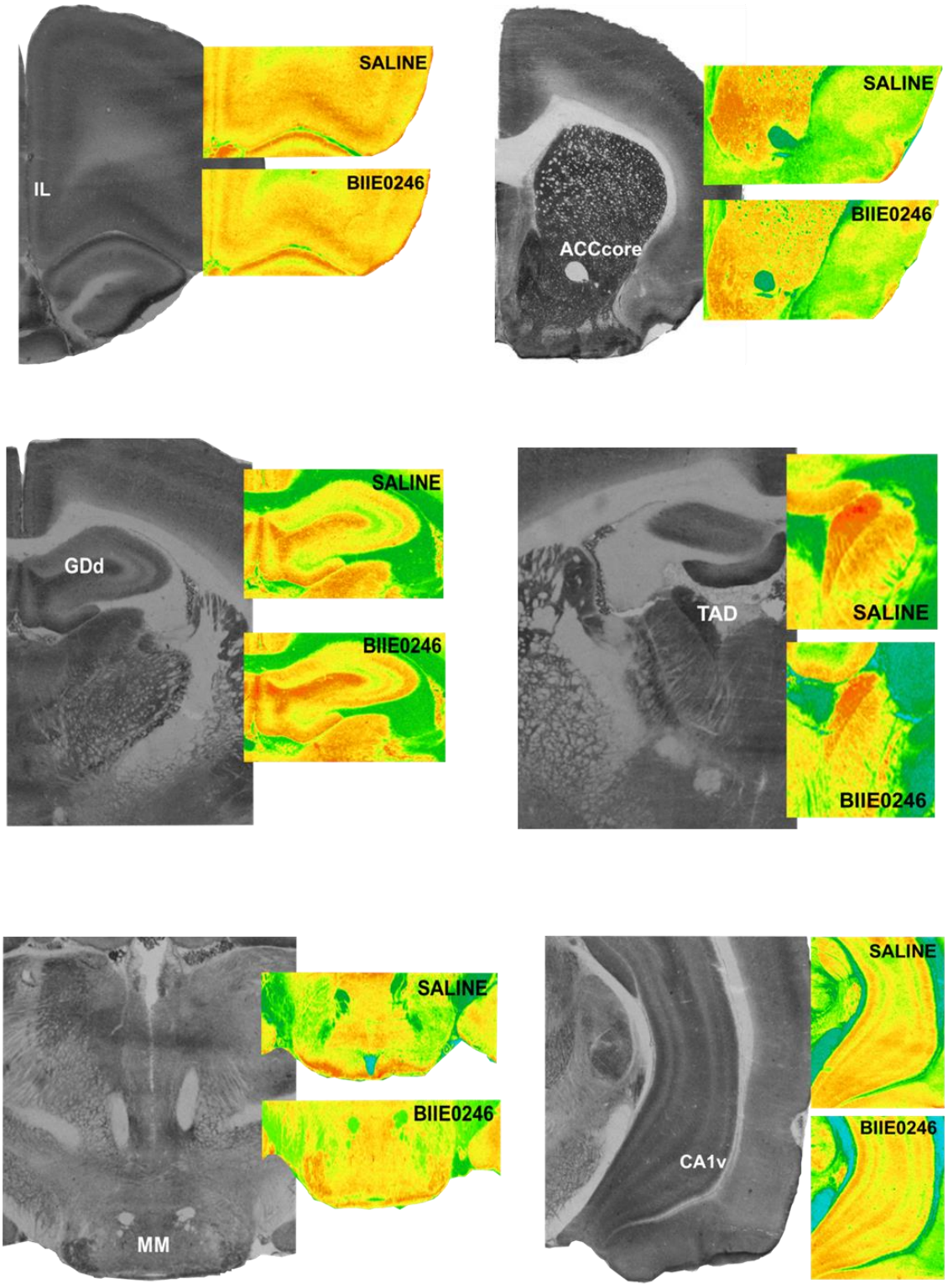

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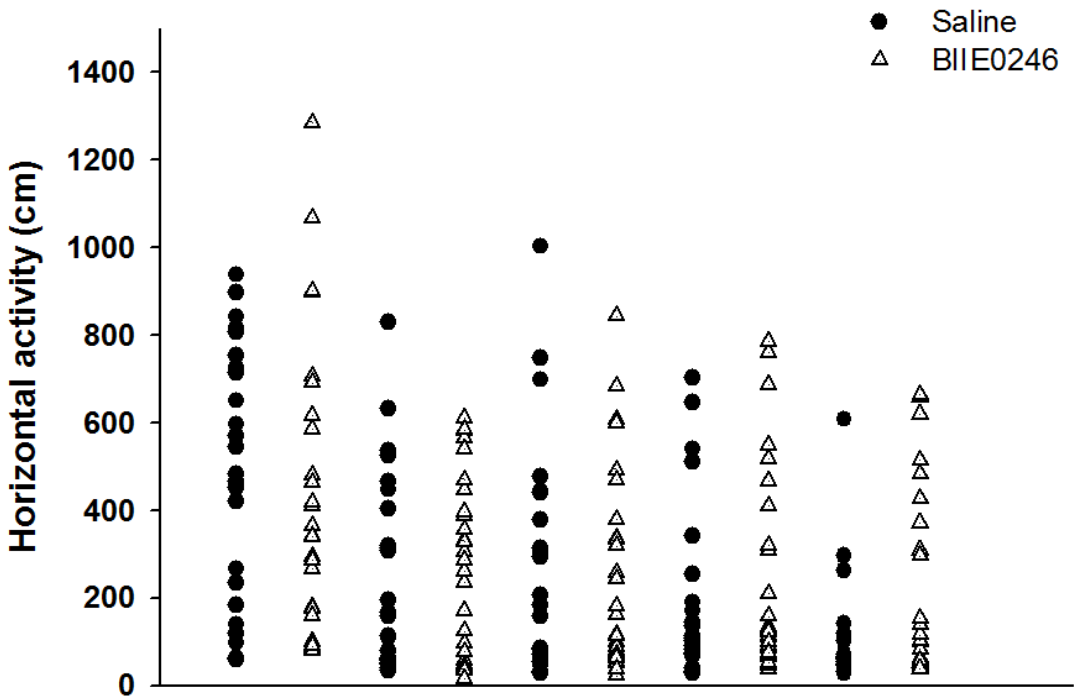
Figure 2

Figure 3


Saline
BIIE0246
Horizontal activity (cm)
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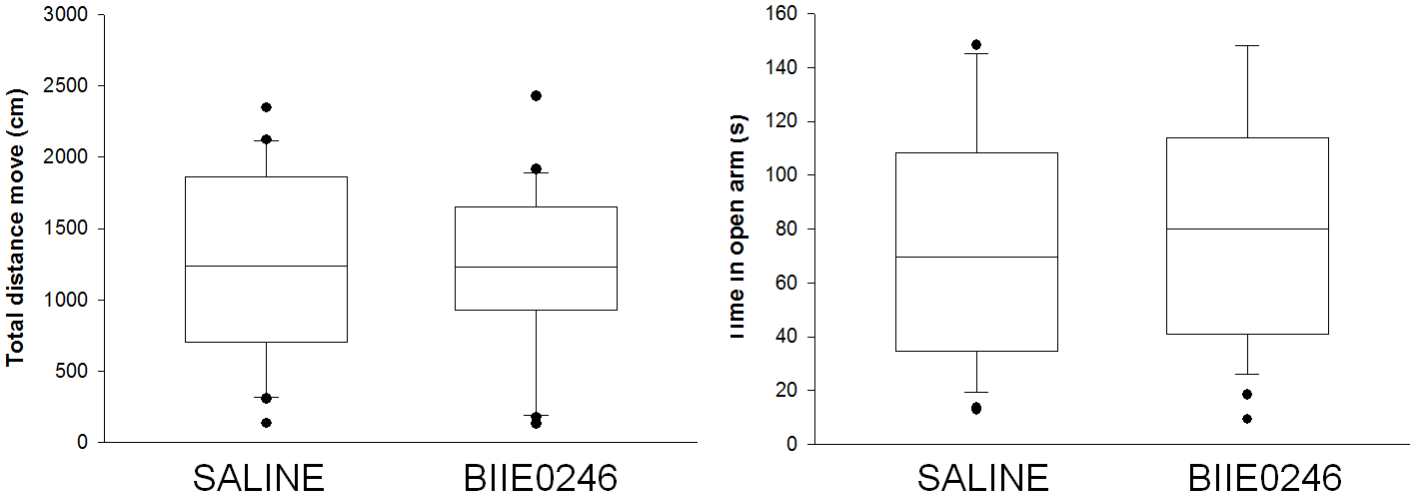
1200
1000
800
600
400
200
0

Figure 4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2500
2000
1500
1000
500
0
SALINE
BIIE0246
Time in open arm (s)
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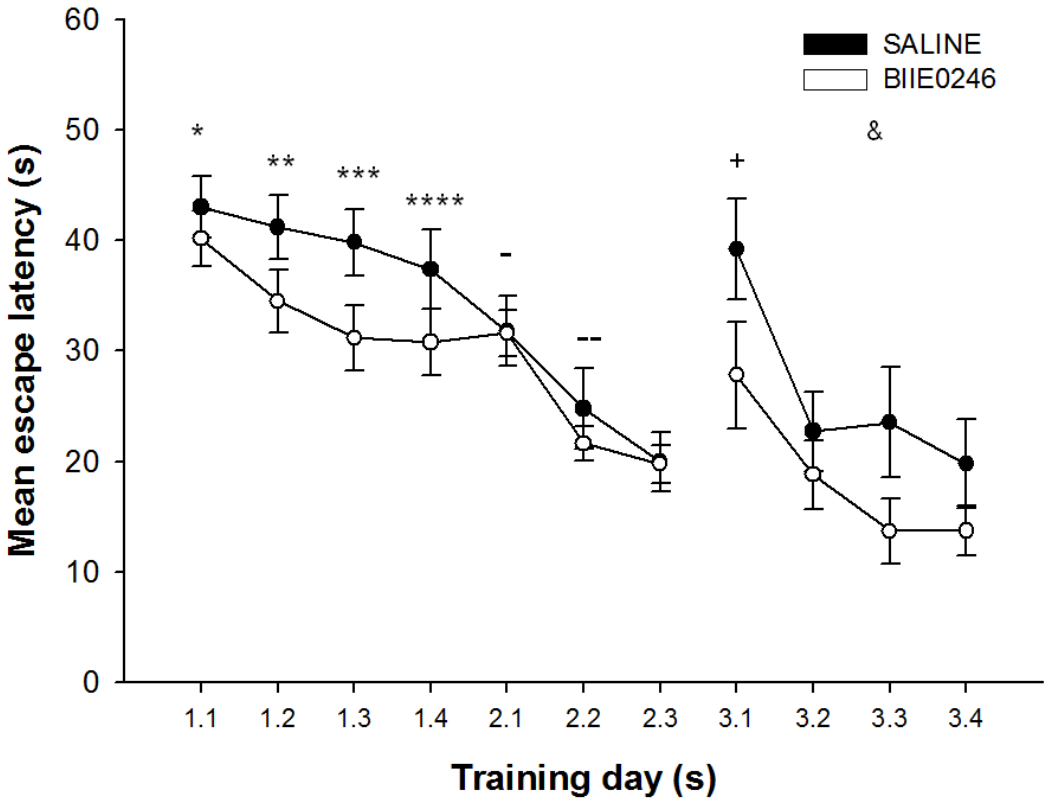
140
120
100
80
60
40
20
0
SALINE
BIIE0246

Figure 5


SALINE
BIIE0246
Mean escape latency (s)
Training day (s)
0
10
20
30
40
50
60
1.1
1.2
1.3
1.4
2.1
2.2
2.3
3.1
3.2
3.3
3.4
*
**
***
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-
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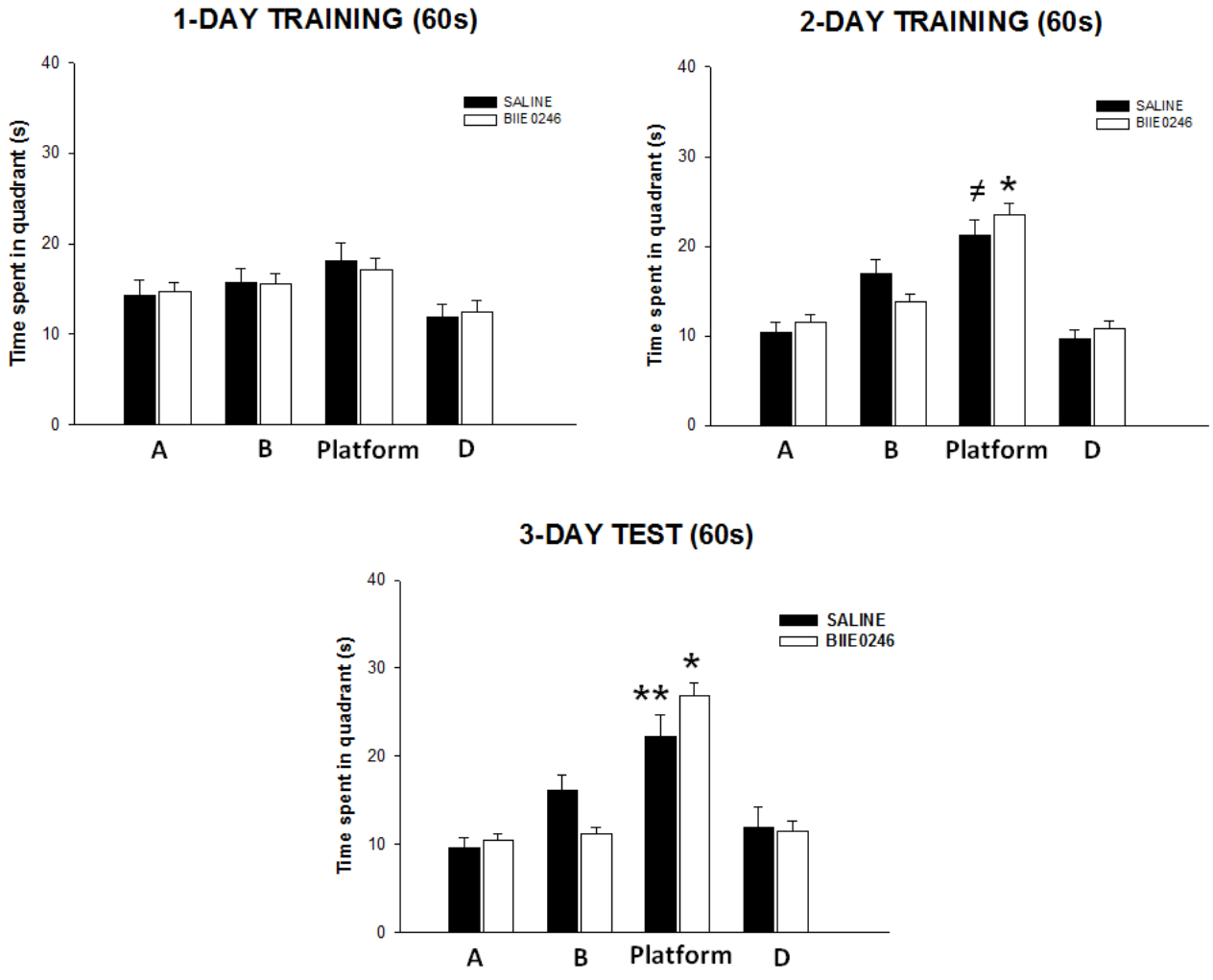
+
&

Figure 6


1-DAY TRAINING (60s)
Time spent in quadrant (s)
40
30
20
10
0
SALINE
BIIE 0246
A
B
Platform
D
2-DAY TRAINING (60s)
Time spent in quadrant (s)
≠ *
3-DAY TEST (60s)
Time spent in quadrant (s)
** *

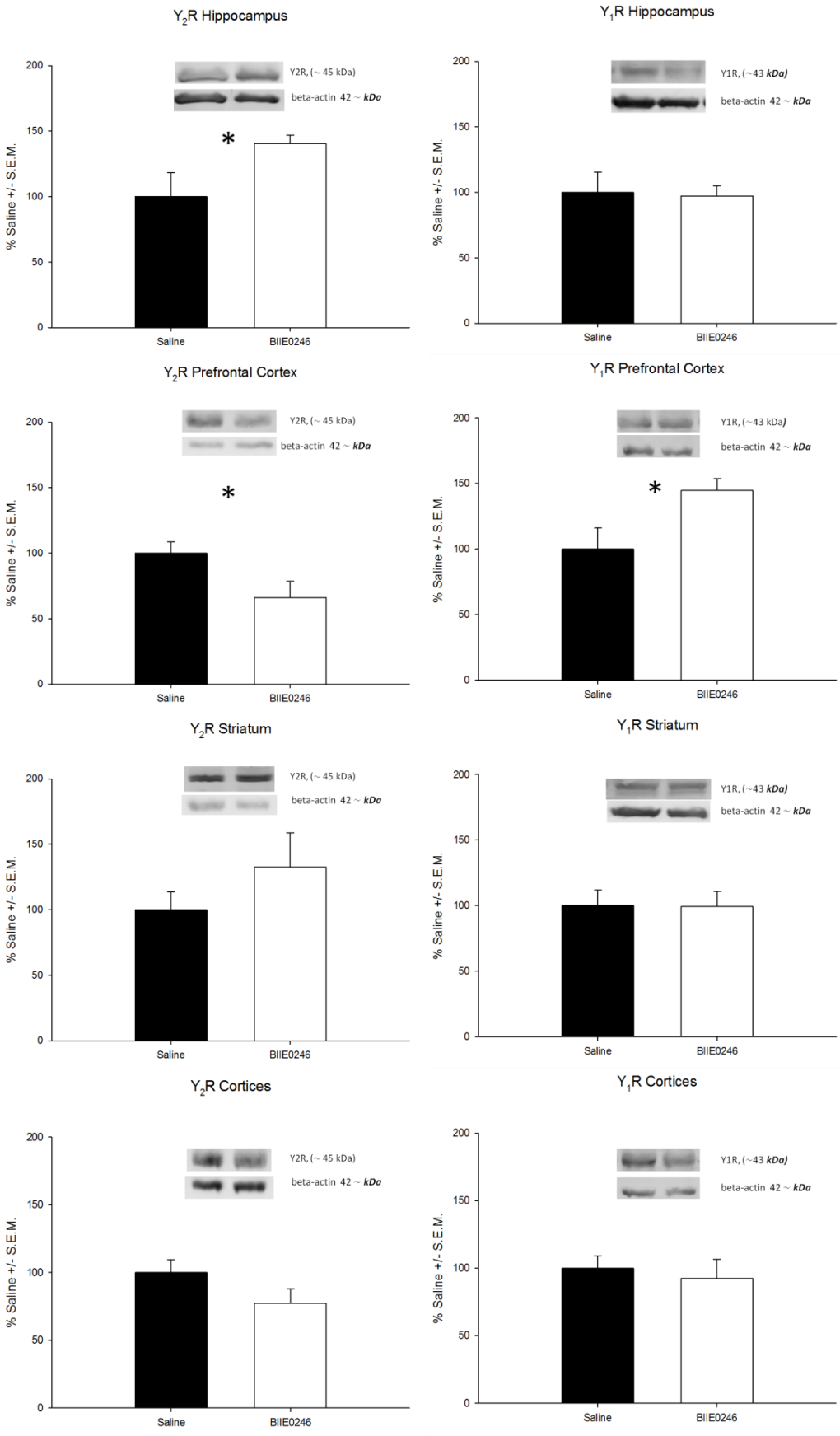

Y2R Hippocampus
Y2R, (~ 45 kDa)
beta-actin 42 ~ kDa
% Saline +/- S.E.M.
200
150
100
50
0
*
Saline
BIIE0246
Y1R Hippocampus
Y1R, (~43 kDa)
beta-actin 42 ~ kDa
Y2R Prefrontal Cortex
Y1R Prefrontal Cortex
Y2R Striatum
Y1R Striatum
Y2R Cortices
Y1R Cortices

Figure 7

Figure 8- A

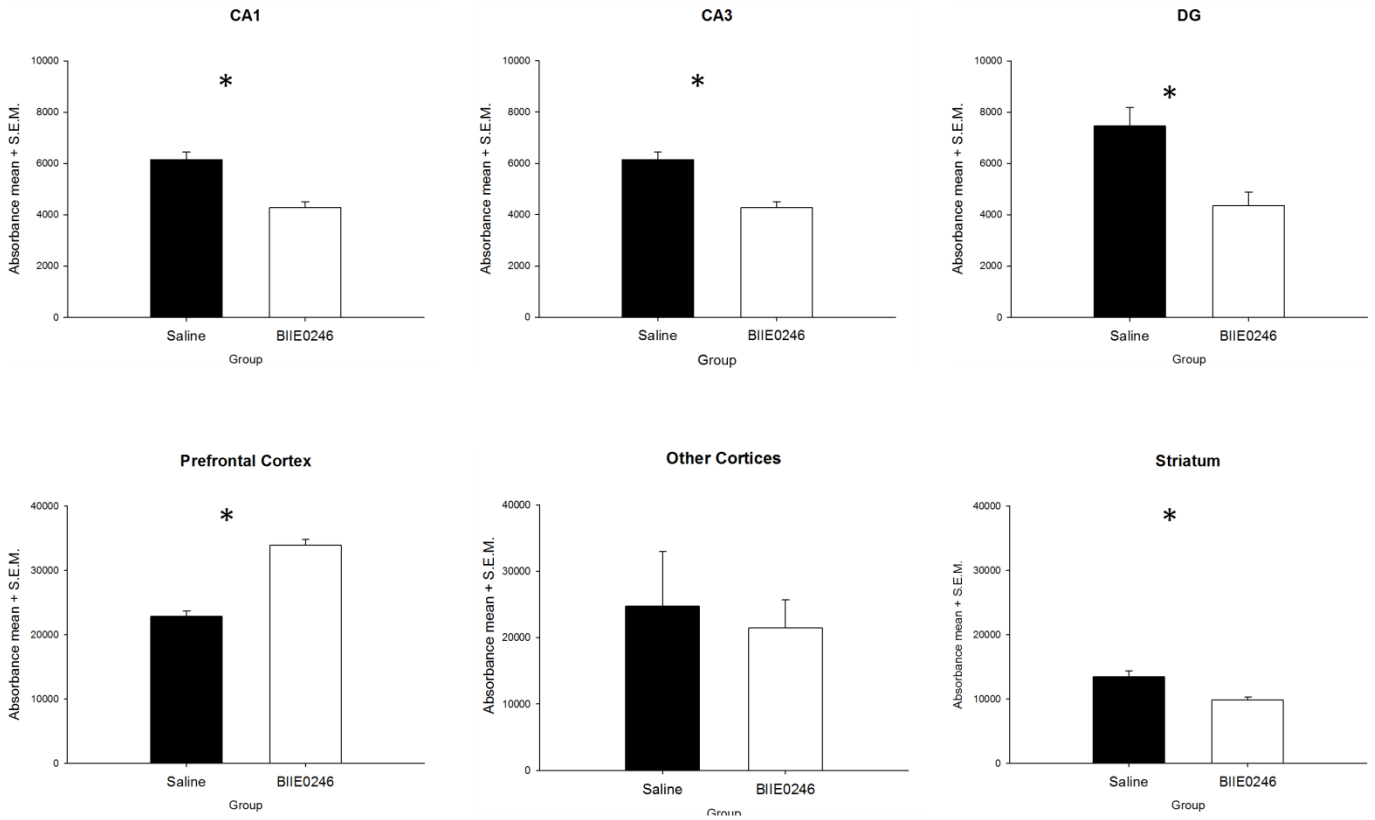
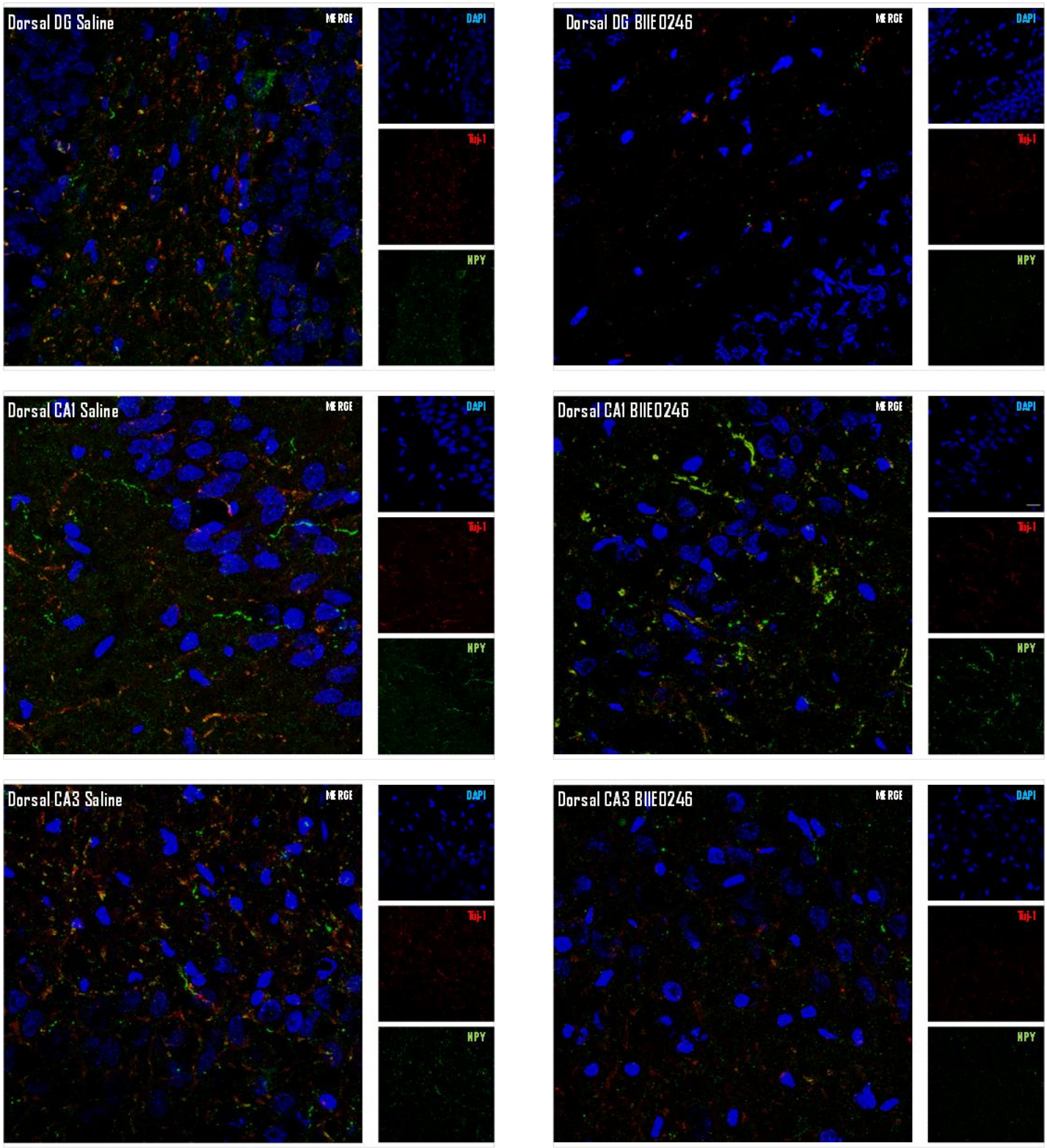

CA1
CA3
DG
Prefrontal Cortex
Other Cortices
Striatum
Absorbance mean + S.E.M.
Saline
BIIE0246
Group
*

Figure 8-B


Dorsal DG Saline
MERGE
DAPI
Tuj-1
NPY
Dorsal DG BIIE0246
MERGE
DAPI
Tuj-1
NPY
Dorsal CA1 Saline
MERGE
DAPI
Tuj-1
NPY
Dorsal CA1 BIIE0246
MERGE
DAPI
Tuj-1
NPY
Dorsal CA3 Saline
MERGE
DAPI
Tuj-1
NPY
Dorsal CA3 BIIE0246
MERGE
DAPI
Tuj-1
NPY

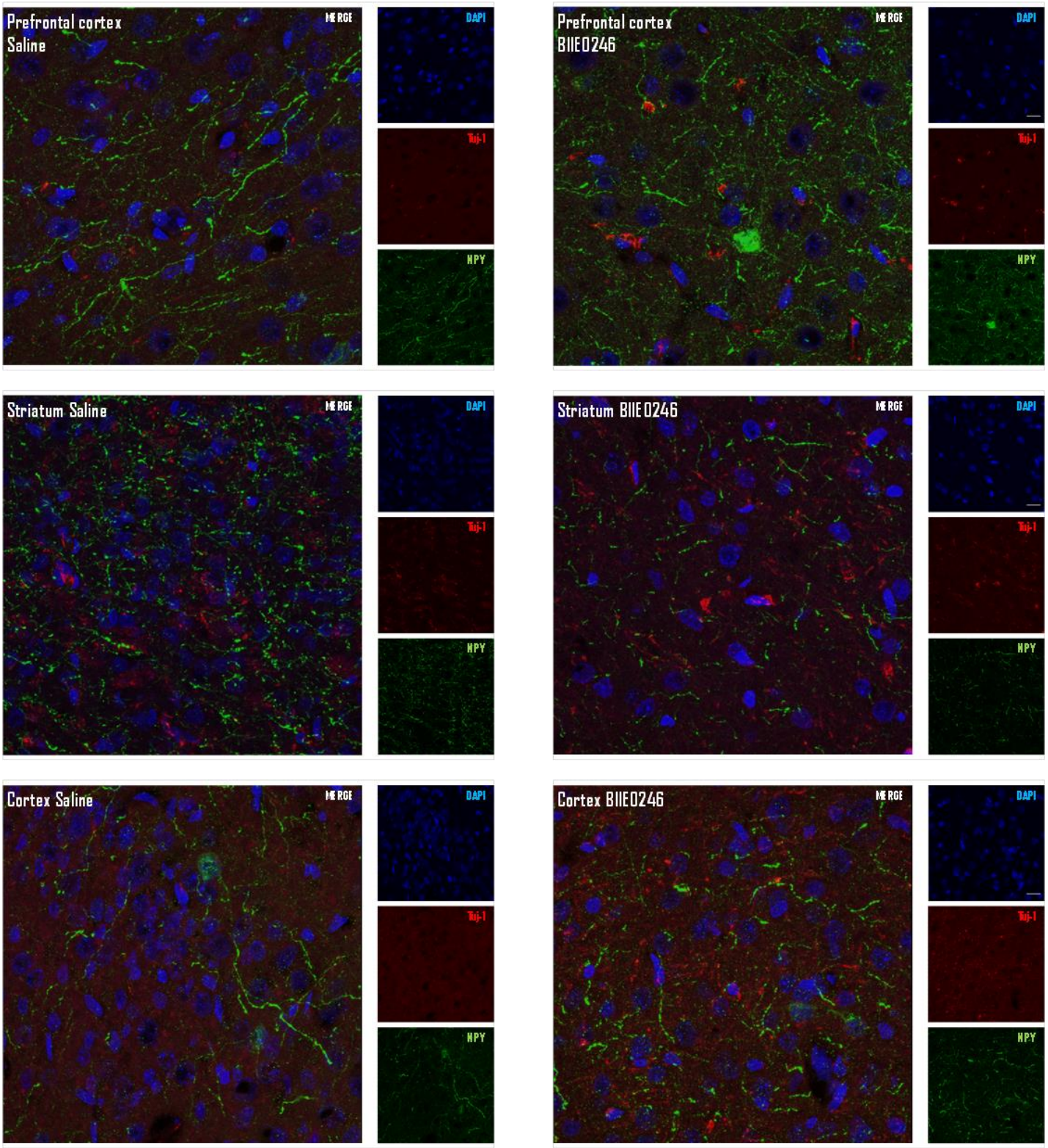

Prefrontal cortex
Saline
MERGE
DAPI
Tuj-1
NPY
Prefrontal cortex
BIIE0246
MERGE
DAPI
Tuj-1
NPY
Striatum Saline
MERGE
DAPI
Tuj-1
NPY
Striatum BIIE0246
MERGE
DAPI
Tuj-1
NPY
Cortex Saline
MERGE
DAPI
Tuj-1
NPY
Cortex BIIE0246
MERGE
DAPI
Tuj-1
NPY

Figure 8-C

Table 1

	n	SALINE	n	BIIE0246
CORTEX				
Prelimbic	8	19.2±0.4	6	18.7±0.3
Infralimbic	8	20.0±0.5	6	17.8±0.4^{+}
Cingulate	8	19.8±0.7	6	19.2±0.5
Motor	8	20.6±0.4	6	19.9±0.3
Parietal	8	18.8±0.2	6	17.8±0.6
Retrosplenial (Agranular)	8	18.4±1.0	6	20.1±1.6
Retrosplenial (Granular)	8	21.3±1.2	6	22.2±1.5
Entorhinal	7	14.0±0.5	6	13.7±0.9
Perirhinal	7	17.2±0.7	6	16.8±0.8
HIPPOCAMPUS				
CA1 dorsal	8	16.0±0.6	6	17.0±1.0
CA3 dorsal	8	19.5±1.0	6	20.6±1.0
GD dorsal	8	22.0±0.6	6	24.6±1.0*
CA1 ventral	8	24.5±0.7	6	22.5±0.5*
CA3 ventral	7	24.8±1.8	6	23.0±0.8
GD ventral	8	23.6±1.1	6	24.7±1.3
DIENCEPHALON				
Anterodorsal Thalamic Nucleus	8	31.6±1.0	6	32.4±0.7*
Anteroventral Thalamic Nucleus	8	21.7±0.4	6	22.3±0.6
Mediodorsal Thalamic Nucleus	8	18.9±0.8	6	17.6±0.6
Medial Mammillary Nuclei	6	30.9±2.0	6	23.0±1.0^{+}
Lateral Mammillary Nucleus	6	36.7±2.9	6	32.5±1.4
AMYGDALA COMPLEX				
Basolateral Nucleus	7	21.7±0.8	6	21.1±1.6
Medial Nucleus	7	15.7±0.8	6	15.3±0.8
Central Nucleus	7	16.7±1.0	6	16.6±1.1
Lateral Nucleus	7	16.7±0.9	6	14.8±6.6
OTHERS				
Dorsal Striatum	8	19.7±0.4	6	20.1±0.8
Accumbens Nucleus (core)	8	24.1±0.9	6	21.7±0.5*
Accumbens Nucleus (shell)	8	26.0±0.8	6	25.8±0.7
Lateral Septum	8	18.8±0.7	6	20.0±1.2
Medial Septum	8	13.6±0.6	6	12.8±0.5
Bed Nucleus Stria Terminalis	8	19.4±0.9	6	19.8±0.9

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Discusión

El objeto de este trabajo es contribuir al esclarecimiento de las estructuras y redes neuronales que subyacen tanto a las fases de recuerdo como a las de extinción de una tarea de memoria espacial de referencia, evaluada haciendo uso del laberinto acuático de Morris (MWM). Con este fin analizamos las estructuras y vías neurales que sustentan a estas dos fases de la memoria espacial en condiciones fisiológicas (Artículos 1, 4 y 5). Posteriormente se inactivaron temporalmente estructuras cerebrales seleccionadas según los resultados de los experimentos previos, y se analizaron los efectos de la inactivación en el recuerdo y la extinción de la memoria espacial, así como las modificaciones producidas en las estructuras cerebrales y reorganización de redes funcionales que sustentan a dichas conductas (Artículos 2, 3, 6 y 7). Adicionalmente, se determinaron los cambios en la expresión de proteínas de señalización intracelular implicadas en los cambios en plasticidad sináptica, que se creen asociados con los procesos de memoria, tales como el neuropéptido Y (NPY), en aquellas estructuras cerebrales que mostraron una relación directa con la conducta evaluada y provocadas por la inactivación cerebral.

5.1. RECUERDO:

5.1.1. ESTRUCTURAS Y REDES IMPLICADAS EN EL RECUERDO DE LA MEMORIA ESPACIAL EN CONDICIONES FISIOLÓGICAS.

En el primer experimento presentado se analizan las estructuras y redes neurales que intervienen en la capacidad de recuerdo de una tarea de memoria de referencia espacial en el laberinto acuático de Morris, que había sido adquirida con éxito con anterioridad. Dicha tarea consististió en la localización de una plataforma que permanece sumergida en uno de los cuatro cuadrantes virtuales en los que se divide el laberinto, y por tanto es invisible para los animales, con ayuda de pistas visuales alocéntricas colocadas en la estancia en la que se situaba el laberinto. La adquisición de la tarea sucede a lo largo de cinco días, tras lo cual se realiza una prueba de retención. Para valorar la adquisición de la tarea se tuvo en cuenta el descenso significativo del tiempo en que el animal tarda en llegar a la plataforma (latencias de escape) así como el tiempo que el animal pasa en el cuadrante virtual previamente reforzado,

en la prueba de retención realizada inmediatamente después de la adquisición. Tras un tiempo de reposo se mide la capacidad de recuerdo de la tarea los **animales se enfrentan en el mismo contexto y condiciones anteriores, a una nueva prueba de retención, llamada prueba de recuerdo.**

Posteriormente se analizan las estructuras cerebrales implicadas mediante un mapeo metabólico cerebral, para ello se lleva a cabo una histoquímica para la citocromo c oxidasa mitocondrial en áreas que se suponen relevantes en otras fases de la memoria espacial, tal como el sistema límbico y la corteza cerebral (Artículo 1).

6.1.1.1. Conducta: los animales adquieren y recuerdan la localización de la plataforma escondida en una tarea de memoria espacial de referencia en el laberinto acuático de Morris.

La primera observación, atendiendo a la medida conductual, es que las ratas evaluadas en el laberinto acuático de Morris fueron capaces de adquirir con éxito la tarea de memoria de referencia espacial (SRM) en el laberinto acuático de Morris en cinco días. Los animales del grupo de recuerdo adquirieron la tarea y fueron capaces de recordar la localización de la plataforma una semana y hasta un mes después. El dominio de la adquisición de la tarea de memoria espacial se puso de manifiesto tanto por el descenso en las latencias de escape necesarias para alcanzar la plataforma de escape a lo largo de los días de entrenamiento, como por la cantidad de tiempo, significativamente más alto, que los animales pasaban en el cuadrante que hacía sido previamente reforzado por la localización de la plataforma de escape.

Por otra parte, se añadió un grupo control, que nadó un tiempo equivalente al de los animales experimentales pero siempre en ausencia de plataforma, para asegurar que los cambios hallados a nivel de las distintas regiones cerebrales, en el metabolismo oxidativo o en la expresión de la proteína c-Fos eran debidos a los procesos de memoria estudiados, y no a otros factores, tales como el cambio de contexto habitual de animal, aspectos de ansiedad, o de actividad motora. A este grupo, que nadó una cantidad de tiempo equipatable a los animales de recuerdo, pero siempre en ausencia de plataforma, se le denominó grupo "Control Nado" o Naïve. En este sentido cabe destacar que ambos grupos, control y experimental,

nadaron la misma distancia y con la misma velocidad. Cuando se evaluó la capacidad de recuerdo de los animales a la semana de haber finalizado la adquisición de la tarea, se observó de nuevo una diferencia estadísticamente significativa en la preferencia de los animales por el cuadrante previamente reforzado, es decir, los animales recordaban la antigua localización de la plataforma que habían aprendido una semana atrás.

Por lo tanto, podemos considerar que la adquisición y el recuerdo de la memoria espacial se dieron con éxito en nuestros experimentos.

6.1.1.2. Análisis de la actividad metabólica: Interacciones funcionales entre el giro dentado, el estriado y el tálamo anterior.

Una vez finalizada la evaluación conductual de los animales, y con el propósito de analizar la implicación de las distintas estructuras cerebrales en las redes funcionales que subyacen a esta tarea de recuerdo de la memoria espacial, llevamos a cabo una técnica histoquímica para la proteína citocromo c oxidasa de la cadena de respiración mitocondrial. De las estructuras relacionadas con la adquisición y la consolidación de la memoria espacial que habíamos analizado, encontramos diferencias entre los grupos experimental y control nado en varias de las regiones analizadas, incluyendo áreas de la corteza prefrontal (cingulada, prelímbica e infralímbica), el córtex motor primario, el estriado dorsal y ventral, el núcleo accumbens tanto en sus porciones de *Core* como de *Shell, y* finalmente en el giro dentado del hipocampo, tanto en su porción dorsal como ventral. Además, los estudios de correlación mostraron diferentes redes neurales entre los animales que habían realizado la tarea de recuerdo de la memoria espacial, grupo Recuerdo, y aquellos que los del grupo Control Nado. En este sentido, el grupo recuerdo presentaba un patrón de conexiones mucho más complejo, que incluía dos redes funcionales independientes. La primera de ellas presenta interacciones entre áreas pertenecientes a la corteza prefrontal medial, como el área cingulada, y el hipocampo dorsal (Conejo, Cimadevilla, Gonzalez-Pardo, Mendez-Couz, & Arias, 2013), y también entre la corteza prefrontal y el estriado. Una segunda red incluía conexiones entre los núcleos talámicos y la porción ventral del hipocampo. Sin embargo, la red de conexiones metabólicas que se encontró en el grupo control nado era mucho más simple, con interacciones entre estructuras que presentan una proximidad y correlación anatómica grande. En este caso las conexiones se limitaron a las áreas prelímbica e infralímbica de la cortea prefrontal por una parte, y a las áreas CA1 y CA3 del hipocampo ventral por otra.

Estos resultados son consistentes con la idea prevaleciente que sugiere una participación a gran escala de la red hipocampo-cortical distribuida en la memoria espacial (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Conejo et al., 2013; Frankland & Bontempi, 2005; Leon, Bruno, Allard, Nader, & Cuello, 2010; Wang & Cai, 2008). Además de la implicación de esta ya bien conocida red, existe un consenso generalizado sobre la reorganización temporal de los circuitos que subyacen a la memoria espacial (Bontempi et al., 1999; Conejo, Gonzalez-Pardo, Gonzalez-Lima, & Arias, 2010; Maviel, Durkin, Menzaghi, & Bontempi, 2004). En otras palabras, se cree que existen diferencias entre las redes neurales que están implicadas en el control de la memoria reciente y aquellas que sustentan la memoria remota. Esta hipótesis se apoya en estudios de lesión cerebral, en los que se sugiere que la inactivación de regiones corticales específicas alteraría la memoria remota sin trastocar una memoria reciente (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Quillfeldt et al., 1996; Takehara, Kawahara, & Kirino, 2003). Específicamente, a la corteza prefrontal medial se le atribuye desde hace tiempo un papel específico de vital importancia en tareas de navegación espacial. Más concretamente, las áreas prelímbica e infralímbica se han relacionado con tareas de memoria de trabajo espacial (Ragozzino, Adams, & Kesner, 1998) y con la memoria espacial reciente (Wang & Cai, 2008), mientras que al área cingulada se le atribuye una participación en tareas de discriminación espacial o discriminación de contexto (Frankland & Bontempi, 2006; Frankland et al., 2006). El potencial del córtex prefrontal para la integración y síntesis de la información recibida de numerosas fuentes (Miller, 1996) podría indicar su habilidad para procesar memorias remotas (Kil et al., 2014), de forma similar al papel del hipocampo para procesar las memorias recientes (Frankland & Bontempi, 2005). En consecuencia, la inactivación del área prelímbica [(Mendez-Couz, Conejo, Vallejo, & Arias, 2015), Artículo 3] o del área cingulada impide el recuerdo de la memoria remota (Frankland et al., 2004; Maviel et al., 2004). En la misma línea, los estudios farmacológicos de desconexión de los circuitos hipocampo-corteza prefrontal medial confirman esta idea (Wang & Cai, 2008).

Además de hallarse un incremento de actividad metabólica en las porciones dorsal y ventral del giro dentado, también se encontró una correlación negativa entre la corteza cingulada y el giro dentado dorsal en los animales que habían realizado la tarea de recuerdo de memoria espacial. Haciendo referencia a este hecho, nuestro grupo de investigación ya había publicado previamente que la coerteza prefrontal medial participa de manera temporal-denpendiente en la adquisisión de una tarea de memoria de referencia espacial en el

laberínto acuático de Morris (Conejo et al., 2010). En el mencionado trabajo se muestra un incremento de actividad citocromo oxidasa mitocondrial en la mPFC durante las fases tardías de la adquisición de esta tarea. Es más, el acoplamiento entre la mPFC y el hipocampo también aparece de forma coherente en las fases de recuerdo de esta memoria (Conejo et al., 2013). En conjunto, estos datos apoyarían la hipótesis establecida por Frankland and Bontempi (2005), en la que se sugiere que la mPFC podría tener un papel muy activo durante el recuerdo, impidiendo al hipocampo la recodificación de las memorias existentes. En este sentido, aunque la ya mencionada relación entre la corteza prefrontal y el hipocampo dorsal ha sido ampliamente abordada en la literatura, el papel que el giro dentado juega en todo este proceso aún no se conoce con certeza. Hasta la fecha, muchos estudios focalizados en el hipocampo dorsal han demostrado que el giro dentado (DG) podría participar en tareas de memoria de trabajo (Emerich & Walsh, 1989; Gilbert, Kesner, & Lee, 2001) y en aquellas que requieren el uso de información que es específica para cada ensayo, durante el recuerdo remoto de tareas de memoria espacial (Talpos, McTighe, Dias, Saksida, & Bussey, 2010). Este último tipo de tareas coincidiría, en cierto modo, con la empleada en nuestro estudio, ya que los animales recibieron un único ensayo que sirvió como prueba de recuerdo, una semana después de haber adquirido la tarea espacial. En lo concerniente a la participación del DG en la memoria espacial, numerosos estudios que lesiones selectivas de esta región en roedores empeora la ejecución de las tareas de memoria de trabajo espacial (McLamb, Mundy, & Tilson, 1988) y altera específicamente tareas de memoria de referencia espacial (A. M. Morris, Churchwell, Kesner, & Gilbert, 2012; A. M. Morris, Weeden, Churchwell, & Kesner, 2012; Nanry, Mundy, & Tilson, 1989; Okada & Okaichi, 2009; Xavier, Oliveira-Filho, & Santos, 1999).

Además del hipocampo y la corteza prefrontal, nos encontramos con un incremento en el metabolismo oxidativo cerebral, de manera regional, en el estriado dorsal y el núcleo accumbens. Estos resultados coinciden con los de otros trabajos presentes en la literatura en los que se sostiene que el estriado dorsal, conjuntamente con el hipocampo dorsal, son necesarios para la navegación espacial en el MWM (Miranda, Blanco, Begega, Rubio, & Arias, 2006; Miyoshi et al., 2012). De acuerdo con estos autores, durante la navegación las relaciones espaciales entre los estímulos ambientales son procesadas en el hipocampo para establecer una especie de mapa del contexto espacial (Telensky et al., 2011), posteriormente, el estriado dorsal usará la información ya procesada para proporcionar un plan de acción motor que permita al animal dirigirse hacia la posición de destino. Por otra parte, el estriado dorsal estaría implicado en la selección de acciones motoras que se requieren para la navegación y

que pueden basarse en información que proviene de las pistas o del contexto (Da Cunha et al., 2009; Opris & Bruce, 2005). Cuando nos referimos al recuerdo de la información espacial en humanos, existen evidencias provenientes de estudios en los que se emplean pruebas de resonancia magnética funcional. En estos experimentos se muestra que los participantes que se consideraban más eficientes en tareas de navegación espacial presentan una mayor activación, tanto del hipocampo como del núcleo caudado, cuando seguían una ruta hacia una meta no visible en un ambiente que les resultaba conocido, y que ya habían aprendido con éxito anteriormente (Hartley, Maguire, Spiers, & Burgess, 2003). Por lo tanto, el estriado dorsal podría ser necesario para utilizar la información espacial que previamente había sido codificada y mapeada por el hipocampo y posteriormente había sido integrada por la mPFC para guiar la navegación hacia el lugar de destino. A pesar de los estudios mencionados, el papel específico del núcleo estriado durante el recuerdo de la RSM no queda del todo esclarecido, por lo que resultarían de ayuda otro tipo de experimentos adicionales tales como la inactivación farmacológica temporal de dicha estructura.

En nuestro estudio nos encontramos con una implicación del tálamo anterior y medio-dorsal. En esta línea, recientes estudios han relacionado a estos núcleos con procesos de recuerdo (Staudigl et al., 2012). Según este autor, la actividad del núcleo medio-dorsal del tálamo y de la corteza prefrontal estaría sincronizada durante el recuerdo en humanos, lo que sugiere que esas regiones cerebrales podrían enviar una señal temprana de los procesos a recordar a la corteza, lo que desembocaría en la búsqueda de esa información almacenada por parte de la corteza y en procesos de decisión. Por otra parte, también se ha señalado que el recuerdo de miedo al contexto estudiado en modelo animal de ratón estaría apoyado en un conectoma funcional en el que el tálamo formaría parte, conformando una red talámica-hipocampo-cortical (Wheeler et al., 2013), lo que se mostró mediante estudios de expresión del gen de expresión temprana c-fos. Los datos del presente trabajo también están respaldados por descubrimientos previos, en los que se demuestra que una lesión del núcleo anterior del tálamo altera tanto la memoria espacial (Lim, Labaree, Li, & Huang, 2014; Lopez et al., 2009; van Groen, Kadish, & Michael Wyss, 2002; Warburton & Aggleton, 1999) como la no espacial (Wolff, Gibb, & Dalrymple-Alford, 2006) en ratas, de una manera muy similar a lo que sucedería bajo una lesión hipocampal. De hecho, varios estudios de desconexión han demostrado la relación funcional entre estas regiones en tareas de memoria espacial (Henry, Petrides, St-Laurent, & Sziklas, 2004; Warburton, Baird, Morgan, Muir, & Aggleton, 2001).

Por otra parte, las células de direccionalidad, del llamado ”sistema de dirección de la cabeza” que informan a la rata de la dirección específica hacia la cual se están enfrentando, se han encontrado en el postsubículum y núcleo anterior del tálamo, y su función parece estar asociada con la integridad de este último núcleo (Shinder & Taube, 2011). En nuestro trabajo, la actividad CO de los núcleos talámicos seleccionados mostró una correlación positiva con el campo CA1 del hipocampo ventral en los animales que habían llevado a cabo la tarea de recuerdo. Por lo tanto, no podemos descartar la posibilidad de que la interacción de los mismos con el hipocampo ventral pudiera estar particularmente implicada en los procesos de memoria espacial.

Siguiendo con la porción ventral del hipocampo, se observó específicamente unos nieveles más elevados de actividad metabólica en el giro dentado en los animales que habían realizado la tarea de recuerdo en comparación con sus controles nado. Tradicionalmente, al hipocampo dorsal y ventral se le han atribuido diferentes funciones en base a sus diferencias en conectividad con otras regiones en distintas redes neurales (Fanselow & Dong, 2010; Pennartz, Ito, Verschure, Battaglia, & Robbins, 2011). Esta diferenciación funcional también se ha encontrado en humanos, lo que implica una división funcional antero-posterior (Doeller, King, & Burgess, 2008; Iaria, Chen, Guariglia, Ptito, & Petrides, 2007). El hipocampo ventral (homólogo al anterior en humanos) se cree más relacionado con emociones y estados corporales (Fanselow & Dong, 2010), en cambio, el hipocampo dorsal (posterior en humanos) se asocia con habilidades visuo-espaciales y cognitivas, lo que incluye a la orientación especial (R. G. Morris, Garrud, Rawlins, & O'Keefe, 1982; Moser & Moser, 1998b). Por lo tanto, la activación del hipocampo ventral hallada podría atribuirse al estrés asociado con la tarea realizada en el laberinto acuático. En este sentido, no se encontraron diferencias de activación en regiones relacionadas con la huida bajo condiciones de estrés, tales como la corteza perirrinal o la amígdala basolateral (Villarreal, Gonzalez-Lima, Berndt, & Barea-Rodriguez, 2002).

Teniendo en cuenta lo anterior, parece posible que el hipocampo ventral pueda tener un papel importante en el recuerdo de una tarea de SRM. Estos resultados están de acuerdo con otros estudios en la literatura, tal como como el de Ruediger, Spirig, Donato, and Caroni (2012) en el que se atribuye un papel esencial al hipocampo ventral en el aprendizaje dirigido a una meta y en las distintas formas de búsqueda, específicamente en el inicio del aprendizaje (SACAR PAPER; QUITAR??). En lo concerniente al recuerdo de una memoria espacial, los

datos expuestos en nuestro trabajo también concuerdan con los de estudios previos en los que evaluando la expresión de genes de expresión temprana o la absorción de 2-desoxyglucosa (Bontempi et al., 1999; Maviel et al., 2004), e incluso en estudios de lesión (Loureiro, Lecourtier, et al., 2012), han demostrado la relación entre las porciones dorsal y ventral del hipocampo en el recuerdo de una memoria espacial evaluada entre uno y cinco días después del aprendizaje.

6.1.2. Efecto de la inactivación del hipocampo en el recuerdo de la memoria espacial y en las redes metabólicas cerebrales asociadas.

6.1.2.1. La inactivación unilateral del hipocampo dorsal en ratas altera el recuerdo de una tarea de SRM tanto como la bilateral

Los resultados de nuestros estudios muestran que una inactivación temporal del hipocampo dorsal, de forma unilateral o bilateral, con la droga tetradotoxina tiene efectos similares en el recuerdo de una tarea de memoria de referencia espacial adquirida cuatro semanas antes en el laberinto acuático de agua (Artículo 2). El periodo de tiempo usado para evaluar la memoria (28 días) se basó en estudios previos (Frankland & Bontempi, 2005; Lopez et al., 2012; Remondes & Schuman, 2004). En este sentido, ambos tratamientos alteraron la ejecución en el test de prueba o test de transfer en el MWM, en la que los sujetos no recordaron la posición de la plataforma escondida. Estos resultados concuerdan, al igual que los del Artículo 1, con otros trabajo en la literatura en los que se muestra que el hipocampo está relacionado con el recuerdo de memorias espaciales en el MWM, evaluada una semana después de la adquisición, como en el caso anterior, o incluso cuando estas habían sido adquiridas varias semanas antes (Broadbent, Squire, & Clark, 2010; Martin, de Hoz, & Morris, 2005; Riedel et al., 1999).

A pesar de que ambos tratamientos produjeron una alteración en la ejecución de la tarea de recuerdo, ésta se alteró de distinta manera en ambos grupos. Así, mientras que el tratamiento bilateral condujo a los animales a una búsqueda uniforme alrededor del laberinto, aquellos que estaban bajo una inactivación de un solo hemisferio presentaron una clara preferencia por el cuadrante lateral al previamente reforzado por la plataforma. Esto probablemente muestra que los sujetos unilateralmente inactivados preservan algunas memorias, (aunque puedan ser no del todo exactas) sobre la meta. Estas mismas alteraciones se han visto en experimentos en los que se usa una modificación del laberinto de Morris, en las que para acceder a la plataforma las ratas

tienen que nadar un tiempo por encima de la plataforma, para que esta emerja (aprendizaje procedimental). En este estudio, los animales que habían recibido el tratamiento de inactivación hipocampal tras la adquisición, que sabían "cómo" tenían que realizar una tarea, pero no exactamente "dónde" buscar (Micheau, Riedel, Roloff, Inglis, & Morris, 2004).

El efecto de la inactivación unilateral sobre la conducta es, hasta cierto punto, causa de controversia. El bloqueo unilateral no siempre perturba las conductas hipocampo-dependientes. Para entender este efecto, probablemente necesitamos tener en cuenta tanto el tipo de tarea como la fase de la memoria afectada por el tratamiento. Por lo tanto, en tareas muy espacialmente-dependientes, tales como generalmente las llevadas a cabo en el MWM, o evitación activa de arenas, la inactivación unilateral altera todas las fases de la formación de memoria, como ya se ha demostrado en diversos estudios realizados desde hace un par de décadas (Cimadevilla, Miranda, Lopez, & Arias, 2005; Cimadevilla, Wesierska, Fenton, & Bures, 2001; Fenton & Bures, 1993). Sin embargo, estas mismas intervenciones no alteraron de forma uniforme la memoria en tareas dependientes de hipocampo en las que la necesidad de navegación espacial es baja, éste sería el caso de tareas de evitación pasiva, donde la orientación y la navegación en el ambiente está limitada, por lo que se requiere, más bien, de un adecuado reconocimiento del contexto (Cimadevilla, Mendez, Mendez-Lopez, & Arias, 2007; Conejo et al., 2010; Lorenzini, Baldi, Bucherelli, Sacchetti, & Tassoni, 1996). Por otra parte, es necesario considerar la fase de la memoria que estamos interrumpiendo durante la inactivación del hipocampo. Concretamente, el recuerdo ha demostrado ser más susceptible a la interferencia que otras fases de la formación de la memoria. En este sentido, tal y como Moser and Moser (1998a) mostraron, la cantidad de tejido hipocampal que se requiere para llevar a cabo tareas de recuerdo es mayor que la necesaria para la adquisición de dichas tareas.

Además del efecto en tareas de recuerdo, también en la literatura aparecen estudios en los que se evalua el impacto de esta lesión, de forma unilateral o bilateral, en otras fases de memoria, en los que se encontraron resultados similares a los aquí expuestos. Por ejemplo, en experimentos en los que se inyectaba tetradotoxina de manera intrahipocampal para bloquear la consolidación, no se encontraron diferencias entre ambos tratamientos (Cimadevilla, Miranda, Lopez, & Arias, 2008). Por lo tanto, aunque la inactivación unilateral en teoría deja al hipocampo contralateral intacto para conservar los procesos mnésicos, un único hipocampo puede no ser suficiente para sustentar y procesar adecuadamente los procesos de

memoria espacial. En este sentido, tenemos que considerar que las alteraciones cognitivas que suceden tras una inactivación unilateral pueden estar causadas por una plausible interferencia entre los hipocampos inactivado y no inactivado. En concordancia con esta idea, desde hace tiempo se conoce que cada hipocampo envía y recibe fibras del hipocampo contralateral (Swanson, Wyss, & Cowan, 1978), por lo que una lesión unilateral podría alterar los procesos fisiológicos que se suceden en el otro (Van Praag, Black, & Stäubli, 1997; Van Praag, Chung, Black, & Stäubli, 1998).

Ya que en nuestro estudio la inactivación unilateral tuvo lugar en el hipocampo derecho, dadas las alteraciones observadas, se podría pensar que la memoria espacial está lateralizada en éste (Klur et al., 2009), y por lo tanto la inactivación de este lado sería la responsable del deterioro del recuerdo de la memoria espacial. Sin embargo, esta idea despierta una gran controversia, ya que entre otros, experimentos en los que se inactivaba el hipocampo izquierdo o el derecho de forma independiente, presentaron efectos muy discretos en la ejecución de la tarea a realizar (Klur et al., 2009), y otros autores no llegaron a detectarlos (Fenton & Bures, 1993). Cuando nos referimos a humanos, el papel que presentan ambos hipocampos en la orientación espacial no se libra de controversia. Por ejemplo, se ha visto que un foco epiléptico unilateral en el lóbulo temporal medial, o una resección unilateral del hipocampo, son suficientes para alterar severamente la ejecución de tareas espaciales en laberintos virtuales, y esto sucede independientemente del lado cerebral implicado (Astur, Taylor, Mamelak, Philpott, & Sutherland, 2002; Canovas, Leon, Serrano, Roldan, & Cimadevilla, 2011).

6.1.3. La actividad metabólica cerebral varía en estructuras relacionadas con la orientación espacial tras la inactivación del hipocampo.

Una vez observados los efectos conductuales en la ejecución de una tarea de recuerdo de memoria espacial tras la inactivación (unilateral o bilateral) del hipocampo, nos propusimos ealuar los cambios en las estructuras y redes cerebrales relacionadas con la memoria espacial que se habían producido. Para ello, al igual que en el experimento anteriormente descrito, utilizamos la técnica histoquímica de la citocromo c oxidasa mitocondrial.

Se observó que la actividad metabólica del giro dentado, CA3 y CA1variaba en función de si la inactivación del hipocampo había sido unilateral o bilateral. En este sentido, el grupo

control salino mostró correlaciones positivas entre los giros dentados derecho e izquierdo y entre lados ipsilaterales del Cuerno de Ammon. También los campos CA3 contralaterales mostraron correlaciones positivas entre ellos. Por tanto, podemos decir que el patrón de activación de estas áreas se altera cuando se inhibe la actividad del hipocampo. Como nosotros, otros autores han propuesto que el giro dentado y el campo CA3 pueden estar involucrados en el procesamiento de la geometría de la estancia (Kesner, 2007), siendo esenciales las fibras de Mossy que llegan a él para codificar la información espacial (Lassalle, Bataille, & Halley, 2000).

A diferencia de los animales unilateralmente inactivados y de los controles salinos, los animales con inactivación bilateral mostraron diferencias en actividad CO en diferentes regiones del hipocampo. En concreto, se encontró un incremento de actividad en las areas CA1 y CA3 durante el recuerdo, mientras que la actividad CO del DG disminuyó de forma notable. Pudiera ser que las áreas CA1 y CA3 y el DG tengan funciones opuestas durante distintas fases del procesamiento de la memoria espacial. En este sentido, algunos autores (Chee, Goh, Lim, Graham, & Lee, 2004; Jerman, Kesner, & Hunsaker, 2006) han demostrado que la vía perforante que penetra en el CA3 es crítica para los procesos de recuerdo (relacionados con los mecanismos de *pattern completion)* mientras que el giro dentado es crítico en los procesos de codificación de memorias (lo que probablemente está relacionado con procesos de *pattern separation*). Esto significa que un deterioro en el aprendizaje o los déficits generales de memoria en un animal que nunca ha sido capaz de completar estas tareas no son indicativos de una alteración en los mecanismos de terminación de patrones (Hunsaker & Kesner, 2013). La diferente actividad CO observada entre el Cuerno de Ammon y el giro dentado podría revelar esta disociación, ya que durante el recuerdo, la terminación de patrones es esencial para recuperar el total de la información almacenada, pero los mecanismos de separación de patrones, que tienen lugar en el momento de codificación, no son indispensables, por esta razón el giro dentado parece estar inhibido durante la expresión o el recuerdo.

Dado que se necesita al hipocampo para una orientación adecuada, la inactivación parcial o bilateral causa alteraciones en otras estructuras que llevan a cabo un papel importante en el sistema de orientación del cerebro. Por lo tanto, las relaciones de correlación cambian ligeramente en los animales unilateralmente inactivados, pero se ven francamente trastocados en los animales que han pasado por una inactivación bilateral. Esta pérdida de

correlaciones positivas apoya la hipótesis de que la droga, en este caso tetradotoxina, perturba la red neural implicada en el recuerdo de la memoria espacial. Este tipo de alteración de la red neural que subyace al proceso, también se ha observado al inactivar esta misma área en fases más tardías de la memoria espacial, tales como los procesos de extinción, experimento que queda reflejado en el Artículo 7, y que se discutirá en detalle más adelante. Asimismo, también se han observado cambios a nivel de red cerebral con la inactivación del área prelímbica de la corteza prefrontal, tanto en la red cerebral asociada a lo procesos de recuerdo (Artículo 3) como aquella implicada en los procesos de extinción (Artículo 6).

Las comparaciones de patrones de correlación entre las distintas areas del hipocampo proporcionan información sobre la red neural que subyace a los procesos de conducta estudiados. En este sentido, se demostró que los análisis realizados a nivel de redes neurales son mucho más sensibles para entender las disfunciones cerebrales que si solo se tienen en cuenta las partes individuales que componen el sistema (Rowe, 2010).

En relación con otras áreas asociadas al hipocampo, nuestro trabajo mostró que un deterioro en la ejecución de las tareas conductuales coincidía con un incremento de la actividad metabólica cerebral en el córtex entorrinal y en los núcleos mamilares. Por contra, los animales control salino mostraron una actividad CO reducida en la corteza entorrinal en comparación con los grupos tratados. En este sentido, es de sobra conocido que la corteza entorrinal mantiene abundantes conexiones con el sistema hipocampal, tal como hemos comentado previamente, y que contiene células que se suponen especializadas en la codificación de la información (Hafting, Fyhn, Molden, Moser, & Moser, 2005). Del mismo modo, se ha publicado que las lesiones del área dorsolateral de la corteza entorrinal alteraba los procesos de recuerdo de tareas que habían sido adquiridas una semana antes (Steffenach, Witter, Moser, & Moser, 2005).

Puesto que la fisiología del sistema hipocampal se altera con las infusiones de TTX, ésto podría dar lugar a un incremento de la actividad de las regiones relacionadas con los procesos de recuerdo. Una hipótesis alternativa sugiere que los intentos fallidos de averiguar la posición de la plataforma incrementarían la actividad exploratoria, y por tanto la actividad CO en el córtex entorrinal, ya que es sabido que la actividad exploratoria puede regular la actividad en la corteza entorrinal (Matrov, Kolts, & Harro, 2007). En este sentido, observamos que los grupos inactivados cambiaron con más frecuencia entre las circunferencias virtuales

en las que se dividió el laberinto que el grupo control; sin embargo, no se encontraron diferencias entre los grupos en la distancia total recorrida.

Además de las ya mencionadas, se encontraron redes metabólicas similares a estas en otras regiones implicadas en memoria espacial. En este sentido, los núcleos laterales de los cuerpos mamilares y el núcleo anterodorsal del tálamo son conocidos por formar parte del Circuito de Papez y por contener el sistema de dirección de la cabeza (Taube, 1995, 2007), lo que contribuye al procesamiento de pistas tanto alocéntricas como geométricas (Vann, 2011). De manera adicional, el núcleo mamilar lateral proyecta directamente al núcleo anterodorsal del tálamo vía el tracto mamilotalámico (Aggleton & Pearce, 2001; Hayakawa & Zyo, 1989). Estudios previos han demostrado que la actividad CO del núcleo mamilar lateral cambia en tareas de memoria de trabajo espacial (Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2004; Mendez-Lopez, Mendez, Lopez, & Arias, 2009b), y nuestros trabajos, también se ha observado una modificación de la actividad metabólica en este núcleo en tareas de extinción de la conducta espacial, tanto en condiciones fisiológicas como bajo los efectos de la inactivación de la corteza prefrontal (Mendez-Couz, Conejo, Vallejo, et al., 2015)(Artículos 4 y 6). Estos cambios también se han visto en la expresión de expresión de genes de expresión temprana relacionados con la conducta espacial (Mendez-Couz, Conejo, Vallejo, & Arias, 2014). Sin embargo, en los grupos inactivados se observa un incremento de actividad en este núcleo durante las tareas de recuerdo de memoria espacial, mientras que, como se verá más adelante, durante la extinción de la tarea previamente adquirida, se observa una menor actividad del núcleo lateral de los cuerpos mamilares cuando se comparan con grupos control salino.

En relación al núcleo anterodorsal del tálamo, se encontraron niveles de actividad CO mayores en los grupos con inactivación bilateral en comparación con los grupos control. Aunque este núcleo recibe una conexión principal desde el subículo (la mayor eferencia del hipocampo), se cree que las lesiones en el hipocampo no distorsionan las señales de las células de dirección de la cabeza (Golob & Taube, 1997). Sin embargo, se conoce que las estructuras mencionadas forman parte del circuito de Papez y que durante los procesos de aprendizaje y memoria estas regiones interaccionan, cambiando su metabolismo (Conejo et al., 2010), por lo que parece factible que la inactivación produzca cambios en la actividad CO de estas estructuras.

También es necesario remarcar que el grupo de inactivación bilateral mostró un incremento de actividad CO en muchas otras estructuras cerebrales relacionadas con los circuitos de memoria. De hecho, los animales bilateralmente inactivados presentaron una actividad CO mayor en la corteza perirrinal, , una estructura que ha sido repetidamente relacionada con el reconocimiento de objetos (Albasser et al., 2011; Hopkins & Bucci, 2010) y discriminación de contextos (Abe, Ishida, Nonaka, & Iwasaki, 2009), así como en tareas de recuerdo de memoria espacial (Ramos, 2008). Tal y como este este último autor demostró, las ratas con inactivación perirrinal mostraban alteraciones en el recuerdo de tareas de memoria espacial que habían sido adquiridas con éxito antes de la intervención.

La actividad de la corteza cingulada también se incrementó en el grupo de inactivación bilateral respecto al grupo unilateralmente inactivado y al grupo control. Esta estructura une estructuras límbicas y corticales y se conoce que está implicada en tareas de memoria espacial en ratas (Sutherland, Whishaw, & Kolb, 1988; Whishaw, Maaswinkel, Gonzalez, & Kolb, 2001). Este resultado concuerda con el incremento de actividad CO en los animales que habían pasado por un proceso de recuerdo una semana después de adquirir la tarea en comparación con sus controles nado

5.2. EXTINCIÓN

La mayor parte de las conductas adaptativas en organismos complejos en comportamiento aprendido, dirigido por la disponibilidad de refuerzos positivos o negativos. Asimismo, una falta de refuerzos positivos, cuando habían estado disponibles, conduce a la extinción del comportamiento previamente aprendido. A lo largo de ensayos de extinción, el organismo aprende que la respuesta que una vez había sido adaptativa ya no conduce de manera efectiva al refuerzo, y por lo tanto gradualmente cesa esa conducta.

Durante las tareas de condicionamiento Pavloviano estándar, las presentaciones repetidas del estímulo condicionado (CS), pero en ausencia del estímulo incondicionado (US) que antes había sido apareado con el Cs dan lugar a la extinción de la respuesta previamente condicionada. Si atendemos a la idea de Huston, Schulz, and Topic (2009) los procesos de aprendizaje y extinción en el laberinto acuático de agua seguirían las reglas clásicas que gobiernan el aprendizaje instrumental. Tal y como hemos como hemos comentado previamente, la adquisición y la extinción del aprendizaje evaluado en el laberinto acuático de Morris seguiría, entonces, los mismas reglas del condicionamiento clásico y operante, luego

los mismos procesos estaría detrás del aprendizaje espacial asociativo y el no-asociativo (Prados, Manteiga, & Sansa, 2003; Prados, Sansa, & Artigas, 2008; Sanchez-Moreno, Rodrigo, Chamizo, & Mackintosh, 1999).

Actualmente se conoce poco de las bases neurales que subyacen a los efectos de diferentes variantes para la extinción de una memoria espacial, tal como el número de ensayos o el contexto espacial o temporal (Delamater & Lattal, 2014; Lattal, Mullen, & Abel, 2003). Luego los primeros experimentos de esta línea tenían como objetivo evaluar el protocolo de extinción a utilizar.

5.2.1. La extinción de la tarea de memoria espacial se consigue tras cuatro sesiones de extinción y no presenta recuperación espontánea a las 24h.

Para evaluar la eficacia del protocolo de extinción (ArtArt.. 5), entrenamos ratas Wistar en una tarea estándar de adquisición de SRM en el laberinto acuático de agua y después seguimos un protocolo de extinción en el que la plataforma que actuaba como refuerzo era retirada del laberinto (grupo Extinción, EX), como control de la variable de estudio utilizamos animales que no adquirieron ninguna tarea y simplemente nadaron por un tiempo equivalente a los anteriores (control nado o Naïve, N). Para analizar la posibilidad de la interferencia de la posible recuperación espontánea de la tarea previamente adquirida en las bases neuronales a estudiar, se añadió un grupo de animales, a los que denominamos Extinction-Retrieval (EXR) en los que se midió la recuperación espontánea al día siguiente de haber ejecutado la tarea de extinción.

Los resultados mostraron que los animales del grupo EX mostraron una buena adquisición de la tarea espacial, de acuerdo a los parámetros ya comentados, mostrando una clara preferencia por el cuadrante de escape, que había sido reforzado. Esta preferencia, sin embargo, disminuyó a lo largo de las sesiones de extinción a las que se sometió a los animales, ya que ésta mostraba valores similares a los que se darían por azar alrededor de la tercera sesión de extinción, lo que indica la eficacia del protocolo utilizado para conseguir la extinción del comportamiento asociado al aprendizaje anterior. Además, la conducta de extinción no mostró recuperación espontánea tras 24 h de haberse producido, tal y como mostró la prueba de retención realizada a los animales del grupo EXR al día siguiente de la extinción, en la que seguían sin mantener la preferencia por el cuadrante virtual del laberinto previamente

reforzado. Por lo tanto, creemos que podemos considerar que la extinción de SRM se ha conseguido con éxito con el protocolo seguido.

Tal y como se ha mencionado en la introducción, según algunos autores (Huston, van den Brink, Komorowski, Huq, & Topic, 2012; Schulz, Huston, Buddenberg, & Topic, 2007) la plataforma de escape actuaría como una recompensa, siendo responsable de la mejora de las tareas de memoria de referencia a través de los ensayos en el MWM. En esta línea, una vez la tarea de memoria de referencia es adquirida, la extinción del aprendizaje de escape puede ser inducido retirando la plataforma del laberinto, exponiendo así al animal a ensayos de nado sin tener presente el refuerzo. Teniendo esto en cuenta, la extinción de la memoria espacial se puede medir mediante los cambios en la conducta a lo largo de las sesiones de extinción, lo que incluye una disminución en la preferencia por el cuadrante de escape (Lattal et al., 2003; Topic et al., 2005). Algunos autores no están de acuerdo con el empleo de este tipo de protocolos en los que se usa el laberinto de agua para provocar la extinción de la memoria espacial. Así, Prados et al. (2003) mostró una recuperación espontánea de la tarea previamente adquirida tras 96 h de su adquisición. Esto ocurriría si el número de ensayos no reforzados fuese insuficiente para inducir una extinción duradera a largo plazo. Por ejemplo, en el mencionado estudio las ratas se sometieron a la mitad de los ensayos de extinción que en el nuestro (8 *vs.* 16). Además, los ensayos de extinción fueron consecutivos, en lugar del retraso de 30 min entre sesiones que nosotros utilizamos, lo que podría explicar la diferencia en ambos resultados.

Por otra parte, algunos autores, entre los que se incluye el grupo del Dr. J.P. Huston consideran que los protocolos conductuales aquí utilizados para inducir la extinción de la memoria espacial podrían producir estados de "desesperación" conductual, basados en la inmovilidad (Huston et al., 2009; Huston, Silva, Komorowski, Schulz, & Topic, 2013; Schulz et al., 2007). Sin embargo, nosotros no encontramos diferencias a este respecto entre el grupo que había tenido presente la plataforma durante la adquisición y el grupo control, en la distancia total nadada y el la velocidad media durante el protocolo de extinción. Por lo tanto, asumimos que el tiempo en que los animales de ambos grupos pasaron inmóviles fue equivalente. En este sentido, la inmovilidad podría estar causada por otros factores distintos a aquellos atribuibles al protocolo *per sé,* tales como la fatiga o la falta de motivación (también conocido como "desesperación conductual"), para buscar la plataforma de escape tras varios ensayos consecutivos de extinción. Por tanto, no podemos descartar la posibilidad de la falta

del reforzador (la plataforma de escape) en el laberinto de agua pueda inducir conductas relacionadas con la desesperación en ambos grupos. En suma, se podría pensar que este tipo de extinción puede ser entendido como un nuevo proceso de aprendizaje, ya que la conducta tras la extinción está, al menos en parte, influenciada por una forma de aprendizaje inhibitorio específico de contexto (Archbold, Bouton, & Nader, 2010; Bouton, Westbrook, Corcoran, & Maren, 2006).

5.2.2. La extinción de la memoria espacial afecta diferencialmente a la actividad metabólica del hipocampo dorsal y ventral y a las redes neurales asociadas

Con el fin de valorar las estructuras y redes neurales asociadas al proceso de extinción de la memoria espacial, estudiamos el metabolismo cerebral regional mediante el uso de la misma técnica histoquímica para la proteína CO mitocondria utilizada en los Artículos 1 y 2 (Artículo 4).

Los resultados obtenidos de nuestro trabajo sugieren que las regiones cerebrales tales como el hipocampo dorsal y ventral, la corteza parietal y la corteza retrosplenial, amígdala y los cuerpos mamilares participan activamente durante la extinción de la SRM.

Basándonos en los diferentes patrones de actividad Co y en las correlaciones entre estructuras que hemos encontrado, podemos decir que regiones particulares median distintos procesos que tienen lugar durante la extinción de esta memoria (Mendez-Couz et al., 2014).

En nuestros estudios se puso de manifiesto la participación de las áreas CA1 y el CA3 del hipocampo, el giro dentado ventral, la corteza retrosplenial y los núcleos medial de los cuerpos mamilares se reclutaron como regiones metabólicamente activas para completar con éxito la extinción de la memoria espacial. Sin embargo, estás ultimas estructuras no parecen tener un papel fundamental en fases más tardías del mismo proceso de extinción (Mendez-Couz et al., 2014).

Estos resultados concuerdan de nuevo, al igual que los resultados obtenidos de nuestros estudios de recuerdo (Artículos1-3), con el acuerdo común prevalente de una distribución hipocampo-cortical de los circuitos implicados en memoria espacial y su reorganización temporal (Bontempi et al., 1999; Conejo et al., 2013; Frankland & Bontempi, 2005; Mendez-Couz, Conejo, Gonzalez-Pardo, & Arias, 2015).

Aunque el estudio de expresión de genes tempranos c-Fos no encontró cambios en el hipocampo dorsal o ventral [Artículo 5, (Mendez-Couz et al., 2014)], la implicación de ambas subdivisiones en las fases tempranas de esta tarea no parece del todo sorprendente, dados los múltiples indicios que implican al hipocampo en la adquisición de la memoria espacial (Conejo et al., 2010; Conejo, Gonzalez-Pardo, Lopez, Cantora, & Arias, 2007; Fidalgo, Conejo, Gonzalez-Pardo, & Arias, 2014; Loureiro, Lecourtier, et al., 2012; Miyoshi et al., 2012). Actualmente, la teoría prevalente postula que la extinción de una conducta en particular puede ser entendida como un proceso de aprendizaje que implica la formación de nuevas memorias, aunque la traza de la memoria original se preservaría (Bouton et al., 2006).De hecho, algunos autores sugieren que los mecanismos moleculares que están detrás de la adquisición o la consolidación de una tarea de memoria espacial serían similares a los descritos durante la adquisición de la tarea original (Delamater & Lattal, 2014; Lattal et al., 2003; Szapiro, Vianna, McGaugh, Medina, & Izquierdo, 2003).

Este mencionado aspecto del sistema hipocampal podría explicar los valores más altos de actividad CO que se encontraron en el hipocampo dorsal CA1 en los animales que habían pasado una tarea de extinción espacial (grupo *Extinción*) en relación con aquellos que solo habían nadado (grupo *Naïve*).

Específicamente, nos encontramos con una mayor actividad CO en el campo CA1 del hipocampo dorsal y correlaciones CO directas entre el CA1 y la corteza retrosplenial. Según la teoría estándar de la consolidación de la memoria, ya comentada anteriormente, las memorias una vez dependientes del hipocampo pasarían a depender de los circuitos neocorticales (Alvarez & Squire, 1994; Squire & Alvarez, 1995), de hecho esta teoría ha sido probada en modelos animales en los que, tras una lesión en el hipocampo, las memorias adquiridas poco después del daño se pierden, mientras que aquellas adquiridas en un pasado remoto permanecen intactas (Milner, Squire, & Kandel, 1998). Por tanto, este resultado que muestra correlaciones funcionales hipocampo-corticales después del procedimiento de extinción apoyaría esta teoría de consolidación.

El giro dentado del hipocampo ventral también mostró una activación CO mayor en el grupo de extinción comparado con el grupo Naïve. El hipocampo ventral, conjuntamente con el parahipocampo y la amígdala se han relacionado con los procesos de aprendizaje bajo condiciones de estrés (Villarreal et al., 2002). Este resultado que encajaría perfectamente con nuestros datos, si tenemos en cuenta la ausencia de plataforma de escape en el laberinto de

agua, lo que fuerza al animal a adquirir nuevas estrategias. En este sentido, la estimulación a baja frecuencia del hipocampo ventral parece facilitar la extinción del miedo condicionado por contexto (Cleren et al., 2013). También es notable el hecho de que tanto los núcleos central como medial de la amígdala mostraron un patrón de actividad CO elevado, lo que reflejaría con esta idea. Siguiendo esta línea, aunque el complejo amigdalino permanece activo durante las fases finales de la extinción, ni el hipocampo dorsal ni el ventral parecen mantener esta activación, tal y como demuestra la técnica de inducción de proteína c-Fos, que es mucho más limitada en cuanto al rango temporal medido (Mendez-Couz et al., 2014). Este hecho parece sugerir una ventana temporal en fases tempranas de adquisición de la tarea de extinción en las que el hipocampo jugaría un papel fundamental, mientras que las nuevas memorias adquiridas pasarían a ser procesadas en circuitos neocorticales, lo que de nuevo encaja con la teoría estándar de la consolidación. De hecho, la corteza prefrontal se ha relacionado con procesos más tardíos de aprendizaje espacial en el laberinto acuático de Morris (Conejo et al., 2010) , y específicamente con la extinción de la una memoria espacial, tal y como revela el incremento de expresión de proteína c-Fos cerebral en las áreas prelímbica e infralímbica (Mendez-Couz et al., 2014), y por la reorganización que subyace a las redes neurales cuando esta tarea se lleva a cabo bajo una inactivación de la corteza prelímbica [Artículo 6(Mendez-Couz, Conejo, Vallejo, et al., 2015)].

Sorprendentemente, no nos encontramos diferencias en la actividad CO entre grupos. Esta falta de diferencias significativas en la región mPFC es coherente con los hallazgos anteriormente mencionados, ya que la inactivación del área prelímbica en ratas no afecta la adquisición de la tarea de extinción. En conjunto, estos resultados apoyarían la idea de una implicación tardía del mPFC, ya que se conoce que actúa como un modulador en la extinción a largo plazo (Milad & Quirk, 2002) , y en el mantenimiento de la extinción (Herry & Garcia, 2002, 2003).

El hallazgo de que la corteza retrosplenial mostrase un incremento de activación CO así como interacciones entre el campo CA1 dorsal en el grupo de extinción comparado con el naïve parece lógico si tenemos en cuenta tanto su conectividad con el hipocampo y con el núcleo anterior del tálamo (Aggleton, Wright, Vann, & Saunders, 2012; Van Groen & Wyss, 2003) y los resultados de los estudios de lesión que apuntan firmemente a una implicación importante de esas regiones en lamemoria espacial (Vann & Aggleton, 2002). Específicamente, el efecto de lesiones en esta área se relacionó con la experiencia previa de la

rata en el laberinto acuático (Cain, Humpartzoomian, & Boon, 2006). De manera adicional, en relación a las tareas de memoria espacial de referencia en el laberinto acuático, los animales ya habían aprendido la localización de la plataforma con referencia a varias pistas visuales usando para ello el mapa cognitivo de una manera flexible (R. Morris, 1981; O'Keefe & Nadel, 1978; Tolman, 1948). Algunos autores sugieren que la corteza retrosplenial estaría ligada a la determinación de una localización cuando las pistas visuales se utilizan de una manera flexible (Hindley, Nelson, Aggleton, & Vann, 2014). Esto coincidiría con la situación en la que los animales se encuentran en nuestro estudio, ya que durante el proceso de extinción los animales son conscientes de la ausencia del reforzador que antes estaba presente, lo que requiere poner en marcha una nueva estrategia de escape, en este caso, el mapa cognitivo previamente adquirido podría ser utilizado para el nuevo aprendizaje necesario durante la tarea de extinción.

Como era previsible, nos encontramos con una correlación directa ente el hipocampo dorsal y los núcleos central y medial del complejo amigdalino. Estos resultados realzan los encontrados en otros estudios previos que demuestran la implicación de la amígdala a través del proceso de extinción. A este respecto, en condicionamiento de miedo Pavloviano es de sobra conocido que la amígdala tiene una función vital en la consolidación de memorias relacionadas con el miedo y las emociones, tal y como se demuestra mediante un modelo de miedo y desórdenes de ansiedad cuyo uso está ampliamente extendido (McGaugh, 2002). Es más, la amígdala basolateral se cree implicada en la formación y en la extinción de las memorias de miedo (Akirav & Maroun, 2007). Así, conjuntamente con los núcleos centrales y mediales de la amígdala, encontramos diferencias en el núcleo basolateral en fases tardías de la extinción espacial (Mendez-Couz et al., 2014), sin embargo, solo los núcleos central y medial mostraron diferencias significativas en la actividad metabólica entre grupos. De hecho, un estudio en el que se emplearon lesiones mostró que la expresión del condicionamiento de miedo se bloqueaa, pero aun así la extinción de la memoria adquirida tuvo lugar (Anglada-Figueroa & Quirk, 2005).

Por otro lado, el grupo de extinción mostró correlaciones de actividad CO entre el hipocampo dorsal y la amígdala, ya que los núcleos lateral y central de este complejo mostraron interacciones con el campo CA3 del hipocampo, mientras que esas conexiones se mostraron con el hipocampo ventral en el grupo control nado.

De acuerdo con los resultados del estudio del Artículo 5, los cuerpos mamilares mostraron diferencias en su actividad metabólica entre los grupos, con una actividad más elevada en el grupo control respecto al experimental. La asociación entre los cuerpos mamilares y el hipocampo no parece sorprendente del todo dadas las conexiones anatómicas y funcionales funcionales entre ellos (principalmente a través del fornix postcommisural). Cabe decir que aunque los núcleos medial y lateral están conectados a las mismas estructuras,estas conexiones se producen entre diferentes subregiones, por lo tanto forman un par de sistemas paralelos (Vann & Aggleton, 2004) que pueden estar detrás de la diferente implicación de ambos en esta tarea.

Así, los cuerpos mamilares han estado tradicionalmente asociados en humanos con la memoria, especialmente debido a la naturaleza amnésica del síndrome de Korsakoff que se relaciona con el daño en esta zona (Hildebrandt, Muller, Bussmann-Mork, Goebel, & Eilers, 2001; Mayes, Meudell, Mann, & Pickering, 1988; Tanaka, Miyazawa, Akaoka, & Yamada, 1997). Además, la conexión directa via fornix entre esos núcleos y el hipocampo ha centrado la mayoría de las investigaciones en aprendizaje espacial (Conejo et al., 2004; Mendez-Lopez, Mendez, Lopez, & Arias, 2009a; Santin et al., 2003; Sziklas & Petrides, 1993; Vann, 2010). Actualmente, la mayoría de los trabajos se refieren al papel de los cuerpos mamilares en la memoria resaltando la importancia de las eferencias del hipocampo a los núcleos (Aggleton & Brown, 1999). Sin embargo, se sabe que el núcleo lateral y el núcleo medial difieren en múltiples aspectos tales como su morfología (Veazey, Amaral, & Cowan, 1982), propiedades electrofisiológicas (Sharp & Turner-Williams, 2005; Sharp, Turner-Williams, & Tuttle, 2006; Stackman & Taube, 1998) y conexiones. En este sentido, el núcleo lateral mamilar está asociado con la navegación espacial, ya que contienen tanto las células de dirección de la cabeza como las células de dirección angular (Stackman & Taube, 1998). Sin embargo, su relevancia en memoria es aún una cuestión en debate, ya que algunos autores observaron que las lesiones bilaterales de este núcleo causaron solo ligeras alteraciones en animales evaluados en tareas de alternancia en un laberinto en T, o en tareas de memoria de trabajo (Vann, 2005). Por otro lado, estos núcleos mostraron niveles bajos de expresión c-Fos relacionados con la tarea de extinción de SRM que nos ocupa, cuando la tarea se realizaba bajo los efectos de una inactivación del área prelímbica , tal como se observa en el Artículo 6 (Mendez-Couz, Conejo, Vallejo, et al., 2015).

Cabe destacar que no solo los núcleos laterales mostraron diferencias entre los animales que habían llevado a cabo una tarea de extinción y sus controles nado, también sucedió en el núcleo medial de los cuerpos mamilares. En este sentido, se cree que los núcleos mediales están relacionados con el sistema de ritmo theta, ya que la actividad tetha de estos núcleos está conducida por el campo CA1 del hipocampo (Kocsis & Vertes, 1994). Este hecho podría explicar la gran conexión entre estas estructuras encontrada en el grupo de extinción comparado con el grupo control. Esto seguiría la línea de los resultados de los trabajos de Kirk and Mackay (2003) que sugieren que los núcleos mediales actuarían como un centro de relevo del ritmo theta hipocampal, proyectando al diencéfalo y de nuevo al hipocampo, lo subyacería a una codificación con éxito. Esto podría aclarar por qué estos núcleos parecen tener un papel relevante en el inicio de la adquisición de la tarea de extinción, mientras que solo los laterales aparecen en las fases más tardías (Mendez-Couz et al., 2014). Además, los cuerpos mamilares mediales y sus proyecciones al núcleo anterior del tálamo se creen necesarias para que tanto el hipocampo como la corteza retrosplenial funcionen con normalidad, tal y como demuestran los trabajos basados en lesión relacionados con deterioros en la codificación rápida basada en estrategias alocéntricas [ver la revisión de Vann (2010)] en los que se remarca el efecto de la lesión en las fases iniciales del aprendizaje. De acuerdo con la revisión escrita por esta autora, la teoría clásica, basada en la que las proyecciones desde el hipocampo al diencéfalo vía el fornix deberían ser revisadas, incluyendo en ella las proyecciones directas desde estas estructuras a la formación hipocampal, para una memoria totalmente integrada. Sin embargo se necesitarían otros estudios de lesiones, para arrojar luz en el papel específico de los núcleos central y lateral de los cuerpos mamilares en la extinción de la memoria espacial.

5.2.3. La extinción de la memoria espacial se relaciona con cambios en la expresión de la proteína c-fos de la corteza prefrontal la, amígdala y mamilar lateral, además,los resultados concuerdan con los de inactivación del área prelímbica.

Los resultados de nuestro trabajo (Artículo 5) sugieren que las regiones cerebrales tales como el córtex prefrontal, amígdala y los cuerpos mamilares están activamente implicados en la memoria espacial durante los procesos finales de la extinción de la misma. La expresión de la proteína c-Fos se elevó en las áreas prelímbica e infralímbica de la corteza prefrontal y del complejo admigdalino en relación con este proceso.

La relación de la amígdala con la memoria espacial ya ha sido explicada anteriormente, además, recordemos que los núcleos central y medial presentaron diferencias de actividad CO (Artículo 4).

Si nos referimos al núcleo lateral de la amígdala, en particular, se cree relacionado con los procesos iniciales de la extinción (Herry, Trifilieff, Micheau, Luthi, & Mons, 2006; Sotres-Bayon, Cain, & LeDoux, 2006), y con la expresión de la extinción a través de la inhibición de las señales salientes (Likhtik, Popa, Apergis-Schoute, Fidacaro, & Pare, 2008; Quirk, Likhtik, Pelletier, & Pare, 2003). En este sentido, la activación del núcleo basolateral parece ser necesaria para la retención a largo plazo del condicionamiento de miedo (Davis & Bauer, 2012). De igual manera, Porte et al. (2011) ha sugerido un papel importante del núcleo basolateral de la amígdala durante la extinción espacial en el laberinto de agua, tanto en fases iniciales como en fases más tardías. Según estos autores, esta estructura actuaría como un modulador entre los aspectos no emocionales (basados en el hipocampo) y los emocionales (basados en la amígdala) de la experiencia de aprendizaje espacial en el laberinto de agua. Al igual que estaría implicada en procesos de aprendizaje bajo condiciones de estrés ya comentados(Dolcos, LaBar, & Cabeza, 2004). En esta misma línea, los estudios farmacológicos sugieren que la amígdala forma parte de un circuito implicado en implicado en la conducta de memoria espacial manteniendo la asociación entre las representaciones de lugar, que son dependientes del hipocampo, y su significado conductual (Vafaei, Jezek, Bures, Fenton, & Rashidy-Pour, 2007). En este sentido, los cambios en actividad neuronal en la amígdala lateral relacionados con la extinción (Quirk, Repa, & LeDoux, 1995; Repa et al., 2001) son modulados por el hipocampo(Hobin, Goosens, & Maren, 2003; Maren & Hobin, 2007) y la inactivación del hipocampo altera la extinción de la memoria espacial (Corcoran, Desmond, Frey, & Maren, 2005). Sin embargo, en nuestro trabajo, los cambios en el hipocampo se encontraron al inicio de la tarea (Artículo 4) y no al final, lo que explica por qué otros autores no parecen encontrar al hipocampo implicado en la tarea de extinción (Archbold et al., 2010; Bonini et al., 2011; Hobin, Ji, & Maren, 2006; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011).

Al igual que en el caso del complejo amigdalino, también se encontraron, en el trabajo representado en el Artículo 5, un incremento de células inmunoreactivas para c-Fos en la corteza prefrontal, que se relacionó con la extinción de la SRM. Tal como hemos explicado, estas áreas se creen implicadas en fases tardías de SRM (Conejo et al., 2013; Conejo et al., 2010; Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2007) e, independientemente, en la extinción

de varias tareas de aprendizaje(Milad & Quirk, 2002; Nic Dhonnchadha et al., 2012; Stafford, Raybuck, Ryabinin, & Lattal, 2012; Thompson et al., 2010; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). También ha de considerarse que al retirar la plataforma los animales han de buscar una estrategia de escape alternativa, por lo que los animales continúan explorando el ambiente. Este cambio de estrategia requiere que los animales integren de manera diferente las posibles configuraciones entre los mismos estímulos para tratar de encontrar la plataforma que ha sido eliminada, y este cambio de estrategia se ve alterados cuando la corteza prefrontal medial está lesionada (Rich & Shapiro, 2007; Yoder & Taube, 2011). Estos resultados coinciden tanto con los estudios de histoquímica CO (Artículo 4) como con los estudios de lesión, en los que se observa una reordenación de las correlaciones de la red metabólica neural que subyace al proceso cuando la corteza prelímbica está inactivada (Mendez-Couz, Conejo, Vallejo, et al., 2015), luego podríamos decir que aunque la corteza prefrontal medial parece estar implicada en todo el proceso de extinción, las funciones específicas de las distintas áreas parecen ser temporalmente dependientes.

Por otra parte, ya hemos dicho que la orientación espacial requiere una representación espacial totalmente actualizada de la dirección de la cabeza, lo que se conduce por las células de dirección de la cabeza (Yoder & Taube, 2011). Éstas se encuentran, conjuntamente con el tálamo, en los cuerpos mamilares. A pesar de que, como hemos comentados, muchos estudios de lesión apuntan a una función importante de los cuerpos mamilares en la memoria espacial y el aprendizaje asociativo (Vann, 2010; Vann & Aggleton, 2003; Vann, Honey, & Aggleton, 2003),hasta la fecha hay muy pocos estudios que hayan llevado a cabo un estudio de mapeo funcional de genes para evaluar la contribución específica de esta estructura. Esto podría deberse, al menos en parte, con los bajos niveles de expresión de los genes de expresión inmediata en esta estructura, tales como c-fos o zip-268(Amin, Pearce, Brown, & Aggleton, 2006; Jenkins, Amin, Brown, & Aggleton, 2006). Sin embargo, cuando se consiguió medir cambios en el nivel de la proteína c-Fos , se encontraron cambios en el núcleo lateral, pero no en el medial, tras un condicionamiento de miedo (Conejo, Gonzalez-Pardo, Lopez, et al., 2007) o tras el entrenamiento de memoria de trabajo(Mendez-Lopez et al., 2009a) espacial. En este caso nos encontramos con un menor nivel de proteína c-Fos en los animales experimentales respecto a los controles, lo que podría dar a entender que el sistema de dirección de la cabeza mencionado, no sería necesario en la parte final de este proceso, aunque podría ser necesario para interaccionar con diferentes esquemas de codificación en estructuras relacionadas para formar representaciones espaciales precisas (Mizumori & Leutgeb, 2001; Valerio et al., 2010).

Este núcleo también presenta una baja actividad CO, respecto a los controles (Artículo 4), lo que estaría de acuerdo con esta teoría.

5.3. ESTUDIO DE LA IMPLICACION DEL NEUROPEPTIDO Y EN LA MEMORIA ESPACIAL

En el Artículo 8 se estudia, la implicación del neuropéptido Y en los sistemas de memoria de referencia espacial que previamente hemos estudiado en los Artículos anteriores. Para ello, se analizan los efectos de la infusión en el area CA1 del hipocampo dorsal de un antagonista del receptor Y2 del neuropéptido Y (NPY), denominado BIIE0246, observándose sus efectos sobre la actividad espontánea, conductas de tipo ansioso y memoria espacial. Adicionalmente se estudiaron mediante distintas técnicas de biología celular y molecular, los cambios a nivel regional que se producían en la expresión del NPY y de sus receptores NPY Y1 y NPY Y2, así como la actividad metabólica cerebral, mediante el empleo de la técnica histoquímica para la CO oxidasa usada anteriormente.

Los resultados obtenidos, que se muestran en el Artículo 8 muestran, según nuestros conocimientos, la primera demostración de la expresión regional de Y_2R en los procesos mnésicos tales como la memoria espacial. Además, mostramos que NPY y la expresión de sus receptores Y_1R y Y_2R cambia de manera regional, y las áreas que previamente habían sido relacionadas con la memoria espacial, tales como las incluidas en el sistema hipocampal extendido, ven modificada su actividad metabólica, tras la inhibición del Y_2R en el hipocampo dorsal.

A nivel conductual, tras la infusión del fármaco nos encontramos con una mejora de la ejecución de la tarea de memoria espacial en el MWM. Sin embargo, no se encontraron diferencias en la evitación del brazo abierto en el laberinto en Zero elevado (conductas relacionadas con ansiedad) ni con la actividad horizontal espontánea. Además, al analizar los cambios cerebrales regionales, se encontraron diferencias, no solo en el nivel del receptor Y_2R, sino también en el del receptor Y_1R y del NPY propiamente, en áreas tales como el hipocampo o la corteza prefrontal. Estos resultados se vieron confirmados por los cambios en actividad metabólica expresados en cambios CO, que no se limitaron a los circuitos hipocampo-prefrontal córtex, sino que también se observaron en otras áreas que han sido relacionadas con la memoria espacial, tales como el tálamo [Artículo 1 (Mendez-Couz, Conejo, Gonzalez-

Pardo, et al., 2015)] y los cuerpos mamilares [Artículos 4-7, (Mendez-Couz et al., 2014; Mendez-Couz, Conejo, Vallejo, et al., 2015)]. En conjunto, nuestros resultados apoyan la idea de que el sistema NPY está implicado en las funciones de memoria espacial, resaltando el papel de la distribución desigual de Y_2R y Y_1R en el proceso.

Desde hace años, se conoce que las lesiones tanto en el hipocampo como en el tracto fimbria-fornix pueden produce hiperactividad en ratas. Esto se cree debido a la pérdida de aferencias glutamatérgicas del hipocampo al núcleo accumbens, lo que produce cambios en los receptores de dopamina de este último.(Bannerman et al., 2001; Bannerman et al., 2004; Douglas & Isaacson, 2014; Kimble, 1963; Whishaw & Mittleman, 1991). Sin embargo, nosotros no encontramos resultados que nos hagan pensar en hiperactividad en los animales experimentales en relación con los animales del grupo control, tal y como se demostró por la falta de diferencias en la actividad espontánea horizontal medida por el test de actímetros, o por la distancia total recorrida en el test de laberinto en zero elevado.

Por otra parte, también se cree que el sistema NPY está asociado con conductas relacionadas con ansiedad y con la regulación del estado de ánimo (dos Santos et al., 2013; Heilig, 2004; Holzer, Reichmann, & Farzi, 2012; Thorsell & Heilig, 2002; Thorsell et al., 2000). A pesar de estos precedentes hallados en la literatura, no se encontraron diferencias entre el grupo experimental y control en la cantidad de tiempo invertido en explorar el brazo abierto en el laberinto en Zero elevado, es decir, el índice de reducción de conductas relacionadas con la ansiedad en este test. En este sentido, aunque se han descrito efectos ansiolíticos de esta droga en este paradigma, o en el equivalente laberinto elevado "en cruz", se cree que los efectos ansiolíticos serían dependientes de la dosis y de la región de infusión del fármaco (Goncalves, Martins, Baptista, Ambrosio, & Silva, 2015; Heilig, 2004). Además, distintos receptores, no soloY_1R o Y_2R sino tambien Y_5R pueden contribuir a los efectos ansiolíticos del NPY (Trent & Menard, 2011). De hecho, los resultados arrojados por el trabajo de Zambello et al. (2011) mostró que los efectos semejantes a ansiolíticos o antidepresivos no se podían confirmar en ratones *Knockout* para el receptor Y_2R.

La última tarea a la que se sometió a los animales de este experimento fue una tarea de memoria espacial en el paradigma del laberinto acuático de Morris. A este respecto, nuestros resultados muestran que ambos grupos de animales adquirieron la tarea de memoria espacial. Para ello se analizaron los resultados de los parámetros utilizados en todos los Artículos anteriores para medir la adquisición de la tarea, es decir, disminución de las latencias de

escape y una buena ejecución de la prueba de retención, en la que debían invertir más tiempo explorando el cuadrante virtual previamente reforzado, que se llevó a cabo tras cada sesión de entrenamiento. Sin embargo, tras la infusión de la droga, en el tercer día en el MWM, el grupo experimental mostró una ejecución mejor, reduciendo el tiempo necesario para alcanzar la plataforma en comparación con los animales del grupo control. Además, la diferencia entre el tiempo medio que invirtieron en el cuadrante de escape en comparación con el resto fue mayor en el grupo experimental. En definitiva, ambos grupos adquirieron la tarea, tal y como se esperaba, pero el grupo experimental cumplió más eficazmente en esta tarea de memoria espacial.

Estos resultados concuerdan con los datos obtenidos por Redrobe, Dumont, Herzog, and Quirion (2004), en los que muestran un empeoramiento de la ejecución de la prueba de retención en el MWM en animales *Knockout* para el receptor Y_2R. Sin embargo otros autores (dos Santos et al., 2013) alegan que, en un modelo murino de alzhéimer, la habilidad del NPY para prevenir los déficits en memoria espacial estarían más bien relacionados con un efecto protector del péptido contra la toxicidad acumulada del péptido AB, y no con un efecto pronemónico por sí mismo. Este concepto sería coherente con la idea del Y_2R como una de las principales dianas que subyacen los efectos neuroprotectores del NPY. En consonancia con lo anterior, estudios previos mostraron que la administración de antagonistas del receptor Y2 eliminaba los efectos de protección del NPy (dos Santos et al., 2013; Silva, Carvalho, Carvalho, & Malva, 2003; Silva, Kaufmann, et al., 2005; Silva, Xapelli, Grouzmann, & Cavadas, 2005; Smialowska et al., 2009).

En cuanto a los estudios moleculares, los análisis de Western blot llevados a cabo mostraron una expresión elevada del gen que codifica para la proteína Y2R en el hipocampo, con una disminución de los niveles de proteína en la corteza prefrontal, en comparación con el grupo control salino. En los que respecta a Y1R, solo se encontraron diferencias entre grupos en la región de la corteza prefrontal, siendo mayores en el grupo experimental que en el control.

Dados estos hallazgos, parece que tras la inyección del antagonista de Y2R BIIE0246, el hipocampo presenta una regulación al alza del receptor nombrado. Esta podría ser una forma de contrarrestar la función del receptor que se ha perdido de manera temporal. En relación, se sabe que este receptor desempeña numerosas funciones en el sistema nervioso, tales como en la excitabilidad neuronal (Silva, Xapelli, et al., 2005) o en los procesos cognitivos (Botterill,

Guskjolen, Marks, Caruncho, & Kalynchuk, 2014), que podrían explicar su función en la memoria espacial. De esta manera, un incremento de los niveles de expresión de Y2R a nivel hipocampal podría estar de la alteración en la ejecución de la memoria espacial observado, aunque se necesitan más estudios para entender el proceso a un nivel molecular. En consonancia, el incremento de los niveles de expresión de Y1R que se encontró en el mPFC deja de ser sorprendente, teniendo en cuenta que la expresión de Y2R fue menor, un incremento en el resto de receptores podría ser un intento de contrarrestar la función perdida. Apoyando esta hipótesis, se sabe que RNA mensajero de Y_1 se encuentra por defecto en mayores cantidades en esta región (Parker & Herzog, 1999). Además, la falta de cambios encontrada en el hipocampo podría guardar relación con las diferentes implicaciones que estos estos receptores tienen sobre diferentes funciones. Así, el Y_1R tiene un papel muy importante en la ansiedad y la depresión (Holzer et al., 2012) , pero no se han encontrado efectos ansiogénicos o ansiolíticos en este experimento. Tal y como mencionamos antes para Y2R, la regulación molecular para Y1R tanto en el hipocampo como en la mPFC podría apuntar a una posible implicación del receptor en las funciones de aprendizaje y memoria estudiadas.

Por otra parte, el estudio de cuantificación de los resultados de la inmunohistoquímica contra la proteína NPY mostraron un descenso general del nivel de la proteína en el grupo experimental a nivel hipocampal (CA1 y CA3) y giro dentado. En contraste, la corteza prefrontal presentó una elevada expresión de NPY en el grupo experimental *vs* el control.

La sola administración del antagonista Y2R en el CA1 del hipocampo dorsal produjo cambios en el sistema NPY, no solo en el área de infusión sino que también cambiaron áreas estrechamente conectadas a ella, como la corteza prefrontal. Como hemos mencionado a lo largo de toda esta tesis, el circuito hipocampo-córtex prefrontal juega un papel crítico en la adquisición y la consolidación de la memoria espacial. Específicamente, las memorias que son inicialmente dependientes del hipocampo pasan a depender de circuitos hipocampo-corticales y finalmente del neocórtex (Frankland & Bontempi, 2005; Leon et al., 2010; Smith & Squire, 2009; Squire & Alvarez, 1995). Sin embargo , se necesitan más estudios en este sentido para explicar los cambios metabólicos para que se den estos cambios funcionales en regiones distales.

Cuando se analizó el metabolismo oxidativo a través de una histoquímica CO, se observaron cambios en diferentes regiones. Según lo esperado, hubo cambio en el área

prelímbica de la cortea prefrontal, que presentó una actividad CO menos, mientras que el hipocampo dorsal vió esta actividad incrementada. De nuevo, estos resultados estarían de acuerdo con la teoría de la consolidación de la memoria y con la implicación tardía de la corteza prefrontal en los procesos de adquisición de memoria espacial (Conejo et al., 2010; Conejo, Gonzalez-Pardo, Vallejo, et al., 2007; Fidalgo, Conejo, Gonzalez-Pardo, Lazo, & Arias, 2012), en el recuerdo (Conejo et al., 2013; Mendez-Couz, Conejo, Gonzalez-Pardo, et al., 2015) o la extinción de la memoria espacial (Mendez-Couz et al., 2014; Mendez-Couz, Conejo, Vallejo, et al., 2015).

Por otra parte, el CA1 del hipocampo ventral presentó una actividad CO significativamente menor en el grupo experimental. Como hemos comentado previamente, la porción ventral del hipocampo ha sido relacionado con emociones (Fanselow & Dong, 2010), en contraste con las funciones visuo-espaciales y cognitivas que se le atribuyen a la porción dorsal (R. G. Morris et al., 1982; Moser & Moser, 1998b). Teniendo esto en cuenta se podría pensar en una activación CO en esta área relacionada con los efectos de la infusión de la droga en situación de ansiedad o estrés, sin embargo, no se encontraron diferencias en otras áreas típicamente relacionadas con la ansiedad, tales como la amígdala o la corteza perirrinal (Villarreal et al., 2002), estos resultados son coherentes con la no alteración de la conducta relacionada con ansiedad observada en el EZM. Luego las diferencias encontradas en la ejecución de la tarea de memoria espacial en el MWM no parecen ser atribuibles a efectos ansiolíticos o ansiogénicaos del fármaco.

Asimismo, las regiones límbicas, asociadas con la formación hipocampal e incluidas en el circuito de Papez, tales como el núcleo anterior del tálamo y los cuerpos mamilares y el núcleo accumbens presentaron igualmente cambios en su metabolismo, mostrando un nivel elevado en el primero en relación al grupo control, y una disminución en actividad CO en los cuerpos mamilares en el experimental respecto al control. Ambos núcleos se creen estrechamente ligados a la memoria espacial (Aggleton & Brown, 1999; Da Cunha et al., 2009; Lopez et al., 2009; Loureiro, Cholvin, et al., 2012; Mendez-Couz, Conejo, Gonzalez-Pardo, et al., 2015; Mendez-Couz et al., 2014; Mendez-Lopez et al., 2009b; Mendez et al., 2008; van Groen et al., 2002; Vann, 2010; Vann et al., 2003; Warburton & Aggleton, 1999), ya que forman parte de las estructuras que contienen el Sistema de dirección de la cabeza (Stackman & Taube, 1998; Taube, 1995; Vann, 2011; Wilton, Baird, Muir, Honey, & Aggleton, 2001) que se supone esencial para la navegación espacial.

Conclusiones

CONCLUSIONES:

1- Tras haber adquirido una tarea de memoria de referencia espacial en el laberinto acuático de Morris, los animales son capaces de ejecutar con éxito una tarea de recuerdo a la semana de haber finalizado el aprendizaje. Asimismo, una vez adquirida la conducta, se observó una extinción completa de la misma tras cuatro sesiones de extinción y no presenta recuperación espontánea a las 24 horas.

2-Tras una tarea de extinción de memoria espacial, se producen cambios a nivel de la expresión de proteína c-Fos cerebral en áreas del sistema límbico tales como las áreas prelímbica e infralímbica de la corteza prefrontal, núcleos central y basal de la amígdala y el núcleo lateral de los cuerpos mamilares.

3-El recuerdo de una tarea de memoria de referencia espacial en el laberinto acuático de Morris produce cambios en los niveles de actividad metabólica cerebral regional a nivel de la corteza prefrontal, en el núcleo estriado, del mismo modo encontramos cambios en el tálamo anterodorsal y en el giro dentadodel hipocampo, tanto en su porción dorsal como ventral.

La evaluación de la extinción de la memoria espacial en el mismo laberinto se asocia con cambios de actividad citocromo oxidasa a nivel de las cortezas pariteal y retrosplenial, así como en el hipocampo dorsal y ventral, en el núcleo central de la amígdala y en los cuerpos mamilares.

4-Los patrones de correlación de actividad funcional entre estructuras implicadas en el recuerdo de la memoria espacial incluyen asociaciones complejas, que implican interacciones entre la corteza prefrontal, el tálamo estriado y el hipocampo en su porción dorsal y ventral.

Las redes neurales que subyacen a la ejecución de la extinción de la memoria espacial incluyen coxiones entre la corteza retrosplenial, el hipocampo, la amígdala y los cuerpos mamilares.

5.A)- La tarea de recuerdo de la memoria especial de referencia se altera por la inactivación temporal del área prelímbica de la corteza prefrontal como por la inactivación, unilateral o bilateral, del hipocampo dorsal. El recuerdo de la memoria espacial depende de la integridad

del sistema hipocampal incluso varias semanas después de la tarea inicial. Esta tarea recluta, de diferente manera, diferentes subáreas del hipocampo, y las redes neurales en el circuito hipocampo-prefrontal varían en función del grado de inactivación hipocampal.

5.B)- La inactivación temporal del área prelímbica cambia las redes neurales implicadas en la extinción de una tarea de memoria espacial, aunque su integridad no parece esencial para la ejecución de la tarea. Los cuerpos mamilares muestran una actividad metabólica elevada después de la lesión, resaltando el papel de esta estructura en el proceso. Asimismo, la inactivación hipocampal altera la tarea, aunque no se pueden descartar alteraciones en los necesarios procesos de recuerdo que interfieran en este deterioro.

6- Los cambios en los niveles de expresión del neuropéptido Y y de sus receptores Y_1R e Y_2R a nivel del hipocampo y la corteza prefrontal están asociados con mejoras en la ejecución de una tarea de memoria espacial el el laberinto acuático de Morris. Esto modifica, además, la actividad metabólica de áreas que previamente habían sido relacionadas con la memoria espacial, tales como corteza prefrontal, el tálamo, el hipocampo tanto en su porción dorsal como ventral y los cuerpos mamilares.

7-CONCLUSIONS

1- Animals that were trained in a hidden platform-reference spatial memory task in the Morris water maze are able to successfully complete a retrieval task one week after acquiring the task. Similarly, the previously learned task is successfully extinguished after four extinction sessions and it does not present spontaneous recovery 24 h later.

2- Extinction of the spatial memory is related with higher numbers of c-Fos positive nuclei in the prefrontal cortex and amygdala; whereas lateral mammillary bodies of animals that have completed a extinction task presented lower c-Fos expression levels than in a control group.

3- Retrieval of the previously learned task is associated with increased levels of oxidative metabolism in the prefrontal cortex, dorsal and ventral striatum, antherodorsal thalamic nucleus and the dentate gyrus of the dorsal and ventral hippocampus.

Extinction of the original spatial learning task significantly altered the metabolic activity in the dorsal and ventral hippocampus, the retrosplenial and parietal cortices, the medial and central amygdaloid nuclei and the lateral mammillary bodies.

4- Retrieval of spatial memory is associated to novel activation patterns of brain networks involving prefrontal cortex, thalamus, striatum as well as dorsal and ventral hippocampus.

Brain networks underlying performance of the extinction of spatial memory included connections among mammillary bodies, amygdala, the hippocampus and retrosplenial cortex.

5.A) Retrieval of the spatial reference memory task is altered both by temporal prelimbic area temporal inactivation and by unilateral or bilateral inactivation of dorsal hippocampus. Retrieval of spatial memories depends on the integrity of the hipocampal system even several weeks after the initial training. Retrieval recruits differentially the hippocampal subregions, and functional brain networks in prefrontal-hippocampus circuitry vary according to the degree of the hippocampal blockade.

5.B) Temporal inactivation of the prelimbic area also changes brain functional networks following spatial memory extinction task, although its integrity seems not to be essential for

the task completion. Mammillary bodies show an increased metabolic activity after the lesion, highlighting the role played by this structure in the extinction process.

Inactivation of dorsal hippocampus alters spatial memory extinction task, although we cannot rule out the possibility of the necessary retrieval process disruption interference.

6- Infusion of NPY Y_2antagonist into the dorsal hippocampus modifies spatial memory task execution in the Morris Water Maze in rats. Additionally, regional NPY and Y_2R-Y_1R receptors expression changes in the prefrontal cortex and hippocampus are associated to this task. Similarly, it modifies metabolic activity in brain areas typically related to spatial memory, as hippocampus, prefrontal cortex, thalamus and diencephalic structures as the mammillary bodies.

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