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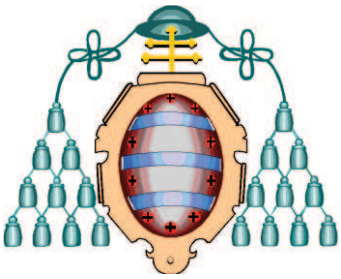

PROGRAMA DE DOCTORADO DE NEUROCIENCIAS

REDES NEURONALES DE LA MEMORIA ESPACIAL: USO DE ESTRATEGIAS EGOCÉNTRICA Y DE GUÍA

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ÍNDICE

LISTA DE TRABAJOS ORIGINALES

Esta tesis se basa en los siguientes artículos

I. Fidalgo C, Conejo NM, González-Pardo H, Arias JL. Functional interaction between the dorsal hippocampus and the striatum in visual discrimination learning. 2012. J Neurosci Res. 90(3):715-20. DOI: 10.1002/jnr.22774.

II. Fidalgo C, Conejo NM, Gonzalez-Pardo H, Arias JL. Dynamic functional brain networks involved in simple visual discrimination learning. (manuscrito).

III. Fidalgo C, Conejo NM, González-Pardo H, Arias JL. Cortico-limbic-striatal contribution after response and reversal learning: a metabolic mapping study. 2011. Brain Res.12; 1368:143-50.

IV. Fidalgo C, Conejo NM, González-Pardo H, Lazo PS, Arias JL. A role for dorsal and ventral hippocampus in response learning. Neurosci Res. 2012 DOI: 10.1016/j.neures.2012.03.011.

V. Fidalgo C, Conejo NM, Gonzalez-Pardo H, Arias JL. Effect of lighting conditions on brain network complexity associated with response learning. (manuscrito en revisión en la revista Brain Structure & Function).

RESUMEN

Tradicionalmente se ha considerado que el hipocampo y el cuerpo estriado forman parte de sistemas de memoria diferentes e independientes. Aunque existen evidencias de que al menos en determinados aprendizajes de orientación espacial existe una interacción entre el hipocampo y el estriado, en el aprendizaje de respuesta y de discriminación visual, aún no se conoce con exactitud el papel exacto de estas estructuras, junto con otras regiones del cerebro anatómicamente relacionadas. Con el fin de esclarecer la contribución de posibles redes cerebrales subyacentes a ambos tipos de aprendizaje, inicialmente ratas macho de la cepa Wistar fueron entrenadas haciendo uso de un laberinto acuático en T en un aprendizaje de respuesta, en el que cual una plataforma de escape se encontraba en la misma posición durante todos los días de entrenamiento, o bien en un aprendizaje de discriminación visual, donde la posición de la plataforma de escape se asoció con una señal visual intralaberíntica. Para determinar la participación de diversas estructuras cerebrales corticales y subcorticales hicimos uso de la técnica histoquímica citocromo c oxidasa (CO) en todos los experimentos, la cual nos proporciona un índice del metabolismo oxidativo cerebral. Del mismo modo, se determinaron los niveles relativos de la subunidad I de la proteína CO mediante western blot para esclarecer la contribución de esta subunidad a la actividad de la enzima y al aprendizaje. Nuestros resultados muestran que las ratas evaluadas en un paradigma de aprendizaje de respuesta requieren menos ensayos para dominar la tarea que los animales evaluados en una tarea de discriminación visual. Por otra parte, encontramos que la actividad metabólica cerebral es diferente de acuerdo a la tarea de aprendizaje espacial realizada por los animales. En el aprendizaje de discriminación visual evaluamos la contribución de distintas estructuras cerebrales, incluyendo el estriado dorsal y el hipocampo en distintos momentos del proceso de aprendizaje. Encontramos que tanto el cuerpo estriado como el hipocampo dorsal son necesarios en la adquisición de una tarea de discriminación visual. Además, regiones asociadas con la novedad, las emociones y la orientación espacial, como son el núcleo accumbens, la amígdala, la corteza parietal y el hipocampo parecen ser relevantes durante la fase inicial del entrenamiento, mientras que la corteza prefrontal se requiere a lo largo de todo el proceso de aprendizaje. Por otro lado, al analizar las redes cerebrales implicadas en el aprendizaje de la respuesta observamos una red córtico-límbico-estriatal, hallándose una mayor actividad metabólica neuronal en la corteza prefrontal, el estriado dorsal, la amígdala, el área tegmental ventral y en el hipocampo dorsal y ventral. Sin embargo, no se observaron cambios en los niveles relativos de la subunidad I catalítica de la enzima CO en el hipocampo. Finalmente se analizó el efecto de las condiciones lumínicas en las redes del cerebro implicadas en el aprendizaje de la respuesta. La corteza parietal y el hipocampo ventral se asociaron con la adquisición de una tarea de aprendizaje de respuesta independientemente de las condiciones lumínicas. Sin embargo, en oscuridad se encontró la participación de un conjunto más amplio de estructuras que incluye regiones corticales, el sistema límbico y el estriado. En resumen, nuestros resultados apoyan que tanto el hipocampo, como el cuerpo estriado, son estructuras clave en los aprendizajes de respuesta y de discriminación visual.

ABSTRACT

The hippocampus and the striatum traditionally have been considered as part of different and independent memory systems. Although there is evidence that supports a functional interaction between the hippocampus and the dorsal striatum at least in particular spatial learning tasks, the precise role of these structures together with anatomically related brain regions on response learning and visual discrimination learning is still unclear. We aimed to assess the contribution of particular brain networks underlying both types of learning, male Wistar rats were trained in a water T-maze during response learning (where the platform was located in the same position across training days) and visual discrimination learning (where the position of the platform was associated with an intramaze visual cue). In order to understand the brain substrates of the memory we used cytochrome c oxidase (CO) quantitative histochemistry in all experiments since this technique allows us to determine changes in brain energy metabolism. Similarly, the relative levels of the catalytic subunit I of CO enzyme were determined by western blot analysis to evaluate the contribution of catalytic subunits to changes in overall CO activity caused by the different learning tasks. Our results show that rats evaluated in a response learning task required fewer trials to master it than animals evaluated in a visual discrimination task. Furthermore, we found different levels of brain oxidative metabolism according to the spatial learning task performed by the animals. Moreover, we evaluated the progressive contribution of different brain regions including dorsal striatum and hippocampus at different time points during visual discrimination learning. Our results show that both the striatum and the dorsal hippocampus are necessary for the acquisition of a visual discrimination task. In addition, regions associated with novelty, emotional and spatial orientation such as the nucleus accumbens, the amygdala, the parietal cortex and the hippocampus seem to be relevant during the earlier phase of training, whereas the prefrontal cortex is recruited at all stages of the learning process. On the other hand, we analyzed the brain networks involved in response learning during the training days. Our results showed that a cortico-limbic-striatal network was related with response learning. Accordingly, increased neural metabolic activity was found in the prefrontal cortex, the dorsal striatum, the amygdala, the ventral tegmental area and both the dorsal and ventral hippocampus. Furthermore, we examined the effect of lighting conditions on the brain networks involved in response learning. The parietal cortex and the ventral hippocampus were associated with the acquisition of the response learning task regardless of lighting conditions. However, under dark conditions a more widespread recruitment of structures involving cortical, limbic and striatal regions was found. In summary, our results support that both the hippocampus and the striatum are key brain regions in response and visual discrimination learning.

1. INTRODUCCIÓN

Los animales son capaces de modificar su conducta como resultado de la experiencia. Este fenómeno, conocido como aprendizaje, es uno de los procesos biológicos que promueven la supervivencia, ya que los seres vivos se enfrentan a efectos adversos del cambio ambiental (cambios climáticos, nuevos depredadores, etc.) que a menudo son minimizados por ajustes conductuales.

Denominamos **aprendizaje** al proceso por el que el sistema nervioso adquiere nueva información, que se observa mediante cambios en el comportamiento, mientras que la **memoria** se refiere a la codificación, el almacenamiento y la recuperación de la información aprendida para responder a las demandas ambientales. Estos procesos dan a nuestras vidas un sentido de continuidad. Aunque desde tiempos remotos nos hemos sentido atraídos por comprender los mecanismos fisiológicos del aprendizaje, no ha sido hasta la segunda mitad del siglo XX cuando los avances tecnológicos y el conocimiento más preciso del funcionamiento del sistema nervioso han permitido comenzar a desvelar los mecanismos que subyacen a los complejos procesos del aprendizaje y la memoria (López-Rojas y cols., 2007).

Comprender los mecanismos cerebrales implicados en la organización de las funciones cognitivas es una tarea extremadamente difícil, dada la enorme complejidad del cerebro en cuanto a las estructuras que lo componen y las interrelaciones entre las mismas. Desde una perspectiva neuropsicológica, el punto de partida de los estudios sobre la anatomía de la memoria se puede situar en los trabajos de Karl Lashley (1890-1958), el cual dedicó gran parte de su vida a la búsqueda del sustrato neural que sirviera de soporte a la memoria y al aprendizaje, a lo que él denominó engrama (huella en el cerebro). Mediante la ablación experimental y el registro conductual postoperatorio, observó que si bien el deterioro de la memoria era proporcional al tamaño de las lesiones cerebrales, no había localizaciones aisladas de memoria, concluyendo así que el engrama no estaba localizado en una región concreta del cerebro sino que estaba distribuido por todo el encéfalo (Lashley, 1950).

El número de neuronas en un individuo a lo largo de su vida es prácticamente constante, de ahí que la sinapsis haya sido un buen candidato de sustrato mnemónico (Matthies, 1989). Fue Ramón y Cajal (1852-1934) el primero en proponer al número y a la fuerza de las conexiones neuronales como la base física del aprendizaje y el soporte de la memoria. Más adelante en 1949, Donald Hebb propuso que la memoria a largo plazo producía cambios en el sistema nervioso resultantes de la activación repetida de circuitos discretos de neuronas, y que estos cambios probablemente ocurriesen a nivel de la sinapsis (Hebb, 1949). Más tarde, Bliss y Lφmo (Bliss y Lomo, 1973) descubrieron que una estimulación de frecuencia moderadamente alta en una misma vía producía incrementos estables y duraderos de respuesta postsináptica, lo que se denominó potenciación sináptica a largo plazo (PLP) que podría constituir la base estructural de la memoria. Además, también se ha identificado un mecanismo denominado depresión a largo plazo (DLP) mediante el cual las sinapsis se vuelven menos eficaces disminuyendo su tasa de respuesta (Bear y Abraham, 1996); se cree que este mecanismo es crítico al menos en la homeostasis de la conexión neural. La sinaptogénesis o formación de nuevas sinapsis, así como la neurogénesis que se produce a lo largo de la vida en el cerebro adulto, también han sido recientemente descritas como procesos implicados en la memoria (Bear y Abraham, 1996; Bruel-Jungerman y cols., 2007; Gil-Perotin y cols., 2009).

1.1. Clasificación de la memoria

Existen diversas clasificaciones de memoria según se atienda a su contenido (*declarativo* o *procedimental*), su duración (*a corto plazo* o a *largo plazo*) o a su naturaleza, es decir aquellas que se mantienen a lo largo del tiempo frente a las que son transitorias (*memoria de trabajo*) (Deiana y cols., 2011). La memoria a *corto plazo* o retención de una información durante un tiempo breve, se basa en cambios efímeros, eléctricos o moleculares en las redes neuronales implicadas (Baddeley, 2012). Por el contrario en la *memoria a largo plazo* los cambios estructurales son persistentes, como por ejemplo la aparición de nuevas espinas dendríticas (O'Donnell y cols., 2011) y la síntesis de nuevas proteínas (Konopka y cols., 2011). Este último es un sistema para almacenar una gran cantidad de información durante un tiempo ilimitado y a diferencia

de la *memoria a corto plazo*, es una memoria estable y duradera, muy poco vulnerable a las interferencias (Morgado, 2005).

La *memoria de trabajo* consiste en una representación consciente y manipulación temporal de la información necesaria para realizar operaciones cognitivas complejas (Baddeley, 2012), como son el razonamiento, la comprensión del lenguaje o el habla. El sujeto memoriza temporalmente la información que le permitirá responder más tarde de manera adecuada. Es por tanto una información transitoria, a corto plazo que continuamente se está borrando y sustituyendo por otra de similar naturaleza.

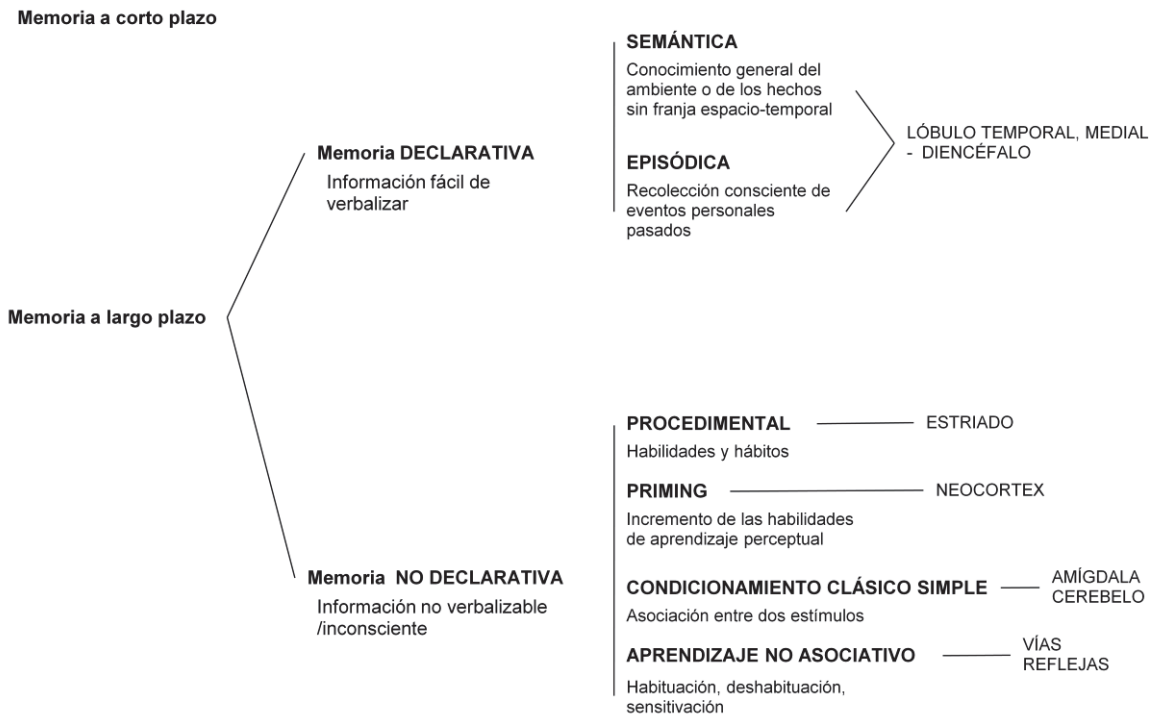

Figura 1: Una de las clasificaciones de los tipos de memoria más empleada en la actualidad en investigación. Modificado de Thompson y Kim, 1996, en la que se hace referencia a las estructuras cerebrales implicadas más relevantes.

Dentro de la memoria a largo plazo se distingue entre *memoria explícita* y la *memoria implícita* (Dew y Cabeza, 2011; Mulligan, 2012), dicotomía que fue propuesta por Squire (Squire, 1992). La *memoria implícita o no declarativa* es aquella que nos permite ejercer hábitos cognitivos o motores, de ahí que también se denomine

procedimental. Es altamente influenciable por predisposiciones biológicas (determinados individuos presentan más habilidad para determinados deportes, conducir, etc.) y se adquiere gradualmente, perfeccionándose con la práctica. Presenta una expresión en gran medida automática e inconsciente y difícil de declarar verbalmente o por escrito. Sin embargo, la *memoria explícita o declarativa* (Tulving, 2002), fácilmente verbalizable, es el almacenamiento cerebral de hechos (*memoria semántica*) y sucesos (*memoria episódica*). Se expresa conscientemente y es flexible y cambiante. La *memoria episódica* resulta del aprendizaje relacional, que consiste en analizar, comparar y contrastar diferentes tipos de información (Crystal, 2010; Ferbinteanu y cols., 2006; Morris, 2001). Un ejemplo es el aprendizaje que nos permite orientarnos en el espacio circundante, de ahí que aunque dado su carácter consciente parezca ser más susceptible de humanos, también esté presente en otros animales, lo que nos permite estudiar el aprendizaje espacial en modelos experimentales (Deiana y cols., 2011).

Los distintos tipos en los que se clasifica la memoria han tenido una gran influencia sobre el estudio de diferentes tipos de aprendizaje. Uno de los más estudiados por su universalidad en el reino animal, pero también por su complejidad es el denominado "aprendizaje de orientación espacial". El estudio de este tipo de aprendizaje se ve favorecido por la facilidad con la que similares paradigmas espaciales pueden ser aplicados tanto a animales como a humanos. Además los hallazgos obtenidos en animales se pueden integrar con descubrimientos no invasivos en humanos, como por ejemplo con la utilización de técnicas de neuroimagen y con el uso de tests en neuropsicología (Bohbot y cols., 2002; Brown y cols., 2012).

1.2. Aprendizaje espacial

Tanto para los humanos como para el resto de los animales tener una buena orientación espacial es clave para la supervivencia, ya que numerosas conductas como las alimenticias o las reproductivas dependen de un buen conocimiento del entorno en el que habitan. El animal debe utilizar una determinada estrategia de navegación, bien innata o bien aprendida, para recordar dónde está su guarida o donde encontrar el

alimento. Podríamos definir la *navegación espacial* como una conducta compleja orientada a una meta que requiere como mínimo conocer el lugar en el que el animal está y el lugar a donde quiere ir. Este conocimiento requiere la codificación de información multimodal concerniente a la posición del cuerpo en relación con el entorno (Wang y Spelke, 2002). A esta capacidad de los animales de emplear distintas estrategias de navegación para encontrar un lugar en un entorno previamente visitado se le denomina *aprendizaje espacial*. Por otra parte entendemos la *memoria espacial* como la habilidad para codificar, almacenar y recuperar información aprendida sobre las localizaciones espaciales (Kessels y cols., 2001).

Como previamente se ha comentado, la memoria espacial se considera un tipo de memoria principalmente declarativa, de tipo episódica, y respecto a su naturaleza temporal, al igual que en el caso de la memoria en general, existen formas de memoria espacial a corto (en la que se encuentra incluida la memoria de trabajo) y largo plazo.

Actualmente se considera que existen tres sistemas de navegación espacial, el *táxico, el cartográfico* y el de *integración de la ruta* (Mittelstaedt y Mittelstaedt, 1980).

El *aprendizaje táxico* se cimenta en el empleo de una estrategia de orientación propioceptiva, situando el espacio en un marco de referencia centrado en el propio sujeto. A su vez este sistema puede desarrollarse a través de dos mecanismos: el *aprendizaje de respuesta u orientación* y el *aprendizaje de guía* (Santin y cols., 2000). En el *aprendizaje de respuesta u orientación* los animales utilizan patrones motores estereotipados localizando el lugar deseado tras aprender series de movimientos, mientras que en el *aprendizaje de guía* el animal aprende a asociar una pista a la meta, como ocurre por ejemplo en las tareas discriminación visual simple, donde los animales distinguen entre pistas visuales disponibles en su entorno para alcanzar la meta (Hu y cols., 2005).

En el *aprendizaje cartográfico* se aprende la localización de un lugar con respecto a la configuración de las pistas disponibles en el entorno circundante. En este aprendizaje, las pistas utilizadas son distales y el animal se forma un mapa cognitivo, es decir, una representación espacial o mapa del entorno en el cual se encuentra la meta. De este modo, los organismos aprenden a anticipar no solo los estímulos particulares,

sino también los elementos interconectados del mapa cognitivo, lo que hace posible establecer inferencias lógicas en la navegación de los sujetos como rutas más cortas y obtención de nuevas soluciones, entre otras (Eichenbaum y cols., 1990; Eichenbaum, 1997; Lafon y cols., 2009).

Actualmente se considera que existe otro tipo de navegación espacial llamado de *integración de la ruta* (*path integration*), *navegación idiotética* (Mittelstaedt y Mittelstaedt, 1980) o *navegación basada en la ruta* (*route-based navigation*) (Baker, 1981). Esta estrategia se basa en que cuando las pistas ambientales no ofrecen suficiente información para solucionar una tarea espacial, el individuo utiliza pistas idiotéticas o propioceptivas (vestibulares, cinestésicas etc.) que proporcionan al animal suficiente información sensorial para encontrar la meta de forma precisa (Issa y Zhang, 2012). En la integración de la ruta el individuo calcula su posición relativa respecto a su localización inicial mediante la integración de pistas generadas por sus propios movimientos. Este sistema se basa en un mecanismo de actualización de la información, que permite al animal en movimiento poder mantener en su memoria la huella sobre la localización de un lugar de salida en relación con su posición actual llamado "dead reckoning". Hay cierta controversia en cuanto a su existencia, ya que para algunos autores este tipo de navegación formaría parte de la navegación normal (Whishaw y cols., 2001).

Por consiguiente, para que se establezca una representación espacial eficiente en el cerebro se requiere que exista una correcta integración de la información sensorial que percibimos, y esta información se ha dividido en información alotética o idiotética. Los estímulos alotéticos son aquellos que proporciona el ambiente, es decir: estímulos olfativos, visuales o sonidos, que aportan información espacial al sujeto. Los aprendizajes basados en esta información se denominan *aprendizajes alocéntricos* (Braun y cols., 2012). Los *estímulos idiotéticos* hacen referencia a estímulos del propio cuerpo, vestibulares, propioceptivos o motores y aportan información de los cambios continuos de la posición u orientación del sujeto. El aprendizaje basado en estos estímulos se denomina *egocéntrico* (Burgess, 2006). Existen evidencias experimentales de que en determinadas situaciones, las ratas intentan aplicar varias estrategias para

solventar problemas espaciales, siendo capaces de cambiar de una a otra cuando es necesario.

1.2.1. Importancia de las redes neuronales en el aprendizaje espacial

Hasta el momento se han realizado numerosos estudios para intentar esclarecer cuales son los procesos mediante los cuales nos guiamos. El cerebro es un órgano de enorme complejidad como consecuencia de las numerosas estructuras que lo componen y las interrelaciones que existen entre ellas. Por ello, un mecanismo eficiente para analizar los cambios que se producen temporalmente en el cerebro al aprender una tarea es el estudio de las redes neuronales que se encuentran implicadas en dicho aprendizaje. En la actualidad existen numerosos trabajos de investigación en los que se analizan las redes neuronales implicadas en un determinado aprendizaje tanto en animales (Gonzalez-Pardo y cols., 2012; Puga y cols., 2007) como en humanos (Lehericy y cols., 2005; Ma y cols., 2010). Estos trabajos muestran como para una determinada conducta por simple que ésta sea, se requiere que existan interacciones entre las estructuras cerebrales y no la simple activación de unas determinadas (Sakurai, 1996). Es decir, una región no actúa de manera independiente al resto del cerebro, sino que para que se produzca una determinada conducta es necesaria la interacción dentro y entre distintos sistemas neuronales creándose de esta forma un contexto neural que será específico de dicha conducta (Conejo y cols., 2010).

A su vez, la memoria ha sido descrita como un proceso complejo formado por distintos estadíos temporales en los que las interacciones entre las estructuras varían a lo largo del proceso de aprendizaje. De esta manera se ha observado que las relaciones entre las regiones varían progresivamente, a lo largo de los días de aprendizaje en cuanto a las estructuras implicadas y en su patrón de conectividad (Conejo y cols., 2010; McIntosh, 1999).

Asimismo es importante destacar que las teorías actuales sobre la memoria asumen la existencia de múltiples formas de memoria, proponiendo la existencia de varios tipos de circuitos neuronales que están adaptados a almacenar distintos tipos de

información (Packard y Goodman, 2012; Poldrack y Packard, 2003; White y McDonald, 2002). Concretamente, en el cerebro de mamíferos, el hipocampo y el estriado dorsal son el sustrato de dos tipos de memoria diferentes. Así, la región hipocampal es importante en la memoria declarativa, mientras que el estriado dorsal lo es en la memoria implícita, es decir, en la memoria de hábitos o memoria procedimental (Packard, 2009). Llamamos memorias de hábitos a aquellas en las que la práctica repetida es la condición más crítica para adquirir la tarea.

Aunque las redes neuronales implicadas en la formación de memoria de hábitos no se conocen con exactitud, numerosos estudios en animales y humanos aportan la importancia de algunas estructuras cerebrales en este tipo de memoria. Tanto el aprendizaje de respuesta como el aprendizaje de guía requieren la formación de este tipo de memoria. Estudios recientes muestran que el **estriado dorsal** es la región cerebral que modula la formación de hábitos (Packard y Knowlton, 2002; Yin y Knowlton, 2006) (Ver figura 1). En roedores el cuerpo estriado incluye el núcleo caudado y el putamen en la región dorsal, y se ha observado que lesiones en esta región se asocian con grandes dificultades en la ejecución de tareas de respuesta en un laberinto complejo en el que el animal tiene que aprender una serie de giros (Pistell y cols., 2009).

En 1978, Graybiel y Ragsdale describieron la presencia de estriosomas (agrupaciones celulares) en esta estructura cerebral, y desde entonces, son múltiples los estudios que han analizado la anatomía del estriado (Bernacer y cols., 2008; Holt y cols., 1997; Prensa y cols., 1999). En el estriado se distingue entre estriosomas y matriz, también llamada esta última zona extra-estriosomal (Goldman-Rakic, 1982). Los estriosomas se encuentran localizados predominantemente en la región medial, mientras que la matriz lo suele estar en la lateral. Aunque ambos compartimentos se localicen a través de toda la estructura (White y McDonald, 2002), estriosomas y matriz difieren entre ellos en sus características químicas y en sus conexiones.

En cuanto a las características químicas, los estriosomas son ricos en encefalina y en sustancia P, mientras que tienen una cantidad inferior con respecto a la matriz de las enzimas acetilcolinesterasa, calbindina y parvalbúmina (Bernacer y cols., 2007; Davis y Puhl, 2011). En ambas se pueden distinguir dos tipos de neuronas en función de la diana de su axón: las neuronas de proyección y las interneuronas. Mientras que las

primeras inervan otros núcleos distintos de aquél en el que se encuentran, en las interneuronas su axón permanece en el mismo núcleo en el que se encuentra su soma. En cuanto a las características conectivas, la matriz recibe proyecciones de la corteza sensorimotora y de capas superficiales de la corteza parietal y occipital (Merello y Cammarota, 2000), mientras que los estriosomas reciben aferencias desde la corteza prefrontal y el sistema límbico (van Domburg y ten Donkelaar, 1991).

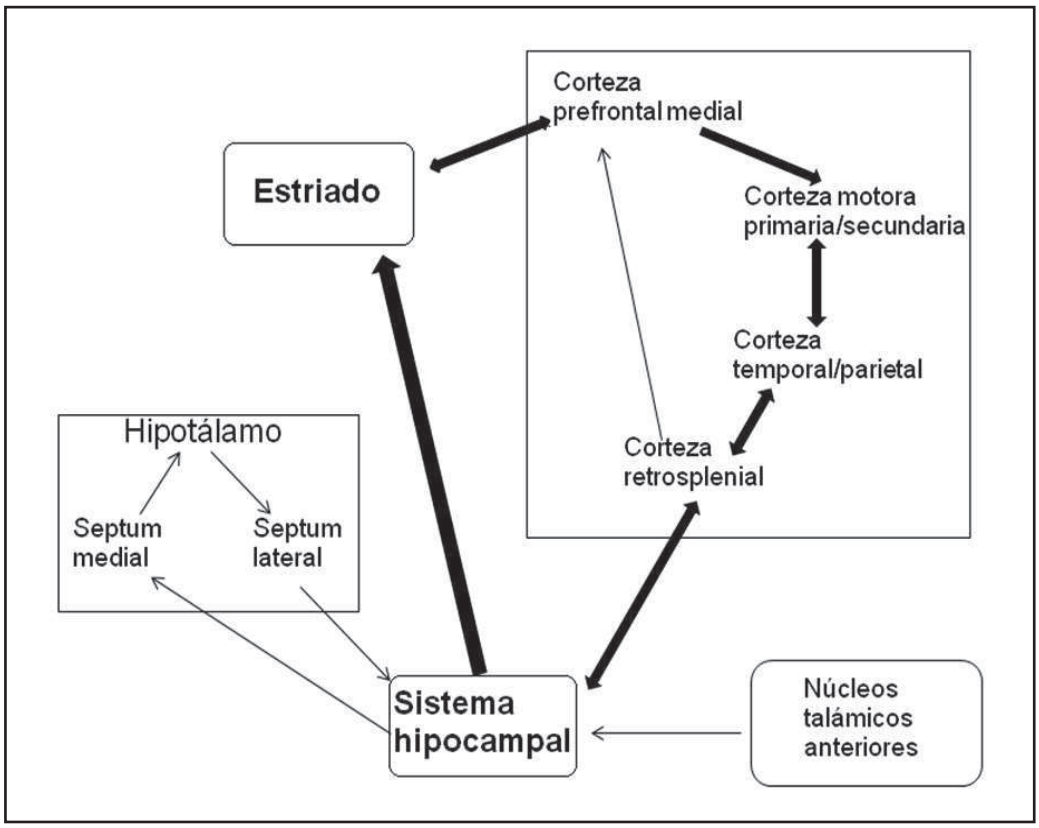

Figura 2: Conexiones neuronales entre el núcleo estriado y algunas estructuras corticales y subcorticales. (Modificado de Mizumori y cols., 2009).

Es posible que las diferencias que se atribuyen generalmente a la separación funcional del estriado dorsal, en estriado dorsal lateral y estriado dorsal medial, se deban en realidad a las diferentes funciones de la matriz y los estriosomas. Así, dependiendo de sus conexiones existe una separación funcional entre ellos. La región dorsolateral recibe aferencias de áreas corticales motoras y sensoriales así como

de núcleos talámicos de la línea media (White y McDonald, 2002). En cambio, el estriado dorsomedial recibe un elevado número de aferencias desde áreas de asociación de la neocorteza (McGeorge y Faull, 1989). La región dorsomedial se encuentra activada metabólicamente durante la realización de tareas de memoria de trabajo espacial (Levy y cols., 1997). Atendiendo a los estudios con lesiones, podemos indicar que la lesión de esta región impide una correcta ejecución de tareas de orientación espacial de respuesta, lo que no ocurre con lesiones del estriado laterodorsal (Devan y cols., 1999). Del mismo modo, las lesiones de la región dorsolateral no provocan alteraciones en la orientación espacial, pero si presentan impedimentos en tareas que requieren flexibilidad conductual (Devan y White, 1999; Palencia y Ragozzino, 2005; Ragozzino, 2007; Tzavos y cols., 2004), es decir, al ejecutar tareas en las que se requiere la capacidad de realizar cambios en la respuesta conductual como consecuencia de cambios en las condiciones ambientales. En este sentido, la corteza prefrontal se ha visto implicada en el aprendizaje *reversal* o de inversión (Contreras y cols., 2008). Este tipo de aprendizaje se puede entender como un caso concreto de flexibilidad conductual porque implica la adaptación del animal a un entorno cambiante en el que los sujetos tienen que revertir una asociación estímulo-respuesta previamente establecida (Floresco y cols., 2009; Ragozzino, 2007).

La región ventral del estriado, formada principalmente por el **núcleo accumbens,** se ha relacionado con conductas de refuerzo (Rolls, 2000). Esta estructura, presenta conexiones con regiones cerebrales que se hayan implicadas en procesos de aprendizaje de refuerzo motivacional, como son la corteza prefrontal, la amígdala y el hipocampo (Friedman y cols., 2002; Wright y Groenewegen, 1996). Concretamente, debido a las fuertes proyecciones entre esta estructura y el hipocampo se ha propuesto recientemente una participación de ambas regiones en la conducta dirigida a una meta (Pennartz y cols., 2011).

Además se ha visto que el estriado ventral está funcionalmente vinculado al aprendizaje espacial. De esta manera, lesiones en el estriado ventral dificultan la ejecución de tareas en el laberinto acuático de Morris y en el laberinto en T (Annett y cols., 1989). Por otro lado, se ha observado una disociación funcional entre la cápsula y

el centro del núcleo accumbens en tareas espaciales y no espaciales como es el reconocimiento de objetos (Nelson y cols., 2010).

Una estructura vinculada en la orientación espacial es el **hipocampo**, el cual se considera clave para el aprendizaje de orientación espacial, aunque no fue hasta los años 50 cuando se relacionó al hipocampo con la memoria. Esta nueva y desconocida función del hipocampo llamó la atención de los investigadores como consecuencia de los déficits de memoria ocurridos en un paciente, conocido como HM, al que se le extirpó quirúrgicamente y de forma bilateral varias estructuras del lóbulo temporal, incluyendo al hipocampo, como tratamiento a la epilepsia que padecía. Así, este paciente perdió su capacidad para *formar nuevas memorias declarativas*. En 1978, O´Keefe y Nadel tomando como partida los trabajos de Tolman, propusieron al hipocampo como la estructura cerebral que interviene en la confección de un mapa cognitivo, es decir, que los animales elaboran una especie de representaciones internas como resultado de la codificación de las relaciones espaciales que guardan entre sí los diferentes hitos del entorno (O´Keefe y Nadel, 1978).

Anatómicamente, la región hipocampal se subdivide en dos partes: la formación hipocampal y la formación parahipocampal. La formación hipocampal está formada a su vez por el hipocampo propiamente dicho (cuerno o asta de Ammon (CA) y el giro dentado (GD)) y el subiculum (incluyendo el prosubiculum) mientras que la formación parahipocampal se compone del presubiculum, el parasubiculum y las cortezas entorrinal, postrrinal y perirrinal. Sin embargo, el término hipocampo es empleado a menudo para designar la formación hipocampal, especialmente para referirse al asta de Ammon y al íntimamente asociado giro dentado. En roedores el hipocampo aparece como una estructura elongada con su eje longitudinal doblado en forma de "C" desde la región dorsal-anterior de los núcleos septales a la región caudo-ventral del lóbulo temporal (Amaral y Witter, 1989).

La mayor parte de los estudios de lesiones en humanos, primates y roedores sugieren un papel primordial del hipocampo en la consolidación de la memoria tanto a corto como a largo plazo. El daño en esta estructura puede producir déficits en la memoria declarativa (Squire y Zola-Morgan, 1991; Squire, 1992), y concretamente en la memoria episódica (Vargha-Khadem y cols., 1997). Además, estudios de lesión

hipocampal ponen de manifiesto su implicación en el aprendizaje espacial (Morris y cols., 1982; O´Keefe y Nadel, 1978; Telensky y cols., 2011).

En 1976 O´Keefe descubre en el hipocampo de la rata la existencia de las llamadas células de lugar "place cells". Estas células varían su tasa de disparo específica y selectivamente cuando una rata ocupa una localización determinada en un entorno (O´Keefe, 1976). Anatómicamente son neuronas piramidales que se han encontrado en las áreas CA1 y CA3 del hipocampo, en el subiculum y en la corteza entorrinal, y que actualmente se han observado también en primates. La existencia de estas células junto con estudios de lesión evidencian el importante papel que juega el hipocampo en el procesamiento de la información espacial. Por esta razón el hipocampo se encuentra en un primer plano en la investigación de las bases biológicas del aprendizaje y la memoria y, aunque la mayoría de los investigadores están de acuerdo en que el hipocampo juega un papel principal en el almacenaje a largo plazo de las memorias explícitas, su función exacta aun no se conoce (Knierim, 2006).

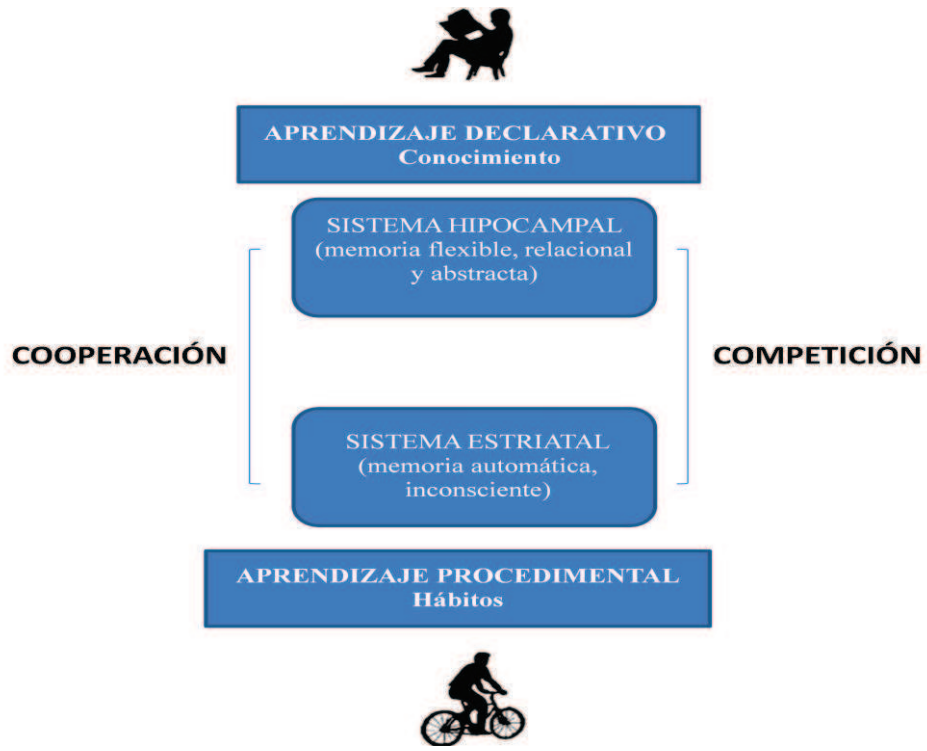

Figura 3: Interacciones entre los sistemas de memoria hipocampal y estriatal. Modificado de Ghiglieri y cols., 2011.

Además, esta estructura se caracteriza por su heterogeneidad funcional a lo largo de su eje longitudinal (Andersen y cols., 1971; Mendez-Lopez y cols., 2009). De esta forma las células de lugar se localizan de forma menos específica en el hipocampo ventral (Jung y cols., 1994), habiéndose descrito por lo tanto que la región dorsal está más relacionada con la memoria espacial (Moser y cols., 1995) y la ventral con otros aspectos como la ansiedad y el miedo (Nascimento Hackl y Carobrez, 2007). Sin embargo, en un estudio reciente se muestra que la inactivación temporal del hipocampo ventral dificulta el recuerdo de la memoria espacial en ratas (Loureiro y cols., 2012).

Como previamente se ha comentado, hipocampo y estriado se consideraban dos estructuras funcionalmente diferentes, siendo el estriado clave en la memoria de hábitos y el hipocampo en la memoria declarativa. Algunos estudios muestran que lesiones hipocampales producen amnesia retrógrada en este tipo de tareas (Driscoll y cols., 2005; Epp y cols., 2008; Sutherland y cols., 2001) sugiriendo que el hipocampo podría ser necesario en el aprendizaje de hábitos. Además, se ha propuesto que al menos en determinadas situaciones ambas estructuras pueden interaccionar y actuar cooperativamente o competitivamente para optimizar la conducta (Ghiglieri y cols., 2011).

Por otro lado, tanto el estriado como el hipocampo presentan conexiones con la **corteza prefrontal**. En estudios con modelos animales se ha visto que la lesión de la corteza prefrontal medial se asocia con grandes dificultades en la ejecución de aprendizajes de respuesta en laberintos acuáticos (de Bruin y cols., 1997; Mogensen y cols., 2005). Además se ha observado que ablaciones bilaterales de la corteza prefrontal en rata dificultan la ejecución de tareas de alternancia demorada espaciales empleando el laberinto en T (Wikmark y cols., 1973; Wortwein y cols., 1993).

Concretamente, la corteza prefrontal y el cuerpo estriado forman parte de un circuito neuronal que es de gran transcendencia en la conducta de humanos y demás animales, estando implicadas estas dos estructuras en conductas dirigidas a una meta, como es el caso del aprendizaje de respuesta y el de guía del que ya hemos hablado. Es decir, estriado y corteza prefrontal son estructuras cerebrales claves en las funciones cognitivas necesarias para elaborar un plan de acción, como son la planificación (Wunderlich y cols., 2012), la memoria de trabajo (Rieckmann y cols., 2011) y la

flexibilidad conductual (Block y cols., 2007; McDonald y cols., 2008; Ragozzino y Choi, 2004; Ragozzino, 2007); asimismo, los daños cerebrales en estas regiones en humanos se han asociado a diversos transtornos como por ejemplo la esquizofrenia (Barch y Ceaser, 2012).

Al igual que la corteza prefrontal y el cuerpo estriado, el complejo amigdalino guarda una estrecha relación con los mecanismos que suscitan la memoria. Se ha observado que tanto el estriado dorsal como el hipocampo y la amígdala son necesarios para el aprendizaje de discriminación visual (aprendizaje de guía) (McDonald y cols., 2007). Además, diferentes estudios realizados con humanos han relacionado la amígdala con los procesos de modulación de la memoria, y se ha observado que un estado de activación emocional produce una facilitación de la misma (Adolphs y cols., 1997). Concretamente, el núcleo basolateral de la amígdala es vital para la consolidación de la misma (McGaugh, 2002).

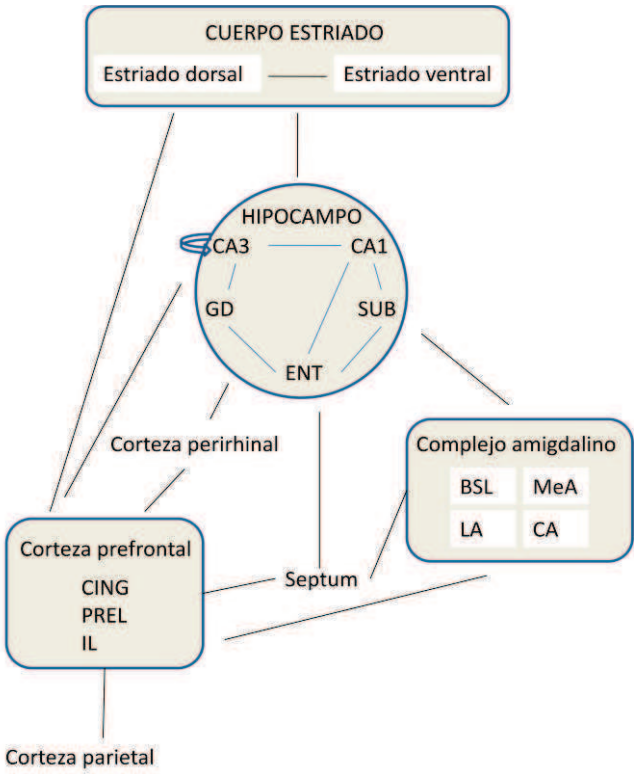

Figura 4: Esquema de las interconexiones existentes entre las estructuras estudiadas. BSL: amígdala basolateral, CA amígdala central, CA1: Cuerno de Ammón 1, CA3: Cuerno de Ammón, CING: corteza cingulada, ENT: corteza entorrinal GD: giro dentado, IL: corteza infralímbica, LA amígdala lateral, MeA: amígdala medial, PREL: corteza prelímbica; SUB: subiculum.

Las estructuras comentadas junto con otras regiones cerebrales como la corteza parietal presentan conexiones neuronales entre sí, participando en el aprendizaje que implica para su correcta ejecución el uso de determinadas estrategias espaciales. De esta manera la corteza parietal se ha visto implicada en el aprendizaje de tareas espaciales (Nitz, 2009) ya que en esta región se procesa tanto la información visual, como auditiva y táctil debido a las conexiones que presenta con regiones sensoriales que incluyen a las cortezas visuales primarias y secundarias, la corteza auditiva y la corteza somatosensorial respectivamente (Save y Poucet, 2009).

Estos datos apoyan de nuevo el hecho de que para que tenga lugar una determinada conducta es necesaria la interacción entre distintos sistemas neuronales. De esta manera, en determinadas ocasiones se observa una red neural determinada en la que ciertas estructuras son comunes y necesarias para la correcta ejecución de conductas diferentes.

1.2.2. Evaluación del aprendizaje de orientación espacial en animales.

La orientación espacial se puede estudiar en roedores mediante el uso de diferentes tipos de pruebas como son el laberinto acuático de Morris (Arias y cols., 2012; Morris, 1984), el laberinto en forma de Y (Ciobica y cols., 2012; Simpson y cols., 2012), el laberinto radial de ocho brazos (Ciobica y cols., 2012) o el laberinto en T, que puede ser usado para analizar la ansiedad (Asth y cols., 2012; Hatano y cols., 2012), en su variante terrestre (Ramkumar y cols., 2012; Rhoads y cols., 2012) o acuática (Del Arco y cols., 2007; Drouin-Ouellet y cols., 2012; Filali y cols., 2011; Maioli y cols., 2012). Concretamente el laberinto acuático en T presenta la ventaja de utilizar como estímulo motivacional el escapar del agua, no requieriendo privación de agua ni de comida. De esta manera evitamos la restricción nutricional, que es utilizada en algunas pruebas y que conlleva cambios emocionales en los animales experimentales. Por otro lado, se pueden realizar gran cantidad de ensayos en un mismo día, sin que la saciedad sea un factor influyente (Locchi y cols., 2007). El laberinto acuático en T es pues una prueba conductual que nos permite desarrollar distintos tipos de entrenamiento espacial en los que se requiera el uso de distintas estrategias en roedores. Básicamente el animal

tiene que elegir un brazo específico para encontrar una plataforma sumergida. En algunas tareas el animal tiene que utilizar pistas del ambiente localizadas bien en el exterior o en el interior del laberinto (aprendizaje alocéntrico) o pistas interoceptivas (propioceptivas, vestibulares o motoras) para localizar la plataforma. En este último caso, nos encontraríamos ante un aprendizaje egocéntrico. La utilización de esta prueba, hace posible a su vez valorar la memoria de referencia, si la plataforma permanece en el mismo lugar durante los ensayos, y la memoria de trabajo, cuando se cambia la plataforma de posición en cada ensayo. Además, gracias a la sencillez del laberinto podremos evaluar las conductas de una manera independiente.

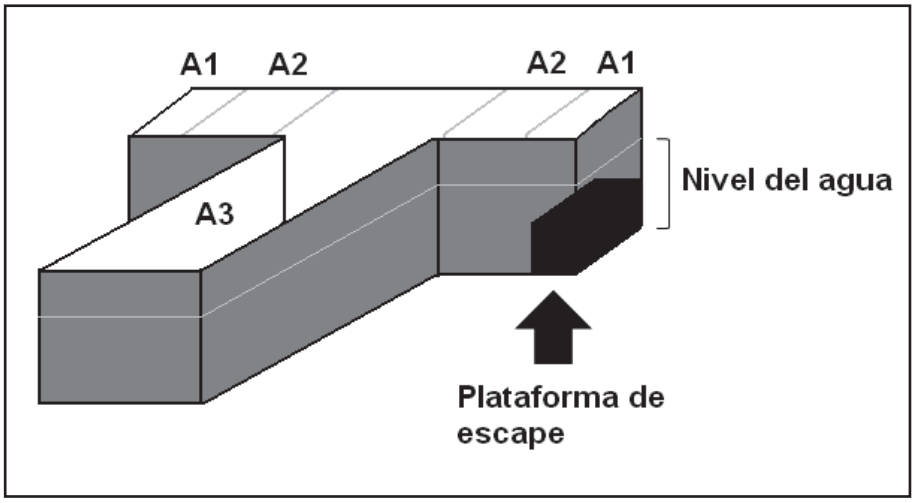

Figura 5: Laberinto acuático en T para estudiar estrategias de orientación espacial. A1: Zona donde se encuentra la plataforma. A2: Región de elección. Una vez que el animal ha entrado en esta zona se considera error si el roedor ha optado por el brazo que no contiene la plataforma. A3: Brazo de salida.

Un posible inconveniente que puede presentar esta prueba, es la tendencia que los roedores suelen tener a girar hacia la izquierda o hacia la derecha lo que puede influir en el aprendizaje de la tarea (Andrade y cols., 2001), es decir presentan una preferencia por uno de los dos brazos de un laberinto en T. A esta conducta se la denomina lateralidad y, en este sentido, se sabe que las ratas Wistar presentan lateralización hacia la derecha (Santin y cols., 1996). Sin embargo, este inconveniente

se puede subsanar si el animal aprende a alternar entre los dos brazos durante la fase de habituación.

1.3. Análisis del metabolismo oxidativo cerebral

Una de las finalidades de las investigaciones en neurociencias es comprender las bases neuronales de la conducta. Para ello, se utilizan diferentes métodos que permitan el estudio de qué estructuras son necesarias y cuál es la interacción existente entre ellas durante una determinada respuesta conductual.

1.3.1. Histoquímica de la citocromo c oxidasa (CO)

En el estudio de los patrones de actividad cerebral, numerosos investigadores recurren a métodos de análisis metabólico, los cuales asumen el principio de que la energía utilizada en el tejido cerebral está determinada por la actividad funcional de un conjunto de neuronas (Hevner y cols., 1995).

Entre los distintos métodos existentes, se encuentran las técnicas de autorradiografía en las que se utilizan análogos de la glucosa como la C14-2-deoxiglucosa y la 18F-fluorodesoxiglucosa (Bontempi y cols., 1999). Los métodos autorradiográficos permiten captar incrementos repentinos en el consumo de la glucosa, lo que condiciona esta técnica a periodos experimentales de duración menor a una hora (Hevner y cols., 1995).

Otro posible método que puede ser utilizado para evaluar la actividad cerebral es mediante el marcaje histoquímico de la enzima citocromo c oxidasa (EC 1.9.3.1). La utilización de esta técnica se basa en el papel esencial que juega esta proteína en la respiración celular. La cadena de transporte de electrones o fosforilación oxidativa es un proceso que se lleva a cabo en la membrana interna mitocondrial y está constituido por una serie de complejos enzima-coenzima de oxidorreductasas y ferroproteínas. El complejo citocromo c oxidasa es el componente final de la cadena (es decir el complejo IV) y está constituido por los citocromos a y a3 y dos iones Cu (II).

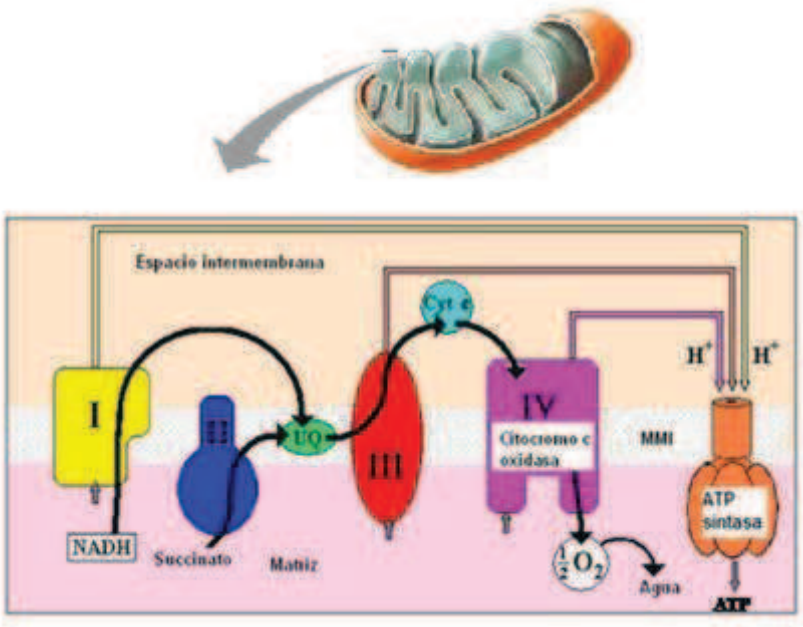

Figura 6 Esquema en el que se representa la cadena transportadora de electrones con la enzima citocromo oxidasa marcada en azul. Esta enzima cataliza la reacción de oxidación del oxígeno a agua. La misión de la cadena transportadora de electrones es la de crear un gradiente electroquímico que se utiliza para la síntesis de ATP. Dicho gradiente electroquímico se consigue mediante el flujo de electrones entre los distintos componentes proteicos de esta cadena.

Una de las ventajas de esta técnica frente a la autorradiografía es su elevada resolución espacial tanto en microscopía óptica como electrónica, lo que permite una delimitación precisa de las regiones cerebrales de interés. De esta forma, el marcaje histoquímico de esta enzima ha sido utilizado en gran cantidad de estudios conductuales como una medida de la actividad metabólica de distintas estructuras cerebrales, relacionadas con, por ejemplo el condicionamiento al miedo (Conejo y cols., 2007), en conductas de extinción (Gonzalez-Lima y Bruchey, 2004), evaluación de la conducta espacial en estudios con modelos animales cirróticos (Mendez y cols., 2009) o estudios farmacológicos (Gonzalez-Pardo y cols., 2006). Esta técnica también ha sido utilizada en estudios no conductuales, para intentar esclarecer los efectos de daño cerebral traumático en la función mitocondrial, observándose una disminución de los niveles de energía en forma de ATP como consecuencia de la inhibición de la enzima citocromo oxidasa en el traumatismo craneoencefálico (Huttemann y cols., 2008).

1.3.1.1. La citocromo c oxidasa como marcador de la actividad cerebral

La citocromo c oxidasa se ha establecido como un marcador sensible de la actividad neuronal. El metabolismo energético refleja la demanda energética de las neuronas y ésta es directamente proporcional a su actividad (Gonzalez-Lima y Bruchey, 2004; Gonzalez-Pardo y cols., 2006). La actividad neuronal consiste principalmente en la síntesis de neurotransmisores y otras moléculas, así como en el transporte axoplasmático y el transporte activo de iones, siendo éste último el mayor consumidor de energía.

1.3.1.2. Regulación de la citocromo c oxidasa en las neuronas

A pesar de la gran cantidad de estudios que se han realizado acerca del metabolismo energético del cerebro, se conoce muy poco de los mecanismos de regulación que tiene la enzima CO.

Son dos los principales mecanismos que se consideran involucrados en la regulación de dicha enzima, la regulación a corto y a largo plazo (Erecinska y Silver, 1989; Hevner y Wong-Riley, 1990). La regulación a corto plazo es una regulación alostérica, es decir, la proteína modifica su estructura en respuesta a cambios locales en la proporción ADP/ATP, el pH u otros metabolitos (Erecinska y Silver, 1989). Estos cambios conformacionales de la proteína van a afectar a su cinética (Reimann y cols., 1988). Un aumento de ADP supone una alta demanda energética que puede actuar como inductor químico, produciendo una agrupación de mitocondrias en regiones de alta demanda energética (Bereiter-Hahn y Voth, 1983). La regulación a largo plazo afecta al número de moléculas funcionales de la enzima. Esta regulación se realiza a nivel de la transcripción o de la traducción del RNA mensajero (Hevner y Wong-Riley, 1990). Aunque como se ha comentado anteriormente, la CO nos permite el estudio de periodos más largos de tiempo que la 2-desoxiglucosa, estudios realizados en nuestro laboratorio muestran que esta enzima también es capaz de responder con gran rapidez (Conejo y cols., 2010; Mendez-Lopez y cols., 2009). Estos datos concuerdan con el ya conocido mecanismo de regulación a corto plazo de la proteína.

La citocromo c oxidasa de mamíferos está compuesta por 13 subunidades, de las cuales tres tienen codificación mitocondrial y las diez restantes la tienen nuclear (Azzi y Muller, 1990; Capaldi, 1990; Kadenbach y cols., 1983), por lo que la síntesis del holoenzima requiere de la acción coordinada de ambos genomas (Azzi y Muller, 1990; Capaldi, 1990). Esta coordinación es compleja ya que en las neuronas la mayoría de las mitocondrias residen en axones y dendritas, es decir, lejos del cuerpo celular. Las tres subunidades mitocondriales de la CO (COm) constituyen el centro catalítico de la enzima.

Las 10 subunidades restantes y los factores de transcripción mitocondriales se codifican en el núcleo celular (Fontanesi y cols., 2006). Los RNAm de las subunidades CO nucleares de la CO (COn) y de los factores de transcripción salen del núcleo y son traducidos en el soma neuronal por los ribosomas, que se pueden encontrar bien libres en el citoplasma o anclados al retículo endoplásmico rugoso (Wong-Riley, 1989). Los precursores proteicos generados son transportados en las mitocondrias y allí se insertan en la membrana mitocondrial interna, donde maduran. En la mitocondria, previamente se habrán sintetizado las tres subunidades que van a formar el núcleo catalítico del holoenzima y se habrán insertado en la membrana mitocondrial interna. Estas tres subunidades y las diez de origen nuclear se ensamblan y dan lugar al holoenzima CO funcional. Los factores de transcripción de origen nuclear también son transportados al interior mitocondrial una vez que han sido traducidos en el soma neuronal. Ya en la mitocondria, inducen la síntesis de las subunidades de la CO mitocondriales.

1.4. Análisis por western blot

La estructura de la proteína citocromo c oxidasa se ha estudiado ampliamente como se ha comentado en el apartado anterior. Aunque la concentración relativa de sus subunidades en relación al aprendizaje no se ha examinado hasta el momento, si que se conoce la regulación de las subunidades I y IV en neuronas in vitro tras añadir KCl que produce la despolarización de las neuronas, o TTX (tetrodotoxina) que las inhibe (Liang y cols., 2006).

La técnica de western blot es muy utilizada en biología molecular y también se ha aplicado a estudios conductuales. Más concretamente, investigaciones recientes

muestran su aplicabilidad en el estudio de la memoria espacial. De esta forma, (Cao y cols., 2012) estudiaron la relación entre la concentración de las proteínas SNAP-25 y Munc18-1 y el deterioro cognitivo que se encuentra asociados a la edad. Estas proteínas, que presentan localización presinática se encuentran asociadas al proceso de transmisión sináptica. Ésto ha permitido estudiar el efecto de la diabetes en la memoria espacial tras determinar mediante western blot la concentración de las proteínas Cyt-c y Bax en el hipocampo de ratas sanas y enfermas de diabetes (Ye y cols., 2011).

Esta técnica nació en la década de 1970, cuando surgió la necesidad de determinar de forma específica una proteína concreta. Esto condujo al desarrollo de un método que permite identificar proteínas separadas por electroforesis en geles de poliacrilamida, transferidas a una membrana de nitrocelulosa y que posteriormente son marcadas específicamente por un anticuerpo. Dicho método fue desarrollado de forma independiente por dos laboratorios a finales de los años 70 y principios de los 80. Uno de ellos usó geles de poliacrilamida-urea (Towbin y cols., 1992; Towbin, 2009) y el otro utilizó geles de (SDS)-poliacrilamida (Burnette, 1981; Burnette, 2009) que son los más utilizados en la actualidad. A partir de entonces, se convirtió en una herramienta muy útil para estudiar la presencia, abundancia relativa, masa molecular relativa así como la modificación post-traduccional de las proteínas, convirtiéndose en una técnica muy atractiva en la investigación dentro del campo de las neurociencias.

2. OBJETIVOS

El aprendizaje y la memoria espacial son vitales en los animales para su orientación tanto en entornos nuevos como conocidos y dependen del uso de distintas estrategias de orientación. Los aprendizajes táxicos requieren el uso de estrategias egocéntricas en las que el animal utiliza información propioceptiva, vestibular, etc. y de de guía, que requieren la asociación de una pista a una meta.

Hasta el momento, se ha propuesto la existencia de varios tipos de circuitos neuronales adaptados a la integración de distintos tipos de información (visual, espacial, motora, vestibular…). Ejemplos de estos circuitos son el sistema hipocampal, que es importante en las memorias declarativas y el sistema estriatal, relevante en la memoria implícita o no declarativa, que nos permite ejercer hábitos cognitivos o motores. Las redes neuronales implicadas en la memoria no declarativa no se conocen con exactitud. Aunque tradicionalmente los circuitos hipocampal y estriatal se han considerado independientes, en la actualidad se defiende que al menos bajo determinadas circunstancias estos circuitos pueden interaccionar entre sí para mediar el proceso del aprendizaje. Por ello, y con el fin de intentar contribuir al esclarecimiento de las vías neuronales que median la memoria no declarativa, estudiamos el uso de dos tipos de estrategias, egocéntrica y de guía, y nos planteamos los objetivos que se describen a continuación. En todos ellos se utiliza la técnica histoquímica CO como índice de los niveles de actividad metabólica cerebral.

Primer objetivo: Se analizará la conducta espacial de ratas macho adultas evaluándolas en un aprendizaje de guía, haciendo uso de un laberinto acuático en T, en el que el animal tiene que asociar la posición de una pista intralaberíntica con la posición de una plataforma sumergida (tarea de discriminación visual). Posteriormente se evaluará la participación del cuerpo estriado y el hipocampo en este tipo de estrategias mediante el uso de la técnica histoquímica de la CO (artículo I).

Segundo objetivo: Como la memoria ha sido descrita como un proceso complejo formado por distintos estadíos temporales en los que la interacción entre las estructuras varían a lo largo del proceso de aprendizaje, nos propusimos analizar las relaciones entre las regiones cerebrales a lo largo de los días de un aprendizaje de guía en cuanto a

las estructuras implicadas y su patrón de conectividad. Por ello empleamos al igual que en el primer objetivo, la técnica histoquímica de la CO. De esta manera intentamos identificar posibles redes neurales que sustenten las distintas fases del proceso de aprendizaje (artículo II).

Tercer objetivo: Se analizará la conducta espacial de ratas macho adultas en un aprendizaje de respuesta y uno en el que se requiere flexibilidad conductual. A continuación, se evaluará la participación de aquellas estructuras que se han visto implicadas bien en aspectos emocionales o en el aprendizaje espacial (artículo III).

Cuarto objetivo: Se analizará la participación del hipocampo en el aprendizaje de respuesta. Asimismo se determinarán los niveles relativos de la subunidad I de la proteína CO mediante western blot para esclarecer la contribución de esta subunidad a la actividad de la enzima y al aprendizaje (Artículo IV).

Quinto objetivo: Se estudiarán los efectos que tiene la iluminación en la adquisición de un aprendizaje de respuesta y cómo estas condiciones afectan a las estructuras cerebrales implicadas en este tipo de aprendizaje. (Artículo V).

3. MATERIAL Y MÉTODOS

3.1. ANIMALES

Todos los experimentos se han realizado empleando ratas (*Rattus norvegicus*) macho de la cepa Wistar (150-250 g) procedentes del bioterio de la Universidad de Oviedo. Los sujetos fueron seleccionados aleatoriamente de diferentes camadas y fueron alojados en grupos de 5 animales por jaula. Los animales se mantuvieron bajo un ciclo de luz/oscuridad de 12 horas (periodo de luz: 08:00-20:00h), temperatura ambiental de 23 ± 2ºC, humedad absoluta de 65 ± 5% y acceso libre a comida y bebida. El uso y la manipulación de los animales se realizó en todo momento de acuerdo con las Comunidades Europeas 2010/63/UE y legislados en nuestro país mediante el Real Decreto 1201/2005.

Según el requerimiento de los experimentos realizados en cada objetivo, los animales fueron divididos en varios grupos, que se explicarán detenidamente tras el protocolo conductual de cada uno de ellos.

3.2. APARATO

Ambos aprendizajes (guía y de respuesta) se evaluaron en un laberinto acuático en T. El equipamiento empleado en todos los casos se compone de los siguientes elementos:

Se utilizó un laberinto acuático en T de fibra de vidrio y situado sobre una base de 40 cm de altura, una cámara (Sony V88E) suspendida del techo colocada sobre la piscina que captaba toda la superficie y un grabador de vídeo (Panasonic NV-HS930). Además, se empleó un ordenador al que se le instaló el programa Ethovision Pro (Ethovision; Noldus Information Technology, Wageningen, Holanda), para visionar la ejecución del animal mediante una conexión con el vídeo grabador y, a su vez, el programa registró los datos pertinentes para su posterior análisis.

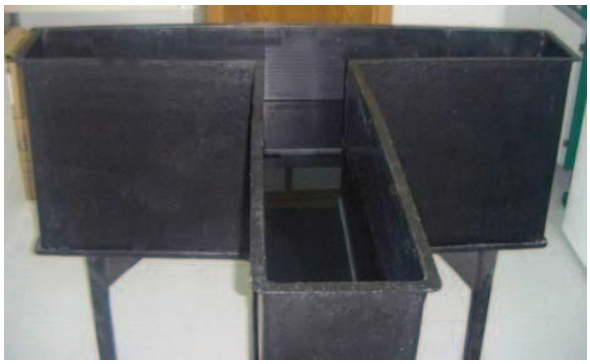

Figura 7: Fotografía del laberinto acuático en T empleado en nuestro estudio.

3.3. PROTOCOLO EXPERIMENTAL

Todos los animales fueron manipulados durante 5 días (5 min/día) y evaluados neurológicamente para descartar aquellos que presentaran algún déficit sensorial y/o motor.

3.3.1. Aprendizaje de discriminación visual mediante el empleo de una pista proximal (objetivos I y II).

El laberinto acuático en T se situó entre dos paneles laterales negros, a los que se adhirieron distintas pistas visuales en forma de figuras de cartón con distintos patrones geométricos que se mantuvieron constantes a lo largo del experimento. La sala en la que se llevó a cabo el procedimiento experimental permaneció aislada de cualquier tipo de ruido o iluminación procedente del exterior y que pudiese interferir en el aprendizaje.

La tarea del aprendizaje espacial se realizó diariamente entre las 09:30 y las 13:00 h. Durante la fase de habituación los animales fueron liberados en el agua con la cabeza orientada hacia la pared de la piscina desde el brazo central y se les permitió nadar durante 60 s por el laberinto en ausencia de plataforma.

Durante la fase de aprendizaje o prueba las ratas recibieron 12 ensayos de entrenamiento por día, separados por un intervalo de 30 s en los que la plataforma se colocó de forma pseudoaleatoria en el brazo izquierdo o derecho, siempre próxima a la pista visual que se localizaba en el interior del laberinto en la pared que se encuentra en

el extremo del brazo. El tiempo de permanencia sobre la plataforma fue de 15s y el tiempo máximo permitido 60s.

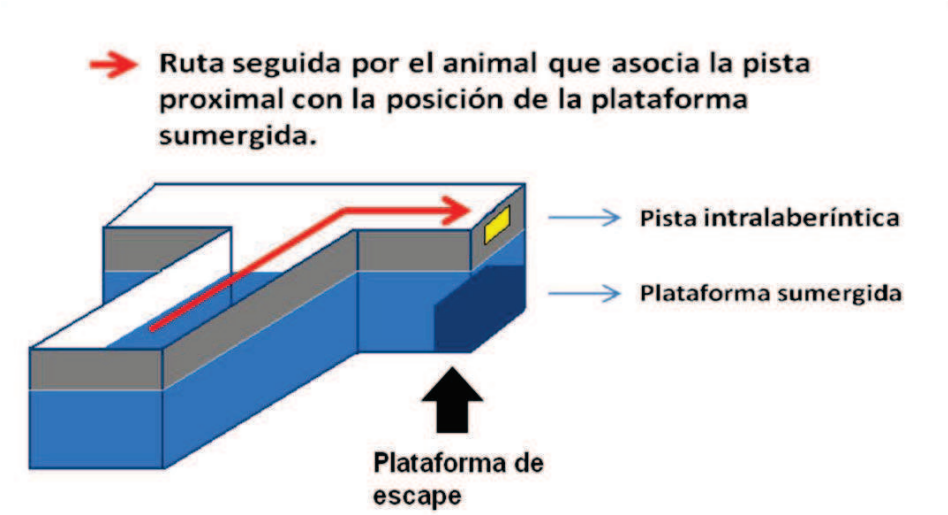

Figura 8: Laberinto acuático en T para estudiar el aprendizaje de guía (discriminación visual). La posición de la pista intralaberíntica es la guía para que la rata encuentre la plataforma sumergida.

Grupos de animales utilizados para estudiar el aprendizaje de discriminación visual

Los animales fueron distribuidos aleatoriamente en dos grupos:

Grupo experimental (n=10): animales que utilizaron una estrategia de discriminación visual durante 6 días consecutivos (12 ensayos por día).

Grupo control nado (n=10): animales que fueron manipulados y se introdujeron en la piscina el mismo número de veces y días que los animales experimentales que adquirieron el aprendizaje, de forma que el tiempo de nado fuera equivalente. Los intervalos entre los ensayos y entre sesiones fueron los mismos que en el grupo experimental, aunque en este caso no se utilizó plataforma de escape. Este grupo se utilizó con el fin de controlar factores como el estrés, la manipulación o la actividad motora.

Grupos de animales utilizados para estudiar el aprendizaje de discriminación visual a lo largo del tiempo.

Los animales fueron distribuídos aleatoriamente en cinco grupos:

Grupo habituación (n=10); grupo día-1 (n=10); grupo día-4 (n=10); grupo día 6 (n=9): los animales fueron sacrificados tras la habituación o tras ser evaluados en un aprendizaje de discriminación visual en los días 1, 4 ó 6 respectivamente.

Grupo control puro (n=10): donde los animales solo fueron manipulados y se utiliza para determinar los niveles de la actividad cerebral a nivel basal.

3.3.2. Estudio del aprendizaje egocéntrico en oscuridad durante 6 días (objetivos III y IV).

El protocolo empleado fue el mismo que en el caso anterior, aunque en éste, la localización de la plataforma fue siempre en el mismo brazo a lo largo de todos los ensayos, siendo éste el contralateral al elegido por el animal durante la habituación para fomentar la alternancia. Para evitar que el animal utilizase pistas visuales para orientarse, el experimento se realizó en oscuridad bajo luz roja, la cual es invisible para los animales de experimentación que empleamos.

Grupos de animales utilizados para estudiar el aprendizaje de respuesta y la flexibilidad conductual

Los animales fueron distribuídos aleatoriamente en tres grupos:

Grupo experimental egocéntrico (n=10): animales evaluados en un aprendizaje de respuesta durante 6 días consecutivos (12 ensayos por día).

Grupo experimental reversal (n=10): animales que utilizaron una estrategia egocéntrica (aprendizaje de respuesta) durante 6 días consecutivos (12 ensayos

por día) con la particularidad de que la plataforma se cambió de posición al lado contralateral del laberinto el último día de aprendizaje (sexto día).

Grupo control nado (n=10)

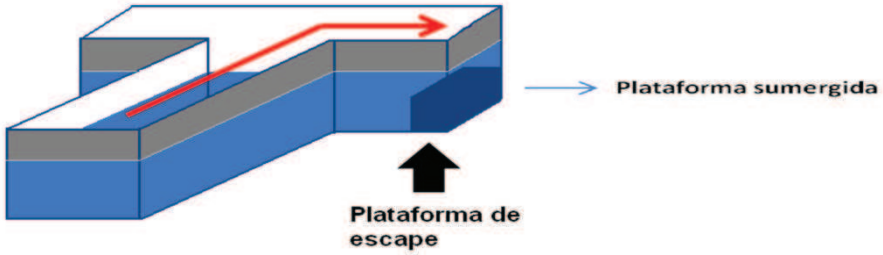

Figura 9: Laberinto acuático en T para estudiar el aprendizaje de respuesta (egocéntrico). La posición de la plataforma se mantiene constante a lo largo de los ensayos.

Grupos de animales utilizados para estudiar la implicación del hipocampo en el aprendizaje de respuesta.

Los animales fueron distribuídos aleatoriamente en dos grupos:

Grupo experimental egocéntrico (n=18): animales que fueron entrenados en una tarea de respuesta durante 6 días consecutivos (12 ensayos por día). De ellos 8 de los animales se seleccionaron para realizar a posteriori el análisis mediante western blot y 10 para realizar la técnica histoquímica CO.

Grupo control nado (n=17): De ellos 8 de los animales se seleccionaron para realizar a posteriori el análisis mediante western blot y los 9 restantes para realizar la técnica histoquímica CO.

3.4. El aprendizaje de respuesta: efecto de la luz (objetivo V).

Para estudiar el efecto que tienen las condiciones lumínicas en la conducta y en el metabolismo cerebral, los animales fueron entrenados durante un único día. Se colocaron dos laberintos idénticos enfrentados y se rodearon de cortinas para evitar que

el animal pudiera utilizar pistas visuales para orientarse. Uno de los grupos conductuales y su control nado fueron entrenados en oscuridad, mientras que otros dos grupos fueron entrenados en una habitación con las condiciones lumínicas habituales. Durante el aprendizaje, los 11 primeros ensayos se realizaron en uno de los laberintos, mientras que el último se realizó en el opuesto, comprobando así que los animales estuvieran utilizando una estrategia egocéntrica y no alocéntrica. De esta forma, si los animales están utilizando una tarea egocéntrica realizarán siempre el mismo giro (izquierda o derecha) independientemente del laberinto en el que se encuentren.

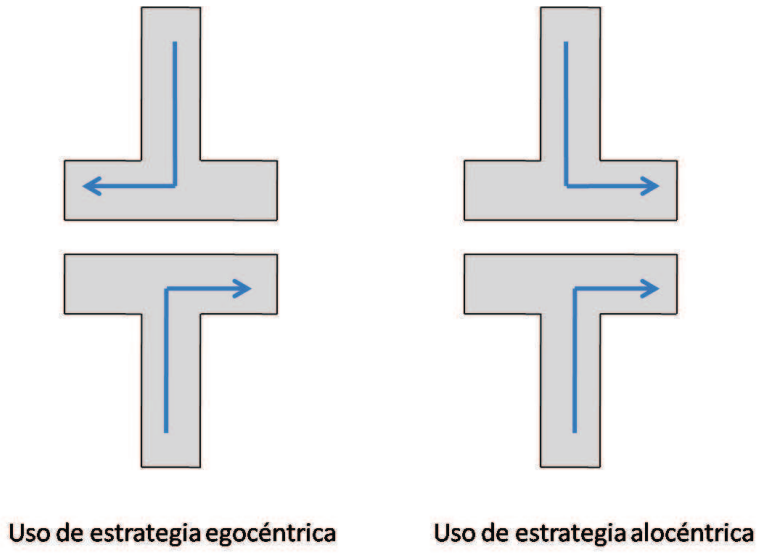

Figura 10: esquema mostrando la conducta que llevaría a cabo un animal que está utilizando una estrategia egocéntrica (izq) o alocéntrica (dcha).

De esta forma, los animales fueron distribuidos aleatoriamente en cuatro grupos:

Grupo experimental egocéntrico en condiciones de oscuridad (n=10): animales que fueron entrenados en una tarea de respuesta en oscuridad.

Grupo control nado en condiciones de oscuridad (n=10)

Grupo experimental egocéntrico en luz (n=10): animales que utilizaron una estrategia de respuesta en condiciones lumínicas normales.

Grupo control nado en luz (n=10)

3.5. OBTENCIÓN DE LOS TEJIDOS

El procesamiento del tejido cerebral (común para todos los objetivos) se realizó empleando los métodos que se describen a continuación.

3.5.1. Determinación de la actividad citocromo c oxidasa cerebral (objetivos I, II, III, IV, V):

Una hora y media después de finalizar las pruebas conductuales, los animales experimentales y sus grupos control correspondientes, fueron decapitados y sus cerebros se extrajeron rápidamente. Los encéfalos se congelaron por inmersión en isopentano a –80º C, durante dos minutos y se conservaron en un congelador a - 40º C, para prevenir el deterioro del tejido con la consiguiente pérdida de actividad enzimática. El cerebro se recubrió con un gel crioprotector "OCT" (Jung, Alemania) para ser posteriormente seccionados coronalmente mediante un microtomo criostático (Microm International GmbH, modelo HM 505 E, Heidelberg, Alemania) en cortes de 30µm de grosor que se colocaron en portaobjetos que previamente habían sido limpiados con alcohol de 100º. Para realizar un muestreo completo y sistemático de las distintas áreas cerebrales a estudiar, se seleccionaron las estructuras a analizar utilizando el atlas de Paxinos y Watson (2005).

3.5.2. Determinación de la concentración relativa de proteína analizada mediante western blot (objetivo IV).

Todos los animales fueron decapitados 90 min después de finalizar las pruebas conductuales. El hipocampo fue extraído en fresco y homogenado en tampón de lisis, para posteriormente ser sonicado y almacenado a -40ºC.

3.6. HISTOQUÍMICA DE LA CITOCROMO C OXIDASA

Esta técnica histoquímica consiste en el marcaje de la citocromo c oxidasa, enzima presente en la membrana interna mitocondrial, que pertenece a la clase óxido-reductasa. Como ya comentamos en la introducción, esta enzima cataliza el último paso

en la cadena de transporte electrónico, en el proceso de fosforilación oxidativa, permitiendo la producción del ATP.

El procedimiento seguido fue el descrito por Wong-Riley (1979) basándonos en ciertas modificaciones propuestas por Gonzalez-Lima y Cada (Gonzalez-Lima y Cada, 1994). Con el fin de corregir las posibles variaciones de la tinción citocromo oxidasa en los distintos baños de incubación, se utilizaron una serie de secciones de diferentes grosores (10, 30, 50 y 70 μm) como estándares, obtenidas de homogenados cerebrales de rata (Figura 11). Este tejido homogenado se obtuvo de la decapitación y extracción de los cerebros de 12 ratas macho adultas Wistar, que posteriormente se sumergieron en tampón fosfato de pH 7,6 a 4ºC. Posteriormente se realizó la homogenización a 4º C y mediante espectrofotometría se determinó la actividad de la citocromo oxidasa del homogenado obtenido. Estos valores de actividad permiten realizar una curva de regresión lineal para convertir los valores de medida de densidad óptica de las estructuras seleccionadas, en valores de actividad citocromo oxidasa. El homogenado cerebral, al igual que las secciones de cerebro de rata seleccionado, se congelaron con isopentano y se almacenaron a -40 ºC.

Para realizar la tinción se emplearon cubetas de 800 ml, en las que se introdujeron gradillas con una capacidad para 60 portaobjetos en las que se incluye el portaobjeto donde se colocaron las secciones del homogenado. Las secciones congeladas se introdujeron en una solución de tampón fosfato con sacarosa (pH 7,6; 0,1M, 100g de sacarosa por litro de tampón) con glutaraldehido al 0,5% durante 5 min para favorecer la adhesión del tejido al portaobjetos. A continuación las secciones se lavaron en tres ocasiones con tampón fosfato con sacarosa durante 5 min antes de introducirlas 4 min en la solución TRIS, cuya composición para un litro es: 363 ml de agua bidestilada, 387 ml de ácido clorhídrico 0,1 N, 5 ml de dimetilsulfóxido (DMSO), 250 ml de trizma base, 100 g de sacarosa y 0,275 g de cloruro de cobalto. Se realizó un lavado en tampón fosfato (pH 7,6; 0,1M) para posteriormente incubarlas en oscuridad durante una hora en la solución de tinción, en agitación lenta y a 37ºC. La composición de dicha solución es la siguiente: 800 ml de tampón fosfato 0,1 M, 0,06 g de citocromo c, 0,016 g de catalasa, 40 g de sacarosa, 2 ml de DMSO y 0,4 g de diaminobencidina (DAB). La DAB se emplea en el revelado y actúa como un cromógeno. Tras haber sido

oxidada por la CO (endógena y exógena), se transforma en un producto marrón insoluble que se fija a la membrana mitocondrial, y da el color que observamos tras la tinción. Esta solución de tinción se prepara en oscuridad ya que la presencia de luz podría dar lugar a una tinción inespecífica como consecuencia de la oxidación espontánea de la DAB.

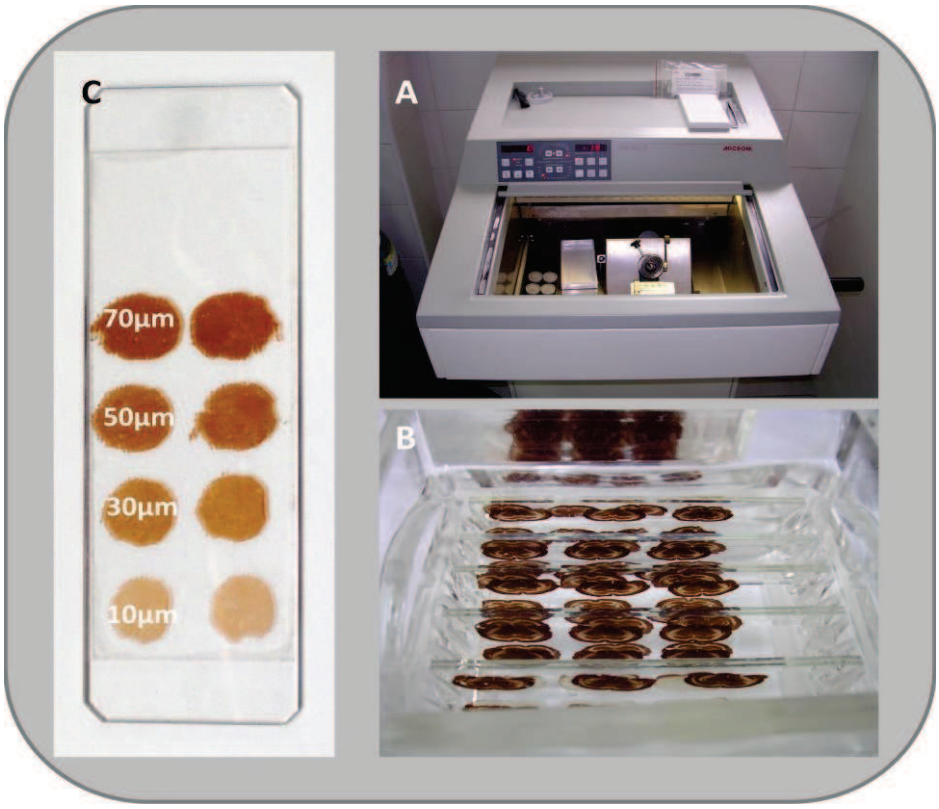

Figura 11: A. Fotografía del criostato en el que se realizaron los cortes coronales de 30 µm de tejido cerebral congelado. **B.** Fotografía de las secciones durante la tinción con la técnica histoquímica CO. **C.** Fotografía de una serie de secciones de diferentes grosores (10, 30, 50 y 70 µm) que se utilizan como estándares para realizar 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.

Posteriormente, y a temperatura ambiente, se bloqueó la reacción con un baño de formaldehido (Prolabo, España) tamponado al 10%, durante 30 min. Finalmente, el tejido se deshidrató mediante baños de cinco minutos en una cadena de alcoholes de concentración creciente. Una vez deshidratado el tejido se realizó el montaje con Entellán (Merck, Darmstadt, Alemania) para la correcta conservación del tejido.

Mediante la utilización de un sistema de análisis de imágenes informatizado (MCID Elite Interfocus Linton, Inglaterra) se analizó y cuantificó la intensidad de la tinción CO mediante densitometría óptica.

Figura 12: Fotografía en la que se muestra el sistema de análisis de imágenes informatizado. A la izquierda se encuentra la pantalla del ordenador donde se observa ampliada la sección elegida y a la derecha se localiza la fuente de luz, conectada a la cámara de alta sensibilidad, donde se coloca el portaobjetos que se pretende cuantificar.

Para el análisis, inicialmente se realizaron las medidas densitométricas de los estándares que acompañan a cada baño de tinción para confeccionar la curva de regresión que va a permitir comparar entre las distintas series/ baños de tinción. A continuación, se seleccionaron las estructuras de interés con la ayuda del atlas de Paxinos y Watson (Paxinos y Watson, 1997), para posteriormente iniciar la medición. Con el fin de evitar errores al delimitar la estructura. se tomaron varias medidas dibujando con el ratón del ordenador cuadrados distribuidos de forma aleatoria dentro de la misma. De cada estructura se realizan cuatro medidas en 3 secciones consecutivas, doce en total.

3.7. ANÁLISIS PROTÉICO POR WESTERN BLOT

El protocolo seguido para realizar la técnica de western blot, se describe brevemente a continuación. Se determinó la concentración de proteína por alícuota mediante el análisis Bradford para posteriormente recogerlas en Laemmly.

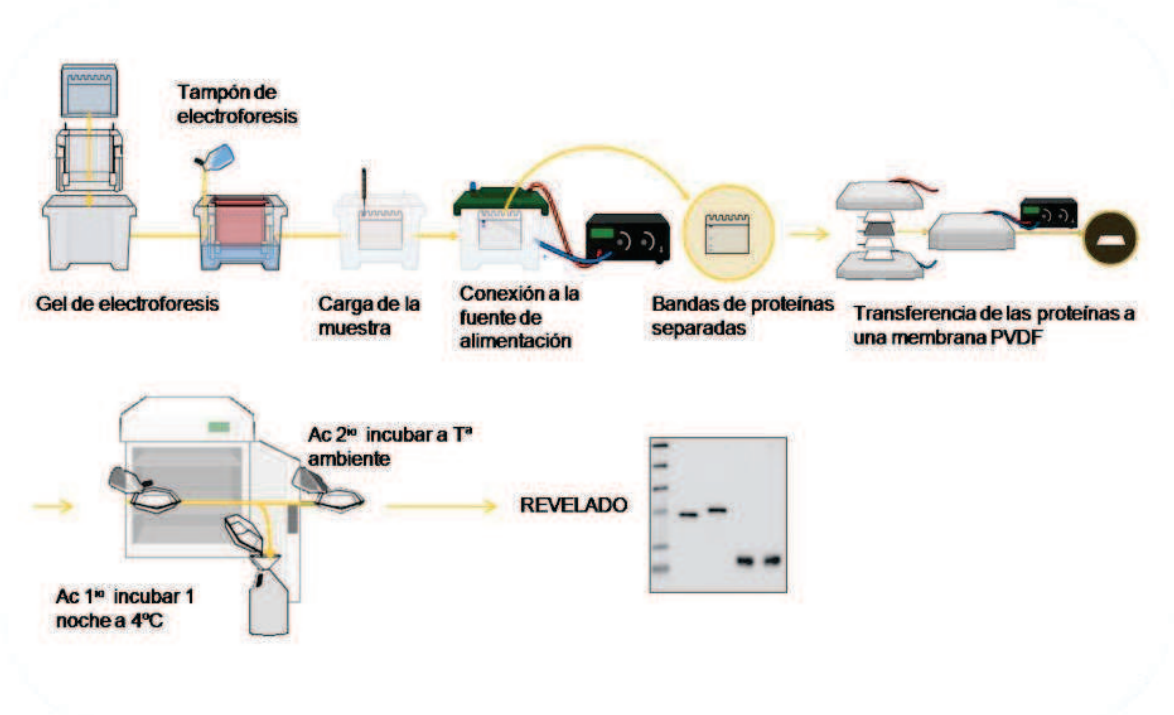

Figura 13: Breve esquema de los principales pasos de la técnica de Western blot.

Las alícuotas se desnaturalizaron por calor y posteriormente 50 µg de proteína se corrieron en un gel SDS-PAGE al 12%. Las proteínas se transfirieron a membranas de polifluoruro de vinilideno (PVDF) (Millipore Iberica, Madrid, Spain) que se bloquearon con TBS/Tween-20 suplementado con leche en polvo al 5% durante 1 h a temperatura ambiente para evitar las uniones inespecíficas del anticuerpo.

Posteriormente, las membranas se incubaron una noche a 4ºC con el anticuerpo (Ac) policlonal anti subunidad CO I (COX1/C-20) a una concentración 1:200 (sc-23982, Santa Cruz Biotechnology, USA). Al día siguiente se lavaron varias veces con TBS/Tween para así eliminar el exceso de anticuerpo. A continuación se incubaron

durante una hora a temperatura ambiente en anticuerpo secundario (1:6000, A8919, Sigma-Aldrich, Madrid, Spain) conjugado con peroxidasa y se volvieron a lavar para eliminar el exceso de anticuerpo secundario. Por último las membranas fueron reveladas.

Como control de carga de la electroforesis se utilizó la proteína β-actina. Para ello se utilizó el anticuerpo monoclonal anti β-actina a concentración 1:2000 (A-5441, Sigma, Madrid, Spain) y el anticuerpo secundario anti ratón conjugado con peroxidasa (Sigma, Cat. A-9044) a concentración 1:10000.

3.8. ANÁLISIS ESTADÍSTICO

Los diferentes datos obtenidos se analizaron estadísticamente utilizando el programa SigmaStat 3.2 y se representaron gráficamente por medio del programa SigmaPlot 8.0 (ambos: Systat, Richmond, EEUU). Los resultados se consideraron estadísticamente significativos cuando $p \leq 0,05$.

3.8.1. Análisis de los procedimientos conductuales

A nivel conductual, se considera que los animales han aprendido la tarea cuando realizan al menos el 70 % de las elecciones correctas, por lo que en algunos trabajos no se ha empleado análisis estadístico para determinar el criterio de aprendizaje (III y IV). En los artículos en los que se estudia el aprendizaje de guía (I y II), el número de elecciones correctas realizadas por los animales entrenados se analizó mediante un test no paramétrico Friedman. Para evaluar las diferencias existentes entre los distintos días, se utilizó, cuando fue necesario, el test a posteriori Newman-Keuls.

Para la comparación entre el número de aciertos realizado por cada grupo experimental egocéntrico dependiendo de las condiciones lumínicas, se utilizó la prueba U de Mann-Whitney.

3.8.2. Análisis de la actividad CO

Con los datos obtenidos de la cuantificación de la actividad CO para cada una de las diferentes regiones cerebrales estudiadas, entre el grupo experimental que realizó un aprendizaje de guía y su control nado (artículo I), se aplicó un test t de Student para muestras independientes.

Cuando se analizaron los valores de la actividad CO en experimentos con más de dos grupos, los datos obtenidos de la cuantificación de la actividad CO en las diferentes regiones cerebrales fueron analizados mediante el análisis de la varianza (ANOVA) de un factor usando como variable independiente los grupos experimentales (control caja, habituación, primero, cuarto y sexto día de entrenamiento) en el artículo II y (grupo egocéntrico, reversal y control nado) en el artículo III. Se utilizó como test a posteriori el test de Tukey para observar diferencias entre pares de grupos experimentales cuando el ANOVA muestra la existencia de diferencias significativas entre grupos. En los casos en los que no se cumplió la normalidad se utilizó el test de Kruskal Wallis.

En aquellos casos en los que deseamos analizar el efecto de dos variables independientes sobre la variable dependiente, como es la actividad CO, (artículos IV y V) utilizamos un ANOVA bifactorial. En el artículo IV se emplearon como variables independientes los factores “grupo” y “hemisferio”, mientras que en el artículo V se utilizaron como variables independientes “grupo” y “condición lumínica”. Como prueba a posteriori se utilizó el test de Tukey en ambos casos.

Por último, 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 experimental. Para ello, previamente los valores de la actividad CO se normalizaron dividiendo la actividad obtenida en cada región cerebral entre la media de la actividad CO de todas las regiones en cada animal, de manera que se reducen las variaciones en la actividad CO que no fueron consecuencia del aprendizaje. A continuación, para evitar errores debidos a tamaños de muestra muy pequeños, se empleó el método “Jackknife” (Shao y Tu, 1995). Este procedimiento consiste en calcular todas las posibles correlaciones que resultan de eliminar un sujeto cada vez

teniendo en consideración únicamente las correlaciones que sean significativas a lo largo de todas las combinaciones.

3.8.3. Análisis estadístico de la concentración relativa determinada por western blot.

La concentración relativa de la subunidad I (COI) se analizó midiendo densitométricamente la intensidad de las bandas mediante la misma cámara y sistema informático que utilizamos para analizar la actividad CO. Para normalizar los datos, se dividió la intensidad obtenida en las bandas de la proteína COI entre las bandas obtenidas de la proteína β-actina. Las diferencias entre los grupos se evaluaron empleando el test estadístico t-Student (artículo IV).

4. RESULTADOS

En este apartado se realiza una breve descripción de los principales resultados obtenidos en cada uno de los experimentos.

4.1. RESULTADOS CONDUCTUALES

4.1.1. Análisis conductual de la tarea de discriminación visual (objetivos I y II):

Los resultados obtenidos en la tarea de discriminación visual muestran un incremento en el número de elecciones correctas realizadas por los animales de los grupos experimentales a lo largo de los días de aprendizaje. Concretamente se observaron diferencias significativas entre el día 6 y el resto de los días de aprendizaje ($p \leq 0.05$).

4.1.2. Análisis conductual del aprendizaje de respuesta:

4.1.2.1. *Aprendizaje de respuesta (objetivos III y IV)*

Los animales alcanzaron el criterio de al menos realizar el 70% de las elecciones correctas el primer día de aprendizaje (objetivos III, IV). El número de elecciones correctas se mantuvo, siendo superior al criterio establecido, a lo largo de los días de aprendizaje. Asimismo, los animales pertenecientes al grupo reversal, es decir, aquellos que tuvieron que revertir una asociación estímulo-respuesta previamente establecida debido a que la posición de la plataforma cambió al lado contralateral el último día, cometieron tan solo entre 1 ó 2 errores en los 12 ensayos realizados el sexto día de aprendizaje.

4.1.2.2. *Aprendizaje de respuesta en dos condiciones lumínicas distintas (objetivo V)*

Como previamente se había observado, todos los animales alcanzaron el criterio de aprendizaje establecido, realizando al menos el 70% de elecciones correctas en los 12 ensayos realizados durante 1 día de entrenamiento.

4.2. ACTIVIDAD METABÓLICA

4.2.1. Uso de una estrategia de discriminación visual

4.2.1.1. *Estudio de las estructuras cerebrales implicadas en el aprendizaje de discriminación visual*

Cuando analizamos la actividad metabólica cerebral de animales que fueron entrenados en una tarea de discriminación visual observamos que el grupo experimental y su grupo control nado difieren en su nivel de metabolismo basal en las áreas CA1 y CA3 dorsal del hipocampo. Además, el análisis de correlaciones interregionales en el grupo experimental que empleó una estrategia de guía para llevar a cabo la tarea de discriminación visual mostró una correlación inversa entre el área CA1 del hipocampo y el estriado anterodorsal.

4.2.1.2. *Estudio de las estructuras cerebrales implicadas en el aprendizaje de discriminación visual a lo largo del tiempo*

Cuando estudiamos la actividad cerebral a lo largo del proceso de aprendizaje de guía nuestros resultados muestran un incremento generalizado de la actividad CO en la mayor parte de las estructuras estudiadas tanto en el día en la que se realizó la habituación, como en los días 1 y 4 en comparación con el grupo evaluado el sexto día. Concretamente, tras la habituación de los animales se observó un aumento de la actividad metabólica cerebral en las cortezas prefrontal, parietal, perirrinal y entorrinal, en las áreas CA1 y CA3 del hipocampo dorsal, en el estriado dorsal y en el complejo amigdalino. La actividad metabólica en estas regiones se mantuvo elevada a lo largo del aprendizaje hasta el día 6, en el que la actividad disminuyó a niveles basales. La corteza del núcleo accumbens se activó en la habituación, para mantenerse a niveles basales el resto del proceso de aprendizaje. Por otro lado, el giro dentado dorsal incrementó su actividad metabólica únicamente durante los días del 1 al 4.

Al analizar las redes neuronales implicadas en el aprendizaje de discriminación visual, observamos la existencia de correlaciones entre las cortezas prefrontal, y

entorrinal en el grupo que solamente fue habituado. Además, se encontró una interacción entre la amígdala lateral, la corteza parietal y la corteza del núcleo accumbens. Por otro lado, el día 1 de entrenamiento se encontraron interacciones implicando a la corteza parietal y CA3 por un lado, y por el otro, una serie de correlaciones intrarregionales entre la corteza cingulada y prelímbica, las cortezas temporales entorrinal y perirrinal y entre el estriado anterodorsal y lateral.

Finalmente, en el cuarto día se encontraron correlaciones entre las distintas regiones de la corteza prefrontal. Estas correlaciones se mantuvieron durante el día 6, añadiéndose una correlación inversa entre la corteza infralímbica y la parietal. Por otro lado, se hallaron altas correlaciones entre los núcleos del complejo amigdalino entre sí al igual que entre el estriado anterodorsal y el estriado lateral.

4.2.2. Uso de una estrategia de respuesta

4.2.2.1. *Análisis de las regiones cerebrales implicadas en el aprendizaje de respuesta y la flexibilidad conductual*

Al analizar las regiones cerebrales implicadas en un aprendizaje egocéntrico que se ha realizado en oscuridad durante 6 días consecutivos, se observó que la actividad CO era significativamente mayor en las regiones corticales (corteza motora, cingulada, prelímbica, infralímbica y orbital), en la amígdala lateral, el estriado anterolateral y el área tegmental ventral de los animales experimentales (grupo día-6) que de sus grupos control nado. En el grupo reversal se observó en comparación con el grupo egocéntrico, un descenso a los niveles basales en la mayor parte de las estructuras analizadas, a excepción de la corteza orbital y el área tegmental ventral, cuya actividad fue significativamente mayor que la existente a nivel basal en el grupo control nado. Sin embargo, el estriado anterodorsal presenta un descenso de la actividad CO respecto al grupo día-6.

Al analizar las correlaciones existentes entre las estructuras cerebrales estudiadas, se observó que el aprendizaje egocéntrico requiere la formación de una red

cerebral formada por subregiones de la corteza prefrontal (corteza motora, cingulada y prelímbica) y una segunda red formada por núcleos del complejo amigdalino (lateral y basolateral). La actividad CO en los animales que realizaron el aprendizaje reversal mostró la existencia de una interacción funcional entre las amígdalas central y basolateral además de una correlación inversa entre la corteza motora y el estriado anterolateral.

4.2.2.2. *Análisis de la implicación del hipocampo en el aprendizaje de respuesta*

Se observó un incremento en la actividad cerebral en el hipocampo dorsal (CA1 y CA3) y en el hipocampo ventral (CA1, CA3 y GD) cuando se compararon animales que habían utilizado una estrategia egocéntrica durante 6 días en oscuridad y sus controles nado correspondientes. Sin embargo, cuando se compararon los niveles relativos de proteína (subunidad I de la enzima CO) (COI) en el hipocampo no se observaron diferencias significativas en el hipocampo izquierdo ni en el derecho.

4.2.2.3. *Estudio del efecto de la luz en el aprendizaje de respuesta*

Los resultados obtenidos muestran que el núcleo geniculado dorsal lateral y la corteza visual disminuyeron significativamente su actividad cerebral dependiendo de las condiciones lumínicas, sin embargo, el proceso de aprendizaje no tuvo ningún efecto sobre ellas.

Se encontraron diferencias significativas en la actividad CO en la corteza parietal y el hipocampo ventral (CA3 y GD) entre el grupo de animales que utilizó la estrategia de respuesta en una habitación iluminada y su control nado. En los animales que realizaron la tarea en oscuridad, también se encontraron diferencias significativas en la corteza parietal e hipocampo ventral (CA1). Además, se observó la activación de otras estructuras cerebrales como la corteza prefrontal (cingulada, prelímbica,

infralímbica), el núcleo del núcleo accumbens, el hipocampo dorsal (CA1, CA3 y giro dentado), la amígdala central, y el cuerpo estriado.

Figura 14

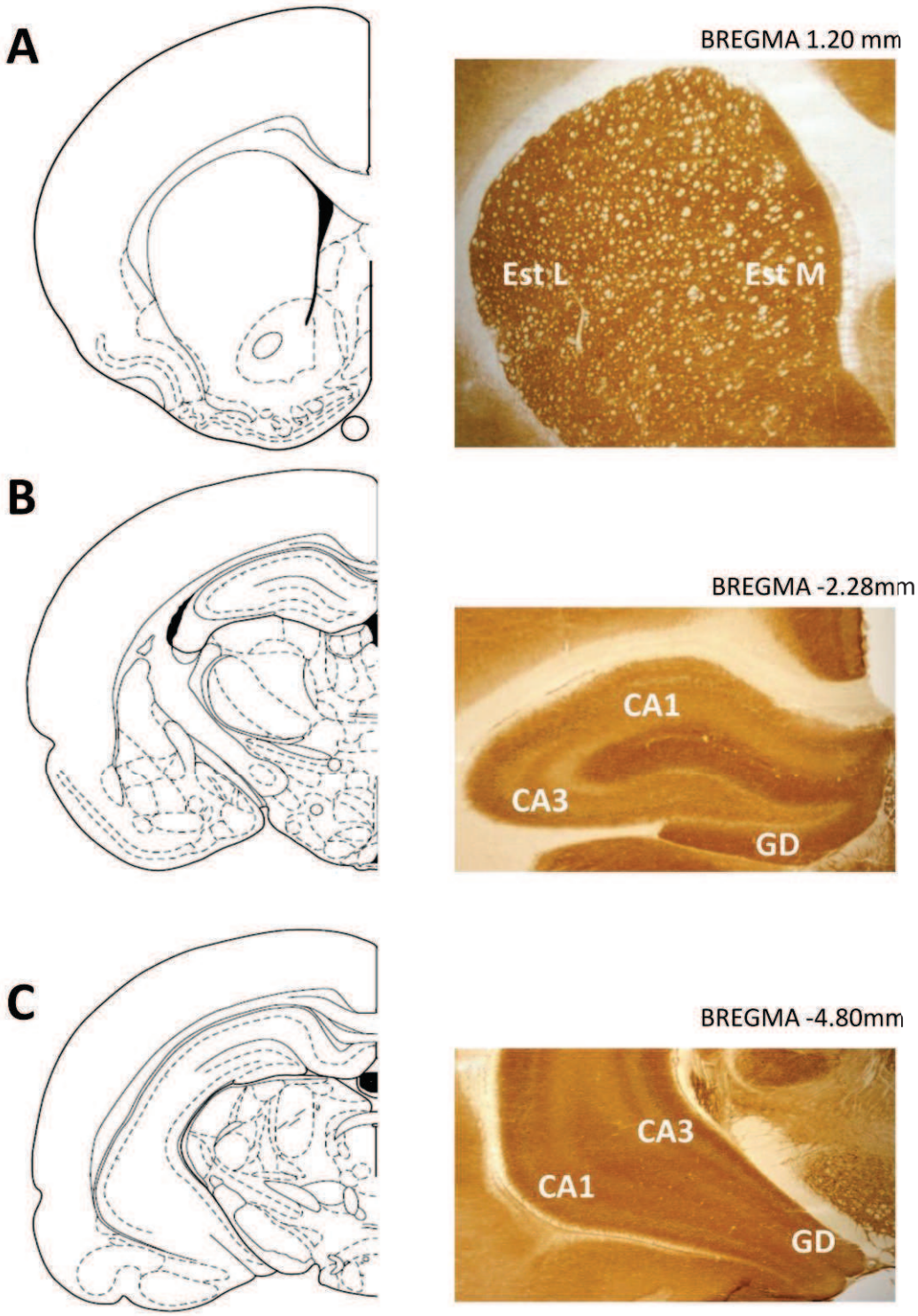

Figura 14: A la izquierda, representación esquemática de las áreas de interés del **A**. cuerpo estriado **B.** hipocampo dorsal y **C.** hipocampo ventral. A la derecha, se muestran microfotografías mostrando secciones coronales teñidas con la técnica CO. **A.** Est M: estriado anteromedial; Est L: estriado anterolateral. **B.** Áreas CA1, CA3 y GD del hipocampo dorsal. **C:** Áreas CA1, CA3 y GD del hipocampo ventral.

5. DISCUSIÓN

El objeto de este trabajo es contribuir al esclarecimiento de las estructuras implicadas en los aprendizajes de respuesta o también llamados aprendizajes de hábitos. Por esta razón, determinamos el metabolismo cerebral en distintas estructuras cerebrales que de una manera u otra se han visto implicadas en estudios de aprendizaje y memoria espacial. Para ello analizamos el uso de dos tipos de estrategias, una de respuesta (objetivos III, IV y V) que requiere el uso de pistas internas del animal y otra de guía, (objetivos I y II) que se evaluó entrenando a los roedores en una tarea de discriminación visual, en la que tenían que asociar la posición de una pista intralaberíntica con la localización de una plataforma sumergida. Ambas estrategias se evaluaron en un laberinto acuático en T durante 6 días de aprendizaje y se empleó como medida conductual el número de aciertos realizados por el animal.

La primera observación atendiendo a la medida conductual, es que las ratas evaluadas en el paradigma de aprendizaje espacial egocéntrico, requirieron un número de ensayos menor para ejecutar correctamente la tarea que los animales evaluados en una estrategia de guía. Concretamente, los animales que utilizaron una estrategia egocéntrica solo necesitaron un día para llegar al 70% de ensayos correctos independientemente de si la tarea se ejecutaba en condiciones de oscuridad o no. Sin embargo, los animales evaluados en la estrategia de guía necesitaron cinco días más. Es decir, que una rata necesita menos de 12 ensayos para localizar una plataforma sumergida, situada constantemente en uno de los brazos del laberinto acuático en T, mientras que una rata entrenada en una tarea de discriminación visual, necesita 72 ensayos. Estos resultados han sido observados en estudios previos donde se han evaluado estrategias de guía, en las que se utilizan periodos largos de aprendizaje (Epp y cols., 2008; Murray y Ridley, 1999), y estrategias egocéntricas, en las que los periodos de aprendizaje son cortos tanto en adultos (12 ensayos requeridos) como en animales viejos, en los que se requirieron 16 ensayos para alcanzar más de un 70% de elecciones correctas (Begega y cols., 2001).

Esta diferencia en cuanto al tiempo requerido por los animales para la correcta ejecución de la tarea, puede deberse a que éstos seleccionan la estrategia que van a utilizar en función del entorno, de tal manera que si existen pistas visuales distales, el

animal tiende a utilizar preferentemente una estrategia alocéntrica (Kealy y cols., 2008). Existen evidencias experimentales de que las ratas intentan aplicar varias estrategias para solventar problemas espaciales, siendo incluso capaces de cambiar de una a otra cuando es necesario. Así, al evaluar ratas entrenadas en un laberinto en T, éstas utilizan inicialmente un aprendizaje cartográfico (de lugar), pero cuando se avanza en el entrenamiento cambian a un aprendizaje táxico (de respuesta) (Packard y McGaugh, 1996). Recordemos que el aprendizaje táxico requiere el uso de patrones motores estereotipados por parte del animal mientras que en el cartográfico el individuo se forma un mapa cognitivo mediante la utilización de las pistas visuales circundantes. Además, cuando existen pistas tanto proximales como distales, los roedores prefieren utilizar, al menos en un principio, las pistas distales (Whishaw y cols., 2001). Dado que la habitación por nosotros empleada contiene pistas distales, es posible que al inicio de la tarea los animales hayan intentado utilizar una estrategia cartográfica, que no es útil para llegar a la plataforma, y posteriormente hayan adquirido la estrategia correcta, que es la de guía. En nuestro experimento, al haber utilizado el laberinto acuático en T, parece poco probable que el animal haya utilizado las pistas distales para orientarse, ya que por las características físicas del aparato, las paredes son muy altas y dificultan al animal ver por encima de ellas. Por ello, nos parece más probable que el animal haya utilizado solamente la pista proximal durante el entrenamiento. De todas formas, sólo el uso de la estrategia de discriminación visual permite al animal realizar la elección correcta, por lo que sí podemos asegurar que los animales estudiados utilizaron la estrategia deseada.

Por otro lado, cuando evaluamos la tarea egocéntrica, el número de ensayos requeridos fue menor, necesitándose únicamente 12 ensayos para alcanzar el criterio preestablecido de realizar al menos un 70% de elecciones correctas, independientemente de si el aprendizaje transcurría en oscuridad o en una habitación iluminada.

En este sentido, debemos considerar que dado que el número de ensayos realizados por los animales que utilizaron una estrategia egocéntrica y una de guía han sido los mismos en los experimentos redactados en los artículos (I, II, III y IV), aquellos que fueron evaluados en la tarea egocéntrica estuvieron durante 5 días repitiendo una actividad que fue aprendida el primer día, por lo que posiblemente, al sexto día, estemos

evaluando una fase de recuerdo. Por otro lado, el grupo que utilizó una estrategia de guía ha necesitado todos los días de aprendizaje para adquirir la tarea y no es hasta el sexto día cuando llegan al nivel requerido. Por lo tanto, estos animales se encontrarían bien en una fase de adquisición del aprendizaje o de consolidación del mismo ya adquirido. Como consecuencia, no podemos comparar el uso de una estrategia egocéntrica con el uso de una estrategia de guía, dado que estos animales no se encuentran en la misma fase de aprendizaje. Como el interés de esta tesis es ahondar en el conocimiento de las bases neurales de la memoria de hábitos, necesitábamos diseñar un nuevo experimento para analizar las estructuras cerebrales que están implicadas en la adquisición de un aprendizaje egocéntrico. Por ello, un nuevo grupo de animales fue entrenado en la tarea egocéntrica, pero únicamente durante un día, que es el tiempo necesario para alcanzar el criterio de aprendizaje.

Por otro lado, la teoría de los múltiples sistemas de memoria propone la existencia de varios tipos de circuitos neuronales que están adaptados a almacenar distintos tipos de información (McDonald y White, 1993; Packard y McGaugh, 1992; Poldrack y Packard, 2003; White y McDonald, 2002; Yin y Knowlton, 2006). Concretamente, en el cerebro de mamíferos, el hipocampo y el estriado dorsal, son el sustrato de dos tipos de memoria diferentes; mientras que la región hipocampal (CA1, CA3, giro dentado y subiculum) y las cortezas adyacentes son importantes en la memoria declarativa, el estriado dorsal lo es en la memoria de hábitos. Sin embargo, en los últimos años este concepto de independencia entre los sistemas de memoria hipocampal y estriatal ha ido cambiando. De esta manera, se ha puesto de manifiesto que disfunciones tanto en el hipocampo como en el estriado contribuyen al déficit cognitivo asociado a la enfermedad de Hungtington (Ciamei y Morton, 2009; Ghiglieri y cols., 2011). Por ello, en este trabajo nos propusimos estudiar la participación de hipocampo y el cuerpo estriado en la adquisición de una estrategia de guía y otra de respuesta, ya que aunque estas estrategias han sido consideradas tradicionalmente como hipocampo independientes, algunos estudios indican déficits en la memoria de hábitos tras lesiones hipocampales (Sutherland y cols., 2001). Para ello, utilizamos la técnica histoquímica de la citocromo c oxidasa. Concretamente, los incrementos en la actividad de esta enzima se interpretan como un aumento de la demanda energética de estas áreas, ya que el metabolismo energético refleja la demanda energética de las neuronas y ésta

es directamente proporcional a su actividad. Por lo tanto, aquellas estructuras con mayor demanda energética presentarán una mayor actividad de esta proteína y como consecuencia estarán relacionadas con el aprendizaje espacial en el que se evalúa a los animales.

Participación de hipocampo y cuerpo estriado en el aprendizaje de discriminación visual.

Al finalizar el aprendizaje de discriminación visual, los animales presentaron un incremento de la actividad metabólica con respecto al grupo control de nado en hipocampo dorsal (concretamente en el área CA1 y en el giro dentado) pero no observamos cambios a nivel del estriado dorsal respecto a este grupo control. Estos resultados, aunque no eran los esperados a priori, dado la importancia del estriado en el aprendizaje de hábitos, son apoyados por estudios que muestran la participación del hipocampo en este tipo de tareas. Concretamente, se ha observado que las áreas CA1 y el GD son claves en el aprendizaje de discriminación visual complejo (Hu y cols., 2005). Asimismo, animales que aprenden este tipo de tarea y posteriormente son lesionados hipocampalmente, desarrollan una amnesia severa retrógrada (Sutherland y cols., 2001; Driscoll y cols., 2005; Epp y cols., 2008; McDonald y cols., 2007), lo que parece indicar una participación del hipocampo en el uso de estrategias de guía tanto en la adquisición como en el recuerdo. Además, hemos observado la existencia de una correlación inversa entre CA1 y el estriado anterodorsal en nuestro experimento, por lo que en este caso concreto, el hipocampo y el estriado parecen estar participando conjuntamente (Mizumori y cols., 2009).

Aunque los resultados obtenidos nos aportan información de la relación existente entre hipocampo y estriado en el uso de una estrategia de guía, no nos permiten deducir cuál es la participación de estas estructuras a lo largo del aprendizaje. Para esclarecer este punto, nos propusimos estudiar las interacciones que se producen entre hipocampo y estriado a lo largo de los distintos estadíos temporales de este complejo proceso. Además, se sabe que el procesamiento de la información espacial en el cerebro requiere que existan conexiones entre estructuras corticales y subcorticales (Conejo y cols., 2010), por lo que nos propusimos estudiar además el papel que juegan otras estructuras cerebrales que presentan conexiones nerviosas con el hipocampo y

estriado, ya que las vías neurales por las que se media el aprendizaje de las discriminaciones visuales no se conocen con exactitud.

Curiosamente, tras el análisis de la actividad CO nos encontramos un incremento generalizado del consumo energético en la habituación, día 1 y día 4 de las estructuras estudiadas para alcanzar niveles basales el sexto día de aprendizaje. Ésto nos hace suponer que durante el aprendizaje se requiere la activación de múltiples regiones cerebrales, pero una vez que la tarea se ha aprendido la actividad cerebral desciende a niveles basales.

Durante la habituación se produce una activación masiva de estructuras cerebrales como las cortezas prefrontal, parietal y temporal, la corteza del accumbens, el complejo amigdalino, el hipocampo dorsal y el cuerpo estriado, que puede ser debida a la novedad del ambiente y al primer contacto con el agua. Concretamente, el núcleo accumbens está muy relacionado con la habituación a nuevos entornos (Campioni y cols., 2009) así como la amígdala y el hipocampo que también responden ante situaciones de estrés (Aguilar-Valles y cols., 2005). Además, observamos que la amígdala y el hipocampo no solo se activan en la habituación, sino que su incremento en la actividad metabólica se mantiene hasta el cuarto día de aprendizaje. En concordancia con nuestros resultados, muchos trabajos muestran que el estrés es un importante mediador de los procesos de aprendizaje. Por un lado, es de gran importancia el papel del complejo amigdalino en los procesos de memoria (Almaguer-Melian y Bergado-Rosado, 2002) y en la consolidación (Guzman-Ramos y Bermudez-Rattoni, 2011; Izquierdo y cols., 2006; McGaugh, 2000). Además, una exposición a una situación de estrés agudo previa a un aprendizaje espacial, mejora la ejecución de ratas de ambos sexos en una tarea espacial que se lleva a cabo en el laberinto acuático de Morris, e incrementa la densidad neuronal en el hipocampo (Uysal y cols., 2012). Por ello, estas estructuras no solo median la primera respuesta del animal a un entorno novedoso, sino que participan en la adquisición del aprendizaje, y sus niveles de actividad descienden a niveles basales a partir del cuarto día, cuando el animal presenta en el análisis conductual un incremento significativo de su rendimiento.

Por otro lado, y en relación con el proceso de adquisición del aprendizaje de guía, el estriado dorsal presenta un incremento de su actividad metabólica durante los

primeros días del aprendizaje, indicando que tanto éste como el hipocampo dorsal parecen ser necesarios para resolver tareas que requieren el uso de estrategias de guía. Estos datos apoyan, una vez más, la participación cooperativa de estas dos estructuras en la discriminación visual lo que permitiría una navegación óptima (Mizumori y cols., 2009).

Sin embargo, el papel de la corteza prefrontal en el aprendizaje de guía es más controvertido, ya que mientras la mayoría de los estudios de lesión en esta región no detectan impedimentos en el uso de este tipo de estrategias (DeCoteau y cols., 2009; Floresco y cols., 2008), Stefani y Moghadam (2005) defienden que el entrenamiento en tareas de discriminación visual requiere de la participación de esta estructura. En los resultados que hemos obtenido en este experimento, la corteza prefrontal se activa durante la adquisición del aprendizaje. Además, el análisis de las correlaciones que nos permite analizar las interacciones existentes entre las distintas estructuras cerebrales evaluadas, muestra una presencia continuada de interacciones entre las distintas áreas de la corteza prefrontal a lo largo de todo el proceso de aprendizaje. Según el concepto de "los sistemas de consolidación de la memoria" la corteza prefrontal sería la región donde se almacenaría la memoria (Squire y Alvarez, 1995). Sin embargo, la activación de la corteza prefrontal en los primeros estadíos del aprendizaje está apoyada por otros estudios en los que se ha observado su implicación en las primeras fases de la formación de la memoria (Squire y Alvarez, 1995). Por ello, en nuestra opinión, es posible que la consolidación esté ocurriendo de manera repetitiva al final de cada día de entrenamiento lo que explica su participación de forma continuada.

Durante la habituación, además de observar interacciones interregionales entre la corteza prefrontal y la entorrinal, se observaron entre la corteza parietal, la cápsula del núcleo accumbens y la amígdala lateral. Estas conexiones entre la corteza prefrontal y la entorrinal ya habían sido descritas previamente (Jones y Witter, 2007), al igual que la existente entre en núcleo accumbens y la amígdala (Newman y Winans, 1980), habiéndose visto implicadas en procesos relacionados con la exposición de un animal ante una situación novedosa (Campioni y cols., 2009) y estresante, respectivamente. La corteza parietal, por otro lado, es una corteza de asociación de información

visuoespacial y motora, lo que explica que sea clave no solo durante la habituación, sino a lo largo de todo el proceso de aprendizaje.

Durante el primer día de aprendizaje se observaron correlaciones entre la corteza parietal y el hipocampo dorsal (CA3). Aunque estas dos regiones no presentan conexiones anatómicas directas entre ellas, si están funcionalmente relacionadas (Save y Poucet, 2009). Además, las distintas subregiones estriatales presentan conexiones entre sí. Sorprendentemente, encontramos una vez más la presencia del hipocampo y el estriado incluso desde las primeras fases del aprendizaje de guía.

En conjunto, estos resultados sugieren una activación progresiva de redes cerebrales que incluyen regiones tanto corticales como subcorticales para la adquisición de un aprendizaje de guía. En los primeros estadíos del aprendizaje, se observa la participación de regiones asociadas con la toma de contacto con ambientes novedosos, conductas emocionales y la integración de información tanto motora como visuoespacial. Por otro lado, la corteza prefrontal participa a lo largo de todo el proceso. Cabe destacar la presencia, tanto del hipocampo como del estriado dorsal en este aprendizaje, lo que demuestra que, al contrario de lo que se defendía anteriormente, ambas estructuras parecen ser necesarias en el uso de este tipo de estrategias.

Participación de distintas estructuras cerebrales en el aprendizaje de respuesta

Cuando analizamos la actividad cerebral de los animales entrenados en un paradigma de orientación egocéntrico, observamos un incremento de actividad CO en la corteza prefrontal (motora, cingulada, prelímbica, infralímbica y orbitofrontal), el estriado dorsal y el área tegmental ventral tras 6 días de aprendizaje en oscuridad. En concordancia con nuestros resultados, el estriado dorsal recibe gran cantidad de proyecciones corticales, formando con la corteza prefrontal el sistema frontoestriatal, de gran implicación en aprendizajes en los que se espera una recompensa (Kehagia y cols., 2010; Mizumori y cols., 2009) (como sería en nuestro caso encontrar la plataforma sumergida). Además, en primates se ha observado un incremento en la actividad de las neuronas de este sistema cuando los animales realizan correctamente aprendizajes que requieren asociaciones simples (Histed y cols., 2009). Por otro lado, la corteza prefrontal a su vez presenta conexiones con la amígdala (McDonald, 1991). Todas estas

conexiones nos aportan el sustrato neuroanatómico necesario para apoyar los resultados obtenidos al hacer el análisis interregional. En este sentido, hemos observado que la corteza prefrontal por un lado y el complejo amigdalino por el otro presentan estrechas conexiones, proponiendo la existencia de un circuito que incluye la corteza prefrontal y la amígdala, necesario para el uso de estrategias egocéntricas.

También observamos que el aprendizaje por nosotros examinado, en el que los animales tienen que adaptarse a un entorno cambiante y revertir una asociación estímulo-respuesta que se había establecido previamente (reversal), se correlaciona con un descenso a niveles basales de la actividad cerebral en la mayor parte de las estructuras analizadas, excepto en la corteza orbitofrontal y el área tegmental ventral, que mantenían niveles de actividad similares a los de los animales entrenados en el aprendizaje egocéntrico. Estos datos, parecen indicar una necesidad de economizar gasto energético por parte del cerebro, de tal manera que para aprender una nueva tarea es necesario el descenso a niveles basales de la actividad energética de las estructuras cerebrales que ya no son necesarias. Por otro lado, la activación de la corteza orbitofrontal y el área tegmental ventral simultáneamente, se ha observado en aprendizajes en los que se somete a los animales a situaciones inesperadas (Takahashi y cols., 2009) como por ejemplo en la extinción de una conducta que previamente había sido reforzada (Nair y Gonzalez-Lima, 1999; Nair y cols., 2001). Asimismo, hoy en día sabemos que el área tegmental ventral es una estructura liberadora de dopamina, la cual se ha relacionado con conductas asociadas a meta (Fields y cols., 2007; Grace y cols., 2007; Kehagia y cols., 2010) lo que explica su activación en ambos aprendizajes. Este experimento en el que un grupo de animales realizó un aprendizaje reversal, permitió además reforzar la idea de que la técnica CO responde rápidamente a cambios conductuales (Conejo y cols., 2010; Mendez-Lopez y cols., 2009). De esta manera, la actividad metabólica de las estructuras cerebrales implicadas en el aprendizaje de respuesta disminuyó a niveles basales cuando la plataforma sumergida se cambió al brazo contralateral el último día de aprendizaje.

Por lo tanto, nuestros resultados muestran la existencia de una red cortico-límbico-estriatal implicada en el aprendizaje egocéntrico tras 6 días de entrenamiento en oscuridad, lo que nos permite proponer que el aprendizaje reversal se puede asociar a la

activación de la corteza orbitofrontal y el área tegmental ventral así como al retorno a niveles basales de actividad metabólica del resto de las regiones del circuito que son necesarias en este aprendizaje egocéntrico.

Tras observar la existencia de un circuito cortico-límbico-estriatal implicado en el aprendizaje egocéntrico, nos preguntamos si al igual que ocurre en las estrategias de guía, el hipocampo juega algún papel en el uso de estrategias egocéntricas, y por ello nos propusimos analizar cuál era el papel del hipocampo a lo largo del eje septo-temporal en el aprendizaje de respuesta. Observando que tanto el hipocampo dorsal (CA1 y CA3) como el hipocampo ventral (CA1, CA3 y GD) presentaron un incremento de su actividad CO respecto al control nado, estos datos continúan apoyando la idea de que en el aprendizaje de hábitos participan tanto el cuerpo estriado como el hipocampo. Además, datos electrofisiológicos muestran la participación del hipocampo dorsal en *la integración de ruta,* una estrategia en la que se requiere el uso de información propioceptiva y vestibular para orientarse en un espacio en ausencia de pistas externas o alocéntricas (Jeffery y O'Keefe, 1999; McNaughton y cols., 2006; Taube, 1998). Asimismo, el hipocampo presenta las denominadas "células de lugar" que descargan selectivamente en determinadas localizaciones espaciales dependiendo de la información vestibular (Stackman y cols., 2002). Por otra parte, se observaron incrementos en la actividad metabólica en el hipocampo ventral, una estructura asociada tradicionalmente con situaciones de estrés y ansiedad (Bannerman y cols., 2004; Engin y Treit, 2007). Sin embargo, nosotros atribuimos este incremento de actividad al aprendizaje de la tarea y no al estrés, dado que los animales controles se encontraron en una situación similar en el mismo ambiente y en contacto con el agua. De acuerdo con esta hipótesis, otros estudios en roedores muestran la participación del hipocampo ventral en la adquisición o el recuerdo (Bontempi y cols., 1999; Gusev y cols., 2005; Maviel y cols., 2004). Concretamente, un artículo recientemente publicado muestra como la inactivación temporal del hipocampo ventral afecta a la recuperación de un aprendizaje de referencia espacial en el laberinto acuático de Morris (Loureiro y cols., 2012).

Una vez que observamos la participación tanto del hipocampo dorsal como del hipocampo ventral en el aprendizaje egocéntrico, no propusimos determinar los niveles

relativos de la subunidad I de la proteína CO mediante western blot, para esclarecer la contribución de esta subunidad a la actividad de la enzima y al aprendizaje. En el ensamblaje de este complejo protéico, la subunidad I juega un papel fundamental en la activación de la proteína (Mick y cols., 2011), por ell fue la seleccionada para nuestro estudio. La técnica western blot ha sido utilizada previamente en otros estudios como la regulación de la CO tras un tratamiento de KCl y tetrodotoxina (Liang y cols., 2006), administración de cafeína (Jones y cols., 2008), isquemia (Racay y cols., 2009). Nosotros no encontramos resultados significativos en cuanto a la cantidad relativa de subunidad I, entre el grupo experimental y el control. En nuestra opinión, puede ser debido a que el proceso de aprendizaje no requiere incrementos de la concentración de proteína. La CO presenta un sistema de regulación muy complejo que no siempre está asociado con incremento de la concentración de proteína, como por ejemplo la *regulación alostérica* o la *modificación postransduccional.* En la regulación alostérica, la fijación de una molécula a una proteína modifica la actividad de ésta mientras que en la *modificación postransduccional* se producen fosforilaciones, metilaciones o glicosilaciones en la proteína que regulan su actividad (Arnold, 2012). Dado que con esta técnica no podemos determinar qué cantidad de las subunidades detectadas eran parte de la enzima activa, es posible que estemos también detectando la subunidad en estado inactiva (es decir sin formar el complejo proteico activo) y por lo tanto esa sea la razón de que observemos diferencias significativas en la actividad de la enzima pero no en la concentración relativa.

Efectos de la luz en las redes cerebrales implicadas en el aprendizaje de respuesta

Los experimentos anteriores nos muestran diferencias en las redes cerebrales implicadas en un aprendizaje de guía y uno de respuesta, por lo que la siguiente cuestión que nos planteamos fue, si las redes cerebrales implicadas en el aprendizaje de una misma tarea serían las mismas independientemente de las condiciones lumínicas en las que ésta se realizó. Para ello, se utilizaron dos grupos de animales experimentales que realizaron un aprendizaje de respuesta, con la diferencia de que uno de ellos fue entrenado en completa oscuridad mientras que el otro lo hizo en las condiciones lumínicas habituales en el laboratorio.

Los resultados obtenidos mostraron diferencias en consumo energético a nivel cerebral dependiendo de si la tarea se realiza en luz o en oscuridad. Es más, se observa que en ambas condiciones lumínicas es necesaria la participación de la corteza parietal y del hipocampo ventral. Sin embargo, cuando el experimento se realiza en oscuridad se requiere la activación de estructuras adicionales como la corteza prefrontal (cingulada, prelímbica, infralímbica), la parte central del núcleo accumbens, el hipocampo dorsal (CA1, CA3 y giro dentado), la amígdala central, y el cuerpo estriado.

Una posible interpretación, es que aunque la estrategia requerida para solucionar la tarea fuera aprender una secuencia de movimientos, los estímulos presentes en luz y oscuridad no son los mismos, por lo que era de esperar que las estructuras cerebrales implicadas tampoco lo fueran. En este sentido, aunque la presencia de luz no facilite la tarea per sé, las pistas visuales presentes en el ambiente podrían facilitar de algún modo la realización de la tarea y permitir un ahorro energético cerebral. Sin embargo, en oscuridad parece que el animal necesita gran cantidad de información adicional para suplir la información visual de la que carece, y como consecuencia se produce por ello el reclutamiento de numerosas estructuras cerebrales.

Por lo tanto, la corteza parietal y el hipocampo ventral son necesarios para el uso de estrategias egocéntricas independientemente de las condiciones lumínicas en las que se realicen. En este sentido, la participación de la corteza parietal en el uso de estrategias egocéntricas se ha observado previamente en estudios de lesión en los que ratas presentan dificultades en la adquisición de tareas egocéntricas tanto en luz (Save y Poucet, 2009) como en oscuridad (Save y Moghaddam, 1996), además de su implicación en la planificación de una ruta para alcanzar la meta durante la navegación espacial. Por otro lado, esta región está funcionalmente relacionada con el hipocampo (Save y Poucet 2009). La participación el hipocampo ventral se ha observado en estudios en roedores utilizando la técnica de la 2-deoxyglucosa o genes de expresión temprana en los que se observa la importancia de esta estructura en la recuperación o la adquisición de un aprendizaje espacial (Bontempi y cols., 1999; Gusev y cols., 2005; Maviel y cols., 2004). Cabe destacar, que previamente ya habíamos observado la participación del hipocampo ventral en la adquisición de una tarea egocéntrica en oscuridad tras un aprendizaje de seis días (artículo IV). Consecuentemente, el análisis

de las correlaciones muestra la existencia de una estrecha interacción entre CA1 y CA3 ventral en los animales que realizaron la tarea con luz, apoyando la participación del hipocampo ventral en la adquisición del aprendizaje egocéntrico.

Por otro lado, en condiciones de oscuridad se requirió el reclutamiento adicional de otras estructuras cerebrales como la corteza prefrontal (cingulado, prelímbico, infralímbico), la amígdala central, y el cuerpo estriado. En este sentido previamente habíamos observado la participación de la corteza prefrontal, el estriado dorsal y la amígdala en un aprendizaje egocéntrico similar que también transcurre en oscuridad (artículo III). Asimismo, en estas condiciones se observa un incremento de actividad en el hipocampo dorsal (CA1, CA3 y giro dentado). Previamente, ya se había observado que lesiones de todo el hipocampo impiden que los animales recuerden los brazos previamente visitados en un laberinto radial de 8 brazos en oscuridad (Allen y cols., 2007), proponiéndose la participación del hipocampo en el uso de estrategias alocéntricas y egocéntricas tanto en roedores (Rondi-Reig y cols., 2006) como en humanos (Igloi y cols., 2010).

Concluyendo, el hipocampo junto con otras estructuras reciben información de forma indirecta desde la corteza prefrontal (Karpova y cols., 2010) y nuestros resultados muestran diferencias significativas que se deben únicamente a las condiciones lumínicas y que son independientes del proceso de aprendizaje en el núcleo geniculado dorsal lateral y la corteza visual. Por otro lado, se ha observado que las neuronas del hipocampo aumentan su actividad cuando se realiza una navegación espacial en condiciones de oscuridad (Gothard y cols., 2001). Por ello creemos que el incremento de la actividad metabólica en el hipocampo se debe no solo al proceso de aprendizaje *per sé*, sino también a la condición lumínica en la que se realizó la tarea.

En concordancia con los resultados antes descritos, el análisis de las correlaciones muestra que en condiciones de oscuridad tanto el hipocampo dorsal como el ventral son necesarios para adquirir un aprendizaje egocéntrico al tiempo que observamos la participación del estriado, lo que una vez más nos lleva a considerar una participación conjunta del hipocampo y estriado.

Adicionalmente, encontramos que el núcleo accumbens también incrementa su actividad cerebral en condiciones de oscuridad. Esta estructura constituye un sistema que integra la entrada de información que proviene de la amígdala, la corteza prefrontal, el hipocampo y el estriado dorsal, facilitando conductas dirigidas a meta (Pennartz y cols., 2011). Por lo tanto, el aprendizaje de respuesta parece requerir un sistema neural que comprende la corteza parietal y el hipocampo, mientras que cuando el experimento se lleva a cabo en oscuridad, es necesaria la participación de una red más compleja en la que se encuentran reclutadas regiones corticales y subcorticales.

En resumen, en nuestro trabajo hemos podido demostrar que al contrario de lo que se defendía tradicionalmente y en concordancia con estudios recientes, el aprendizaje de hábitos no es exclusivamente estriatal, sino que tanto en los aprendizajes de respuesta como en los de guía, el hipocampo y el estriado parecen ser necesarios y actuar conjuntamente. Por otro lado, las interacciones entre hipocampo, cuerpo estriado y otras regiones cerebrales varían a lo largo del proceso de aprendizaje, y son sensibles a cambios lumínicos, dado que estos afectan a los estímulos que percibe el animal para orientarse en el espacio.

CONCLUSIONES

1. Las ratas evaluadas en el paradigma de aprendizaje de respuesta, requieren un menor número de ensayos para ejecutar correctamente la tarea que los animales evaluados en una estrategia de discriminación visual.

2. El hipocampo dorsal es necesario en el uso de estrategias de discriminación visual. Además, la existencia de una correlación entre hipocampo dorsal y el estriado el último día de entrenamiento, refleja la participación de ambas estructuras en el aprendizaje.

3. El uso de estrategias de discriminación visual requiere una activación progresiva de redes cerebrales que incluyen regiones tanto corticales como subcorticales, para la adquisición de un aprendizaje de discriminación visual. El núcleo accumbens, la amígdala, la corteza parietal y el hipocampo son más relevantes en los primeros estadíos del aprendizaje, mientras que la corteza prefrontal es necesaria a lo largo de todo el proceso.

4. En el aprendizaje de respuesta en oscuridad, tras 6 días de aprendizaje, se observa la activación metabólica de la corteza prefrontal (motora, cingulada, prelímbica, infralímbica y orbitofrontal), el estriado dorsal y el área tegmental ventral. Sin embargo, un aprendizaje reversal produce un descenso a nivel basal de la actividad metabólica en la corteza motora, cingulada, prelímbica, infralímbica, el estriado dorsal y amígdala, manteniéndose activas únicamente la corteza orbitofrontal y el área tegmental ventral.

5. Tanto la región dorsal del hipocampo como la ventral incrementan su actividad metabólica en un aprendizaje de respuesta. Sin embargo, no se encontraron diferencias en la concentración relativa de la subunidad I del complejo proteico de la citocromo c oxidasa.

6. Durante la adquisición de un aprendizaje egocéntrico tras un día de entrenamiento, cabe destacar la participación del hipocampo ventral y la corteza parietal en la adquisición de la tarea, independientemente de si ésta se lleva a cabo en condiciones de luz o de oscuridad. Asimismo, la participación del estriado, la amígdala y la corteza prefrontal son necesarias en condiciones de oscuridad.

CONCLUSIONS

1. Rats assessed in a response learning paradigm, require fewer trials to master the task than the animals evaluated on a visual discrimination strategy.

2. The dorsal hippocampus is necessary in visual discrimination strategies. Moreover, the existence of a correlation between dorsal hippocampus and striatum, on the last day, reflects the participation of both structures in the learning process.

3. Using visual discrimination strategies require a progressive activation of brain networks which include both cortical and subcortical regions. The nucleus accumbens, amygdala, parietal cortex and hippocampus are more relevant in the early stages of learning, while the prefrontal cortex is necessary throughout the hole process.

4. There is a metabolic activation of the prefrontal cortex (motor, cingulate, prelimbica, infralimbic and orbitofrontal), the dorsal striatum and ventral tegmental area in response learning after 6 days of learning under dark conditions. However, reversal learning causes a decrease to baseline levels of metabolic activity to basal level in the motor cortex, cingulate, prelímbica, infralimbic, dorsal striatum and amygdala, remaining active only in the orbitofrontal cortex and ventral tegmental area.

5. Both the dorsal and ventral hippocampus increase their metabolic activity in response learning. However, no differences were found in subunit I levels of cytochrome c oxidase.

6. During acquisition of an egocentric learning task after one day of training, the participation of the ventral hippocampus and parietal cortex in the acquisition of the task could be highlighted, whether it is carried out under conditions of light or darkness. Moreover, the involvement of the striatum, amygdala and prefrontal cortex are necessary in dark conditions.

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8. TRABAJOS ORIGINALES

I

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Functional Interaction Between the Dorsal Hippocampus and the Striatum in Visual Discrimination Learning

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The hippocampus and the striatum have traditionally been considered as part of different and independent memory systems. However, there is evidence that supports a functional interaction between the hippocampus and the dorsal striatum at least in particular learning tasks. Here, we evaluated the functional contribution of both brain regions in a visual discrimination learning task using cytochrome c oxidase (CO) quantitative histochemistry. Compared with other brain metabolic mapping techniques, CO activity reflects steady-state neuronal energy demand. Rats were trained for 6 days in a water T-maze to find a hidden escape platform associated with an intramaze visual cue. A control group of animals swam for an equivalent amount of time compared as the trained group but without any escape platform available. After finishing the behavioral task, CO activity was measured in subdivisions of the dorsal hippocampus and the dorsal striatum in both groups. Results show significantly higher CO activity in the CA1 area and the dentate gyrus of the dorsal hippocampus in the trained rats compared with the control group. In addition, a significant negative functional cross-correlation between area CA1 of the dorsal hippocampus and the anterodorsal striatum was found. Our results support current theories on competitive interaction of different memory systems during visual discrimination learning.

Key words: memory systems; cytochrome c oxidase; water T-maze; rat

There is actually growing evidence that different types of memory are mediated by distinct brain regions, supporting the concept of ''multiple memory systems,'' but it is still a matter of debate which brain regions are relevant for each memory system. In particular, the hippocampus seems to be involved in both relational/configural learning (Sutherland and Rudy, 1989) and the declarative memory system (Squire and Zola-Morgan, 1991), whereas the striatum is considered a key brain region for procedural, implicit, or habit memory systems (Packard et al., 1989).

However, recent studies have reported interactions between brain regions involved in different memory systems during learning of particular memory tasks, both in animals and in humans (McDonald and White, 1994; Poldrack and Packard 2003). In addition, it has been found that these functional interactions between memory systems are required for normal behavior in humans and that abnormal interaction may underlie psychiatric disorders such as schizophrenia, depression, or drug addiction (McDonald et al., 2004).

Although it has been established that mnemonic processes in mammals are probably organized in multiple memory systems, there is actually little knowledge about their interactions even in simple memory tasks involving visual discrimination. It has traditionally been considered that the striatum is required for the acquisition of habit learning. However, hippocampal lesion studies showed the development of retrograde amnesia in these tasks (Sutherland et al., 2001; Driscoll et al., 2005; Epp et al., 2008), suggesting that the hippocampus would be involved in the acquisition of habit learning. On the other hand, several authors have proposed that the participation of the hippocampus depends on the complexity of the task, especially when the salience of the visual cues is not high (Murray and Ridley, 1999). In addition, coordinated electrophysiological activity between the dorsal hippocampus and the dorsal striatum has been reported during a procedural learning task (DeCoteau et al., 2007). However, the contribution of the hippocampus and the striatum to visual discrimination learning is a matter of debate.

Most studies performed to characterize the brain substrate of visual discrimination learning are based on lesion methods, which have several known limitations caused by nonspecific brain tissue damage and the interpretation of disconnection effects. Although certainly

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lesion methods have contributed extensively to our knowledge of brain function, we think that complementary methods developed to analyze neuronal activation specifically would enhance our knowledge of brain function. Quantitative cytochrome oxidase (CO) histochemistry was used as a reliable marker of neuronal metabolic capacity, because CO activity represents an index of the energy demands of neurons after prolonged stimulation or training in behavioral tasks (Wong-Riley, 1989; Gonzalez-Lima and Cada, 1994). CO histochemistry has a high anatomical resolution, and it provides a measure of steady or sustained changes in oxidative metabolism associated with brain function (Wong-Riley, 1989). CO histochemistry has been used extensively in previous studies of learning and memory (González-Pardo et al., 2008; Conejo et al., 2010). One of the important advantages of CO histochemistry is that it allows not only determination of the changes in neuronal metabolic demands associated with a particular behavioral task but also evaluation of the functional relationship between brain regions using correlation analysis of CO activity between different brain regions (Puga et al., 2007; Fidalgo et al., 2011).

Although the precise molecular mechanisms relating CO activity and memory are not fully understood, it has been demonstrated that increasing CO activity using pharmacological treatments directly improves memory retention (Gonzalez-Lima and Bruchey, 2004; Riha et al., 2005). In particular, methylene blue increases cellular respiration and therefore CO activity by inducing CO enzyme levels and oxygen consumption (Riha et al., 2005). This dye also improves memory retention in rats, acting as a "metabolic enhancer," insofar as it increases specifically CO activity in brain regions associated with memory tasks in rats (Gonzalez-Lima and Bruchey, 2004; Riha et al., 2005, 2011; Wrubel et al., 2007). This study sought to evaluate the functional involvement of the hippocampus and the striatum in visual discrimination learning using quantitative CO histochemistry as a reliable method to evaluate neuronal energy metabolism.

MATERIALS AND METHODS

Subjects

In total, 20 male Wistar rats weighing between 150–250 g were used in this experiment. All the animals had ad libitum access to food and tap water and were maintained at constant room temperature (20–22°C), with a relative humidity of 65–70% and an artificial light-dark cycle of 12 hr (08:00–20:00/20:00–08:00 hr). The animals were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain) and were randomly divided into two groups, a swim control group (n = 10) and an experimental group (n = 10). All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and Spanish legislation (R.D. 1201/2005). The study was approved by the local committee for animal studies (University of Oviedo). All efforts were made to minimize the number of animals used and their suffering.

Apparatus

Rats were tested in a water T-maze made of black fiberglass filled with tap water (23°C ± 1°C). The main alley (100 cm × 20 cm × 40 cm) was connected to two side arms (right and left; 45 cm × 20 cm × 40 cm). A submerged platform (Plexiglas, 15 cm × 18 cm) was placed in one of the two arms always near a visual intramaze cue. Animals were trained daily (during 6 days) using a single 12-trial session. Each trial was recorded and the paths of the animals were analyzed later using a computerized video-tracking system (Ethovision Pro; Noldus Information Technologies, Wageningen, The Netherlands).

Behavioral Training

To discard possible motor and sensory deficits, animals were tested in a neurological assessment battery. Rats were handled daily for 5 days. The spatial memory task was daily executed between 09:30 and 13:00 hr. During the habituation day, rats of the visual discrimination group (n = 10) were gently immersed in the water escape T-maze for 1 min, without platforms. The training phase started on the next day. The position of the platform and the associated visual cue changed from side to side (left or right arm) following a pseudorandom sequence. A visual cue (a rectangular yellow card with a printed horizontal thick black line in the middle) was attached to the end wall of the goal arm. Each animal received a single 12-trial session per day for 6 days. In each trial, the rats were allowed to swim to locate the platform or were placed on it after 60 sec, where they remained for 15 sec before being placed in a cage for 30 sec. All subjects in the experimental group reached the learning criterion of 80% correct arm choice.

A free-swimming group (swim control) was also used. The rats swam in the maze for the same number of daily trials and training days as those from the experimental group. This swim control group swam during a time equal to the mean of the escape latencies recorded for the experimental group but without an escape platform available.

CO Histochemistry

Ninety minutes after the end of the last trial, the animals were decapitated. Brains were removed, frozen rapidly in isopentane (2-methylbutane) at −70°C (Sigma-Aldrich, Madrid, Spain), and stored at −40°C. Coronal sections (30 μm) of the brain were cut with a cryostat microtome (HM 505 E; Microm International GmbH, Heidelberg, Germany), mounted on slides, and conserved at −40°C until processing with quantitative CO histochemistry, as described by Gonzalez-Lima and Cada, 1994. Some sections from a few subjects could not be used as a result of tissue processing, although the final number of sections available for histochemistry was enough in all cases. To quantify enzymatic activity and to control staining variability across different staining batches, sets of tissue homogenate standards obtained from Wistar rat brain were included with each batch of slides. These standards were

cut at different thicknesses (10, 30, 40, and 60 μm) and included with each batch of slides.

In brief, sections and standards were incubated for 5 min in 0.1 M phosphate buffer with 10% w/v sucrose and 0.5% v/v glutaraldehyde, pH 7.6. After this, slides were 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/liter cobalt chloride, 10% (w/v) sucrose, and 5 ml dimethyl sulfoxide. Once the sections had been rinsed in phosphate buffer (pH 7.6; 0.1 M), they were incubated at 37°C for 1 hr in the dark and with continuous stirring in a solution containing 50 mg 3,3′-diaminobenzidine, 15 mg cytochrome c (Sigma, St. Louis, MO), 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 for 30 min at room temperature with 10% (w/v) sucrose and 4% (v/v) formalin. The slides were dehydrated, cleared with xylene for 10 min, and coverslipped with Entellan (Merck, Darmstadt, Germany).

CO activity is an index of neuronal oxidative metabolic capacity related to neuronal activity. Quantification of CO histochemical staining intensity was done by densitometric analysis using a computer-assisted image analysis workstation (MCID; InterFocus Imaging Ltd., Linton, United Kingdom) composed of a high-precision illuminator, a digital camera, and a computer with specific image analysis software.

Twelve measurements were taken per brain region. These measurements were averaged to obtain one mean per region for each animal and initially measured as relative optical density (ROD) units. To establish comparisons and consider possible staining variations between brain sections from different staining batches, additional measurements were taken from CO-stained brain homogenate standards. Regression curves between section thickness and known CO activity measured in each set of standards were calculated for each incubation bath. Finally, average ROD measured in each brain region was converted into CO activity units (μmol cytochrome c oxidized/min/g tissue wet weight) using the calculated regression curve in each homogenate standard. We measured in all animals the neuronal metabolic activity in the dorsal hippocampus (dentate gyrus, CA1 and CA3 areas) and the anterodorsal, anteromedial, and anterolateral subdivisions of the striatum. The selected brains regions anatomically were defined according to the atlas by Paxinos and Watson (1997).

Statistical Analysis

Behavioral data. The numbers of correct arm choices measured daily in the trained group were analyzed using a nonparametric Friedman one-way repeated measures ANOVA on ranks, using Newman-Keuls post hoc tests to evaluate differences between training days where appropriate.

Cytochrome oxidase activity. Group differences in CO activity of each brain region were assessed by Student's *t*-tests. Data were analyzed in SigmaStat 3.2 (Systat, Chicago, IL) software and were expressed as mean ± SEM. The results are considered statistically significant at $P < 0.05$.

Because training experience in visual discrimination learning could be manifested as neural changes in functional connectivity, the functional relationships among the regional brain activity data were analyzed in terms of pairwise correlations within each experimental group. For the interregional correlation analysis, Pearson's product-moment correlations between pairs of brain regions in each experimental group were computed. 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. This was done to reduce variation in the intensity of the CO staining not resulting from the experimental manipulation. In addition, to avoid errors resulting from an excessive number of significant correlations using small sample sizes, a "jackknife" procedure was used (Shao and Tu, 1995). This procedure is based on the calculation of all possible pairwise 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.

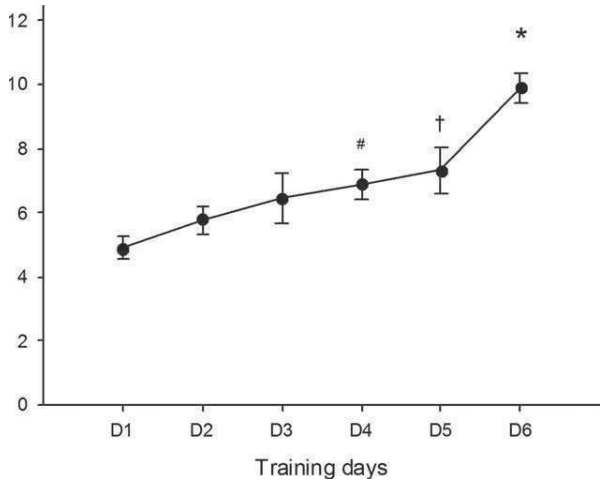

Fig. 1. Number of correct choices across training days in the discrimination group. $^{\star}P < 0.05$ compared with all the training days. $^{\dagger}P < 0.05$ compared with the second training day. $^{\#}P < 0.05$ compared with the first training day.

RESULTS

Visual Discrimination Task

With regard to the number of correct choices made in the training phase, significant increases were observed across training days ($\chi^2[5] = 29.16$, $P < 0.001$). In particular, significant differences in the number of correct choices were found between the sixth training day compared with the rest of the training days (Newman-Keuls test, $P < 0.05$). In addition, significant differences were found between training day 5 and day 2 ($P < 0.05$), between day 4 and day 1 ($P < 0.05$), and between day 2 and day 1 ($P < 0.05$; Fig. 1).

CO Activity

On the other hand, results of CO histochemistry quantification showed that CO activity was higher in the CA1 area ($t[16] = 2.603$; $P = 0.019$) and the dentate gyrus ($t[16] = 2.410$; $P = 0.028$) of the dorsal hip-

pocampus in trained animals compared with the swim control group (Table I). No significant differences were found between the visual discrimination group and the swim control in the rest of brain regions studied.

In addition, significant interregional correlations of CO activity were found in the visual discrimination group. A high cross-correlation between the CA1 hippocampal area and the anterodorsal striatum was found in this group ($r = -0.8$, $P = 0.009$). See Table II for a detailed correlation matrix of the CO activity data.

DISCUSSION

Our results show that both the dorsal striatum and the dorsal hippocampus had significant CO changes after visual discrimination learning. In particular, the CA1 area and the dentate gyrus showed increased CO activity in the visual discrimination group compared with the control group. These hippocampal regions seem to be relevant for difficult visual discrimination learning (Hu et al., 2005). In this regard, it has been reported that the CA1 hippocampal area is required for visual discrimination learning in a water maze task in which extramaze visual cues were hidden using black curtains, similar to our experimental conditions (Carli et al., 1999). The contribution of the hippocampus to habit learning has been a matter of debate, because hippocampal lesions do not impair the acquisition of habit learning in simple visual discrimination tasks (Alvarado et al., 1995; Broadbent et al., 2007). Conversely, it has been demonstrated that lesions of the hippocampus can cause retrograde amnesia for simple visual discrimination tasks (Epp et al., 2008). In addition, visual discrimination learning in a Y-maze is correlated with CO activity in the CA subfields and the dentate gyrus of the hippocampus, especially in difficult visual discrimination tasks (Hu et al., 2005, 2006).

Interestingly, we did not find significant group differences in any regions of the dorsal striatum. This result was not expected, because the striatum is considered to be a key brain region for habit learning as shown by lesion studies. However, it must be considered that we used a different experimental approach to evaluate the neural correlates of learning in intact animals. A limitation of many studies is the assumption that a particular aspect of brain function is mediated by a single brain region, but there is extensive evidence of brain networks associated with particular brain functions. In fact, disconnection effects in corticostriatal circuits required for habit learning (Balleine and O'Doherty, 2010) would explain this apparent disagreement between lesion studies and our results. In agreement with our results, Teather et al. (2005) reported that habit learning of a visible cued task in the water maze was not associated with a significant increase in the expression of immediate-early genes in the dorsal hippocampus or even the lateral striatum. However, these authors described a nonsignificant increase in the expression of c-Jun cells in the dorsal striatum after the cued task. Probably, methodological differences between immediate-early gene expression and CO histochemistry could explain this apparent discrepancy. In particular, changes in CO activity reflect a more stabilized state of brain metabolism during longer periods of time compared with stimulus-evoked expression of immediate-early genes that takes place acutely and transiently after stimulation (Wong-Riley, 1989).

On the other hand, analysis of functional correlations of CO activity revealed a highly significant nega-

TABLE I. Cytochrome Oxidase Activity Units (mean ± SEM; μmol cytochrome c oxidized/min/g tissue) Measured in Selected Brain Regions

	n	Discrimination group	n	Swim control group
CA1 dorsal	9	21.7 ± 0.5★	9	19.7 ± 0.5
CA3 dorsal	9	19.5 ± 0.50	9	18.3 ± 0.6
GD dorsal	9	33.6 ± 1.3★	9	29.9 ± 0.7
Anterodorsal striatum	9	28.1 ± 1.40	9	27.7 ± 1.3
Anteromedial striatum	9	28.6 ± 1.10	9	28.2 ± 1.3
Anterolateral striatum	9	29.4 ± 1.30	9	30.0 ± 0.8

★$P < 0.05$ compared with the swim control group.

TABLE II. Matrix Correlation Showing the Significant Interregional Correlations of CO Activity Calculated in Experimental Group[†]

	CA3	GD	Anteromedial striatum	Anterolateral striatum	Anterodorsal striatum
CA1	0.59	0.74	−0.47	−0.11	−0.80
	0.097	0.02	0.20	0,78	0.00★
CA3		0.53	−0.35	0.25	−0.06
		0.14	0.35	0.51	0.86
GD			−0.25	−0.35	−0.54
			0.51	0.35	0.13
Anteromedial striatum				0.52	0.22
				0.15	0.56
Anterolateral striatum					0.19
					0.63

★$P < 0.05$ significant pairwise correlations after correction for multiple comparisons.
[†]Each cell shows the calculated Pearson's correlation r value followed by the P level for the calculated correlation coefficient.

tive cross-correlation between the anterodorsal striatum and the dorsal CA1 hippocampal area in the visual discrimination group. In this regard, a previous study reported the interaction between the dorsal hippocampus and the striatum during visual discrimination learning (McDonald et al., 2007), suggesting that both brain regions contribute to the acquisition of these kinds of tasks. Therefore, interactions between brain regions supporting different memory systems could be required for visual discrimination learning. Accordingly, some authors suggest that competitive or cooperative interactions between different brain regions involved in particular memory systems would be required depending on the type of learning task (Poldrack and Packard, 2003). However, it has been proposed that different memory systems represented by the hippocampus and the dorsal striatum would participate in parallel or independently in spatial learning tasks (Mizumori et al., 2009). Conversely, other authors suggest that the hippocampus and extrahippocampal regions show inhibitory interactions when the hippocampus is intact (Driscoll et al., 2005). Moreover, a neuroimaging fMRI study in human subjects also showed that a simple rule-learning task involving visual discrimination learning was associated with reciprocal inhibition between the dorsal hippocampus and the dorsal striatum (Seger and Cincotta, 2006). The negative functional correlation between the dorsal hippocampus and the dorsal striatum found would therefore support a competitive interaction between the two brain regions.

In summary, our results show that visual discrimination learning was associated with significant changes in CO activity of the dorsal hippocampus. In addition, a functional interaction between the anterodorsal striatum and the dorsal hippocampal CA1 area supports the concept of competitive interaction between different memory systems. Further research is required to determine the functional relevance of particular brain networks involved in apparently simple forms of associative learning such as visual discrimination learning.

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II

TITLE: Dynamic functional brain networks involved in simple visual discrimination learning

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ABSTRACT

There is increasing knowledge of brain networks involved in different types learning. However, the functional interactions between brain regions at different stages of learning remain unclear. We used cytochrome oxidase histochemistry to evaluate the contribution of different brain networks during visual discrimination learning in a water-T maze at different time points (habituation, 1, 4 or 6 days). As compared with a naive control group, the results of the present study reveals a progressive activation of functional brain networks involving cortical (prefrontal and temporal cortex) and subcortical brain regions (including striatum and hippocampus) associated to the mastery of a simple visual discrimination task. On the other hand, the brain regions involved and their functional interactions changed progressively over days of training. Regions associated with novelty and emotional, visuo-spatial orientation and motor aspects of the behavioural task seem to be relevant during the earlier phase of training, whereas a brain network comprising the prefrontal cortex and the parietal cortex was found at later stages. This study highlights the relevance of taking into consideration functional interactions between brain regions to investigate learning and memory processes.

HIGHLIGHTS: Cortical, striatal and hippocampal regions are linked to discrimination learning; The hippocampus showed a time-limited involvement during early discrimination learning; A brain network comprising prefrontal areas was related with discrimination learning;

KEYWORDS: visual discrimination learning, funcitonal brain networks, rat, cytochrome oxidase.

1. INTRODUCTION

There is increasing knowledge about the neural networks involved in several learning tasks, both in humans and in animals (Ma et al., 2010; Conejo et al., 2010; Puga et al., 2007; Lehéricy et al., 2005). In this regard, it has been shown that particular brain regions do not participate in learning and memory tasks as unitary structures. In fact, brain regions dynamically interact at different stages of the learning process.

The hippocampus and the striatum have been traditionally considered as key brain regions associated with different and independent memory systems. However, there is evidence that supports a functional interaction between the hippocampus and the dorsal striatum at least in particular learning tasks. This is in agreement with recent research that emphasizes the relevance of functional interactions between different brain regions (Vann and Albasser, 2011; Henson and Gagnepain, 2010).

Although there are numerous studies using water mazes to investigate the neural substrates of visual discrimination learning, the neural networks involved in this type of learning are not clearly understood. In addition, numerous studies in animals and humans reported the importance of several brain structures in this kind of memory. Visual discrimination learning shares many features of stimulus-response habit learning (McDonald and White, 1993). In addition, it has been traditionally considered that the striatum is specifically required for the acquisition of stimulus-response habit learning (Yin and Knowlton, 2006; Packard and Knowlton, 2002). However, hippocampal contribution to visual discrimination learning is still a matter of debate. It has been reported that hippocampal lesions do not impair the acquisition of habit learning in simple visual discrimination tasks (Alvarado and Rudy, 1995; Broadbent et al., 2007). However, hippocampal lesion studies showed the development of retrograde amnesia in these tasks

(Sutherland et al., 2001; Driscoll et al., 2005; Epp et al., 2008) suggesting that the hippocampus would be involved in the acquisition of visual discrimination learning.

On the other hand, memory has been described as an active and complex process composed of multiple temporal stages. In addition, it has been suggested that memories might be gradually reorganized over time (Ribot, 1982; Frankland and Bontempi, 2005). The functional connectivity in local and distal anatomical brain pathways has mostly emerged from neuroimaging studies in humans (Guye et al., 2008). However, dynamic interactions between brain regions during the learning process have also been found in rodents analysing correlations in neuronal oxidative metabolism (Conejo et al. 2010). In this context, studies in humans and rodents indicate that interactions among cortical and noncortical brain regions are necessary during memory formation (Zimmer, 2008). Moreover, there is an actual vigorous debate about the traditional concept of 'systems memory consolidation' stating that memory is initially hippocampus-dependent but it is later represented in a distributed cortical network independent of the hippocampus (Takashima et al., 2009; Winocur et al., 2010; Lesburguères et al., 2011).

In the present study, we evaluated the contribution of different brain networks during visual discrimination learning in a water-T maze. Previous studies reported the involvement of the hippocampus and the striatum during a visual discrimination task (Fidalgo et al., 2011b in press). However, the role of the hippocampus and the striatum together with anatomically related brain regions on discrimination learning is still unclear. For this purpose, cytochrome c oxidase (CO) activity was analyzed in the dorsal hippocampus, the dorsal striatum, prefrontal, parietal and temporal cortex as well as the nucleus accumbens and amygdala complex. We used quantitative CO histochemistry as a metabolic brain mapping technique because it has a high anatomical resolution and it provides a measure of steady or sustained

changes in oxidative metabolism associated with brain function (Wong-Riley, 1989). CO histochemistry has been successfully used in previous studies to map changes in brain oxidative metabolism involved in several learning tasks in rats (Bruchey and Gonzalez-Lima, 2008; González-Pardo et al., 2008; Mendez-Lopez et al., 2010). In addition, the CO method can be also used to investigate the functional interactions between brain regions. In this context, it has been previously demonstrated that brain regions that are functionally coupled show dynamical and coordinated changes in their metabolic capacity, expressed as changes in the strength of correlation in CO activity between regions (Sakata et al., 2000; Puga et al., 2007; Conejo et al., 2010; Fidalgo et al., 2011a).

2. EXPERIMENTAL PROCEDURES

2.1. Animals

A total of 49 male Wistar rats weighing between 150-250 g were used in this experiment. The animals were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain) and were randomly housed in groups of five under standard conditions (12-h light/dark cycle with lights on from 08:00–20:00 h) at constant room temperature of 21±2 °C with *ad libitum* access to food and water. Care and use of laboratory animals were done in accordance with the European Communities Council Directive (*2010/63/UE*) and the Spanish legislation (RD 1201/2005). All efforts were made to minimize the number of animals used and their suffering.

2.2. Apparatus

Rats were trained in a water T-maze made of black fibre-glass filled with tap water (23±1 ºC). The main alley (100 × 20 × 40 cm) was connected to two side arms (right and left)

measuring 45 × 20 × 40 cm. A submerged platform made of Plexiglas (15 × 18 cm) was placed in one of the two arms always near a visual intra-maze cue. The position of the platform and the associated visual cue changed from side to side (left or right arm) following a pseudorandom sequence. Each trial was recorded and swim paths of the animals were 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 deficits, animals were tested in a neurological assessment battery. The neurological tests used include the following tests: abduction response of hindlimbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking reflex, pupillary reflex, negative geotactic response, and righting reflex (according to Bures et al., 1976). No animals were discarded due to abnormal neurological responses.

The visual discrimination task was performed between 09:30 and 13:30 h. After daily handling during five days, rats were randomly assigned to five different experimental groups. Four groups were trained in a visual discrimination task using a water T-maze. During the habituation day, animals were gently immersed in the water T-maze for 1 min, without any escape platform available. A group of animals (*habituation group*; $n = 10$) was killed by decapitation 90 minutes following habituation to the water maze task In addition, one group of animals was only gently handled (*naïve group*; $n = 10$) and it was decapitated 90 min later.

Training proceeded during the following six days for the rest of groups trained during 1 day (1-*day group; n = 10*), 4 days (4-*day group; n = 10*), or 6 days (6-*day group; n = 9*). Each rat received a single daily 12-trial session Training consisted of a hidden escape platform underneath the water level associated with a visual cue (a card with a printed

vertical striped pattern) attached to the wall of the arm where the platform was located. In each trial, rats were allowed to swim to locate the platform or they were placed on it after 60 s, where they remained for 15 s before returning them to the cage for 30 s. The position of the escape platform and the adjacent visual cue was randomly changed between trials. The learning criterion used for the *6-day group* was eight or more correct choices out of 12 trials.

2.4. Tissue preparation

90 min after finishing the behavioral procedure the animals were decapitated. Their brains were quickly removed, frozen in isopentane at -70 ºC (Sigma–Aldrich, Madrid, Spain) and stored at -40 ºC to preserve the brain tissue and enzyme activity. Next, 30 µm-thick coronal sections were obtained from the brain tissue using a cryostat microtome (Microm HM 505 E, Heidelberg, Germany). These sections were mounted on slides and stored at -40 ºC until processing with quantitative CO histochemistry. Some sections from a few subjects could not be used as a result of tissue processing, although the final number of sections available for histochemistry was enough in all cases.

2.5. CO histochemistry

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 (1994). To control staining variability across different baths, sets of brain tissue homogenate standards of known CO activity from Wistar rat brain were cut at different thicknesses (10, 30, 50 and 70 µm) and included with each bath of slides. In brief, slides were fixed for 5 min with a 05% glutaraldehyde solution, rinsed three times in phosphate buffer and preincubated 5 min in a solution containing 005 M Tris buffer, pH 76, with 275 mg/l cobalt chloride, 10% (w/v) sucrose, and 5 ml dimethyl-sulfoxide. Once the sections had been rinsed in phosphate buffer (pH 76; 01 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), 4 g sucrose per 100 ml phosphate buffer (pH 74; 01 M). The reaction was stopped by fixing the tissue in buffered formalin for 30 min at room temperature with 10% (w/v) sucrose and 4% (v/v) formalin. The slides were dehydrated, cleared with xylene 10 min 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) composed of a high precision illuminator, a digital camera and a computer with specific image analysis software. A total of twelve measurements were taken per region. These measures were averaged to obtain one mean per region for each animal and were expressed as arbitrary units of optical density (OD) in the prefrontal cortex (anterior cingulate, prelimbic and infralimbic areas), parietal cortex, entorhinal and perirhinal cortex, striatum (anterodorsal, anteromedial and anterolateral regions), amygdala complex (lateral, basolateral, medial and central nucleus) and dorsal hippocampus (CA1, CA3 and dentate gyrus). The selected brains regions anatomically were defined according to the Paxinos and Watson´s (1997) atlas. Regression curves between section thickness and known CO activity measured in each set of standards were calculated for each incubation bath. Finally, average ROD measured in each brain region was converted into CO activity units (μmol of cytochrome c oxidized/min/g tissue wet weight) using the calculated regression curve in each homogenate standard.

2.6. Statistical analysis

CO activity measured as relative OD values of each region were evaluated by one-way ANOVA using experimental group (cage control, habituation, 1-, 4-, 6-day spatial learning experience) as the independent variable. Data were analyzed by SigmaStat 32 software (Systat Software, Chicago, USA) and were expressed as mean ± SEM. The results

were considered statistically significant when $p < 005$ Tukey's HSD test was used as a post hoc test to assess differences between means when the ANOVA revealed significant differences between groups. When the normality test failed, Kruskal-Wallis tests were used. In order to evaluate changes in functional connectivity among brain regions, regional CO activity data were analyzed in terms of pair-wise correlations within 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 all structures measured for each animal. This was done to reduce variation in the intensity of the CO staining not resulting from the experimental manipulation. In addition, in order to avoid errors due to an excessive number of significant correlations using small sample sizes we used a 'jackknife' procedure (Shao and Dongsheng 1995) based on the calculation of all possible pairwise correlations resulting from removing one subject each time, and taking into consideration only those correlations that remain significant (p<002) across all possible combinations.

3. RESULTS

3.1. Behavioral results

As regards to the number of correct choices made in the training phase, significant increases were observed across training days (H(5)=39.0 , p<0001). In particular, significant differences in the number of correct choices were found between the sixth training day as compared to the rest of training days (Newman-Keuls method, p<005). In addition, significant differences were found between training day 5 and day 1 (p<005), day 4 versus day 1 (p<005) and day 3 versus day 1 (p<005) (Fig 1).

3.2. CO activity

Significant differences in CO activity among groups were found in the anterior cingulate cortex ($F(4,43)=6.43$ $p<0.001$), prelimbic cortex ($F(4,43)=8.1$ $p<0.001$), infralimbic cortex ($F(4,43)=8.74$ $p<0.001$), parietal cortex ($F(4,46)=7.84$ $p=0.001$), entorhinal ($F(4,42)=7.85$ $p<0.001$) and perirhinal cortex ($F(4,43)=6.81$ $p<0.001$), accumbens shell ($F(4,45)=3.72$ $p=0.011$), in the anterodorsal ($F(4,47)=5.22$ $p=0.002$), medial ($F(4,47)=6.53$ $p<0.001$) and lateral striatum ($F(4,47)=7.01$ $p<0.001$). In addition, significant metabolic differences have been obtained in CA1 area ($F(4,48)=12.43$ $p<0.001$), CA3 area ($F(4,47)=19.49$ $p<0.001$) and the dentate gyrus ($F(4,48)=6.65$ $p<0.001$) of the dorsal hippocampus and in the following nuclei of the amygdala complex: basolateral ($F(4,46)=13.52$ $p<0.001$), lateral ($F(4,46)=13.64$ $p<0.001$), central ($F(4,46)=31.69$ $p<0.001$), and medial ($F(4,46)=9.77$ $p<0.001$) nuclei.

Table 1 shows the mean CO activity values measured in the 18 regions of interest of the different experimental groups, including a naïve control group as a baseline for comparison. Figure 2 shows microphotographs of the brain regions of interest stained with CO histochemistry.

CO activity measured in the habituation, 1-day and 4-day learning groups was greater than the 6-day and the naïve group in most of the measured structures suggesting increased and sustained metabolic neuronal demands throughout the training days and a return to baseline levels when the animals learned efficiently the task. CO activity increased only in the habituation group as compared to the naïve group in the accumbens shell and the anterodorsal striatum. In contrast, CO activity increased between 1-day and 4-day trained groups in the dentate gyrus of the dorsal hippocampus.

3.3. Interregional within-group correlations in CO activity

Interregional correlations in CO activity of the habituation group were found between the prefrontal and the entorhinal cortex and between the parietal cortex, the nucleus accumbens shell and the lateral amygdala. In addition, the 1-day trained group showed pairwise correlations between the parietal cortex and the dorsal CA3 area as well as between subregions of the prefrontal cortex, the temporal cortex and the dorsal striatum. The 4-day trained group had a more limited reciprocal network of cross-correlations involving only the prefrontal cortex subregions. This correlation network is maintained in the 6-day trained group, but with the additional presence of a correlation between the parietal cortex and the infralimbic cortex as well as a correlation between lateral and anterodorsal striatum. In addition, CO activity correlations between amygdala nuclei were found in the latter group (See Figure 3).

4. DISCUSSION

Our results show a widespread increase in CO activity measured in habituation, 1-day and 4-day learning groups as compared to 6-day and naïve groups in most of the brain regions measured. Other studies have shown similar results, finding a general increase of activity in brain structures during the habituation followed by a more specific activation of particular brain structures as learning progresses (Rinaldi et al., 2010; Bertaina-Anglade et al., 2000). Our results may indicate that the activation of multiple regions is necessary during the learning process, but once the task is well mastered and the consolidation process was effectively finished, brain activity returns to basal levels. Increased CO activity after habituation was found in the prefrontal, the entorhinal and the perirhinal cortex, dorsal CA1 and CA3 hippocampal areas, the nucleus accumbens shell, the dorsal striatum, the amygdala

nuclei and in the parietal cortex. This initial general increase is probably a result of the novelty of the environment and the stress caused for the first contact with the water.

In addition, increased CO activity was found in the amygdaloid complex of habituation, 1-day and 4-day groups. The involvement of the amygdala in fear or anxiety is well known, but studies in humans have also reported that the amygdala enhances the acquisition of declarative knowledge regarding emotionally arousing stimuli (Adolphs, 1997). In particular, the amygdala has been linked to memory processes (Almaguer-Melián and Bergado-Rosado, 2002) and memory consolidation (McGaugh, 2000). Therefore, the participation of the amygdala in visual discrimination learning seems to be important not only during stress situations associated with the habituation process but also during learning in trained animals.

On the other hand, our results show a differential contribution of subregions of the prefrontal cortex in visual discrimination learning. However, the role played by the prefrontal cortex during simple visual discrimination learning is still unclear. Although most lesion studies have found that either prelimbic-infralimbic lesions or cingulate lesions seem to cause impairment in the acquisition of simple visual discriminations (Floresco, et al., 2008, DeCoteau et al., 2009) other authors (Stefani and Moghaddam, 2005) suggest that visual discrimination learning is dependent on the prefrontal cortex. Our results would thus support that the medial prefrontal cortex in general is relevant for visual discrimination learning.

Furthermore, according to the scientific literature available, we confirmed a participation of the striatum in visual discrimination learning (McDonald et al., 2007). Accordingly, our results show a differential contribution of striatal subregions to the visual discrimination task. In particular, the lateral striatum was mostly involved in the early stages of the learning process whereas the medial striatum was recruited at later stages. In this

regard, the functional heterogeneity of the striatum has been previously reported. In fact, particular striatal subregions may differentially contribute to several cognitive functions (DeCoteau and Kesner, 2000; Featherstone and McDonald, 2004; Yin et al., 2004; Ragozzino et al., 2009) being consistent with the differences in the anatomical connections present in the lateral and medial subdivisions of the dorsal striatum.

Finally, visual discrimination learning was related with activation of the dorsal hippocampus. In particular, CA1 and CA3 areas showed increased CO activity in the visual discrimination group as compared to the control group and the 6-day group whereas the dentate gyrus showed a similar increase in CO activity during the first four days. It has been proposed that optimal navigation requires the combination of hippocampal and striatal processing. In this regard, the hippocampus could transform cortical representations according to detected changes in the expected spatial context, while the striatum updates cortical representations based on the most recent reinforcement consequences of previously learned sensory/motor associations (Mizumori et al., 2009). Consistent with this theory, it has been demonstrated that lesions of the dorsal hippocampus can cause retrograde amnesia for simple visual discrimination tasks (Epp et al., 2008).

Although lesion studies shows that perirhinal cortex seems not to be required in simple discriminations (Aggleton et al., 2010, Eacott et al., 2001) damage of this region has been related to learning impairment in a two-choice visual discrimination task (Winters et al., 2010) and object recognition test (Aggleton et al., 2010). The increased metabolism of the entorhinal cortex reported here during the first 4 days of learning could be linked to the participation of the perirhinal cortex in tasks that require visual stimulus information processing in collaboration with other brain regions.

On the other hand, numerous interregional correlations in CO activity were found in the experimental groups. One important advantage of CO histochemistry is that it enables not only to determine the changes in neuronal metabolic demands associated with a particular behavioral task, but also it can be used to evaluate the functional relationship between brain regions using correlation analysis of CO activity between different brain regions (Fidalgo et al., 2011a; Puga et al., 2007). In this study, interregional correlations of CO activity in the habituation group were found between the prefrontal and the entorhinal cortex and between the parietal cortex, the nucleus accumbens shell and the lateral amygdala. Anatomical studies have reported connections between prefrontal and entorhinal cortex (Jones and Witter, 2007) as well as between the nucleus accumbens and the amygdala (Newman and Winans, 1980). As discussed above, the nucleus accumbens shell plays an important role under novel situations (Campioni et al., 2009) and the amygdala has been associated with stress conditions. In addition the parietal cortex is involved in the association between visuo-spatial and motion-related information (Save and Poucet, 2009) which could be important during the habituation process.

Our results show that after 1-day training, pairwise correlations between parietal cortex and CA3 dorsal were observed. The parietal cortex has no direct anatomical connections with the hippocampus. However, these regions were functionally related supporting the idea that the parietal cortex is part of a functional network that allows continuous dialog between the neocortex and the hippocampus (Save y Poucet, 2009). An interesting study that analysed the effects of the stage of learning of an appetitive operant conditioning tasks, reported increases in c-fos positive neurons compared with the same control in the dorsal hippocampus (CA3), the cingulate cortex and the parietal cortex (Bertaina-Anglade et al.. 2000). Indeed, other studies show that the hippocampus is needed

during spatial memory formation (Blum et al.. 1999; Leon WC et al., 2010). Although traditionally the hippocampus was not related to visual discrimination learning, the role of this structure and the striatum to the acquisition of these tasks is still a matter of debate. In our results, both dorsal striatum and hippocampus are involved in the first stages of the learning task. In this regard, a previous study reported the interaction between the dorsal hippocampus and the striatum during visual discrimination learning (McDonald et al.., 2007) suggesting that both brain regions contribute to the acquisition of these kinds of tasks. In addition, it has been proposed that different memory systems represented by the hippocampus and the dorsal striatum would participate in parallel or independently in spatial learning tasks (Mizumori et al.., 2009).

In addition, functional correlation between the perirhinal and the entorhinal cortex was found. Both regions are considered part of the parahippocampal formation. The perirhinal cortex projects directly, as well as indirectly (via the entorhinal cortex) to the hippocampus (Kealy and Commins, 2011). These anatomical connections are in agreement with the correlations that we have found. Although it has been observed that situations involving spatial novelty may require the activation of the hippocampus and the perirhinal cortex (Van Elzakker et al., 2008) we think that this correlation is probably linked to the learning process because the animals were exposed to the maze the day before, during the habituation.

Changes in the correlations between the different subregions of the prefrontal cortex have been found during the learning period. Correlations were found between prelimbic, infralimbic and entorhinal cortices during habituation, and between prelimbic, infralimbic cortex during the 1 day of training. In addition, a local reciprocal network of cross-correlations involving only prefrontal cortex subregions has been found in the 4-day group

being maintained in the 6-day learning group, but including a new correlation between the parietal cortex and the infralimbic and the cingulate cortex.

Although the concept of 'memory systems consolidation' postulates that hippocampus-dependent memories become independent of the hippocampus and stored in the neocortex over the time (Squire and Alvarez, 1995), our results support the participation of the prefrontal cortex in the early stages of the learning process. These data are in concordance with other studies that found a neocortical involvement in the early stages of memory formation (Leon et al., 2011). For example, Blum et al. (2006) found PFC activation in remote and recent trace fear conditioning memory. According to our data, memory consolidation may occur repeatedly at the end of each training period, probably explaining why we found the participation of this structure in all training days.

Finally, correlations between amygdala nuclei have been found in the 6-day training group. In fact, the amygdala together with the prelimbic cortex and the anterior cingulate cortex seem to be important for goal-directed behavior that involves learning a response-outcome contingency (Cardinal et al., 2002) Therefore, the functional coupling of amygdala nuclei supports their role on emotional modulation of response learning. In addition, we reported previously an inverse functional correlation between the dorsal hippocampus and the striatum after the sixth training day in the same visual discrimination task (Fidalgo et al., 2011b in press). Although this functional correlation was not found in the present study, it must be taken into consideration that many brain regions were included here resulting in fewer significant results given the high number of possible comparisons.

In summary, the results of the present study suggest that progressive activation of functional networks involving cortical and subcortical brain regions are required for mastery of a simple visual discrimination task including prefrontal and temporal cortex, striatum and

hippocampus We have also observed a time-dependent involvement of distributed brain networks associated with visual discrimination learning A network including mainly regions associated with novelty, emotional behaviour and integration visuo-spatial and motion information has been established during the earliest phase of the learning task. The prefrontal cortex seems to be necessary along the learning process. The present findings highlight the relevance of dynamic functional interactions between brain regions in learning and memory processes.

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FIGURE 1

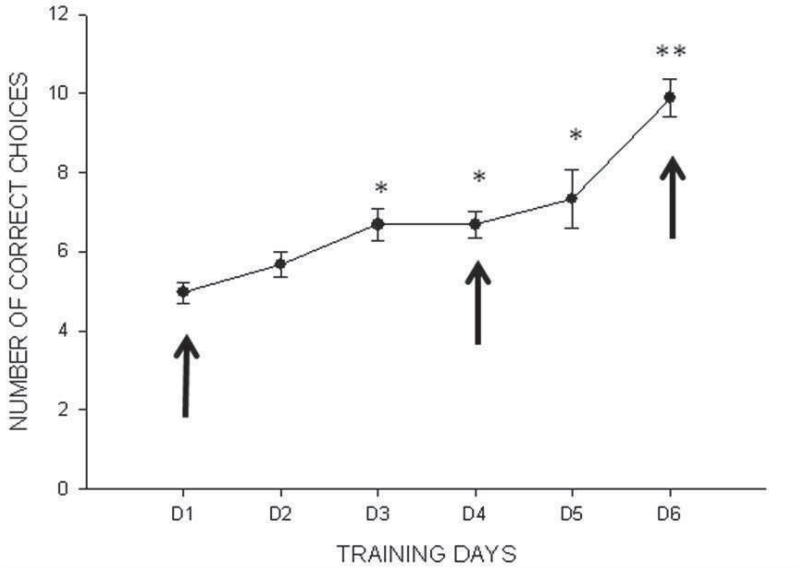

Figure 1: Number of correct choices across training days in the discrimination group. Arrows indicate three time points selected to obtain the different experimental groups. ***p*<005 as compared to the first, second and fourth day. ***p*<005 as compared to the first training day.

FIGURE 2

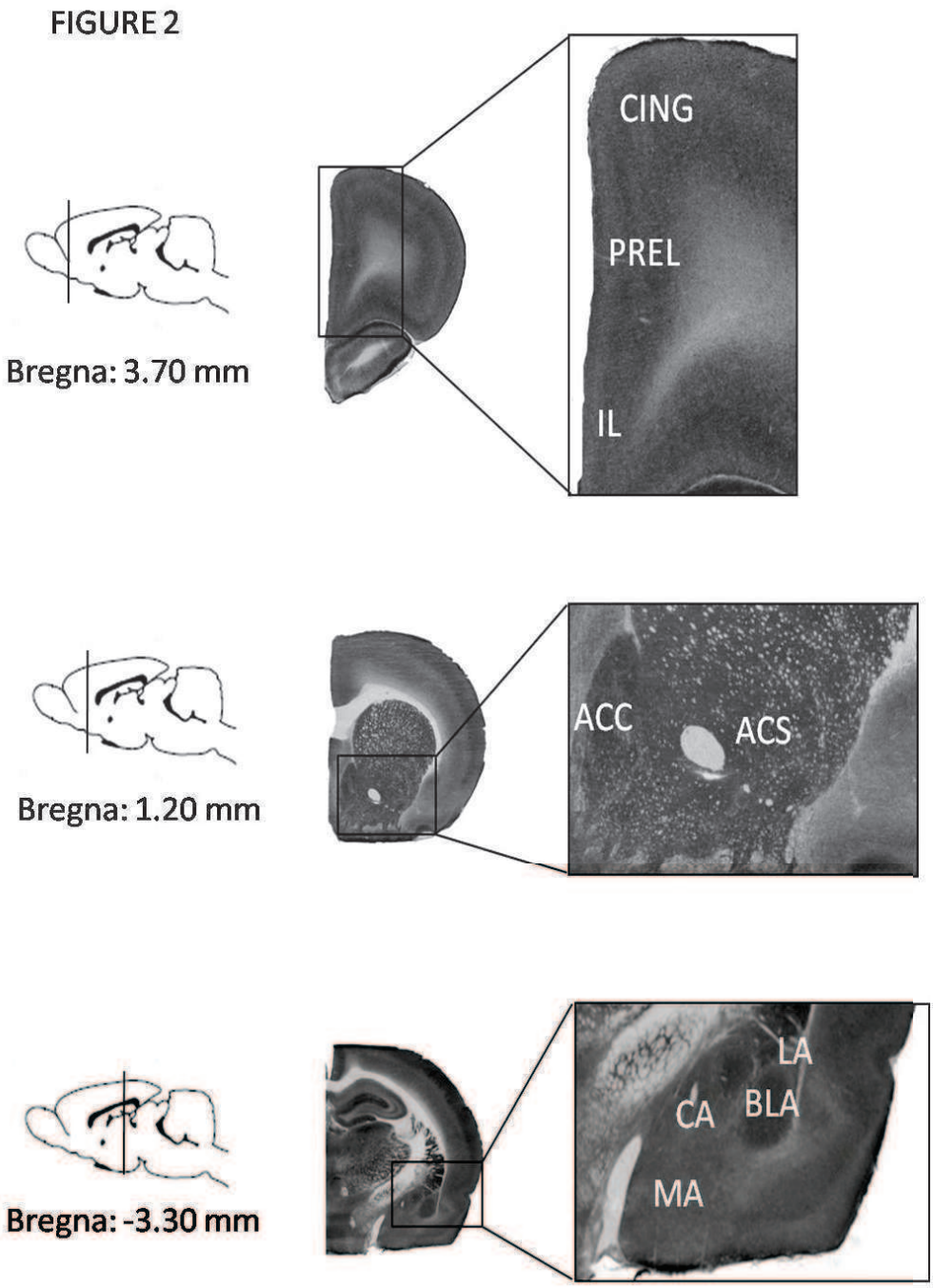

Figure 2: Cytochrome oxidase (COX) histochemical staining of coronal sections (right) at the level of prefrontal cortex (A), accumbens (B) and amygdaloid nuclei (C). CING= cingulate cortex, PREL= prelimbic cortex, ACC= accumbens core; ACS=accumbens shell; LA= Lateral amygdala; BLA= basolateral amygdala; CA= central amygdala; MA= medial amygdala.

FIGURE 3

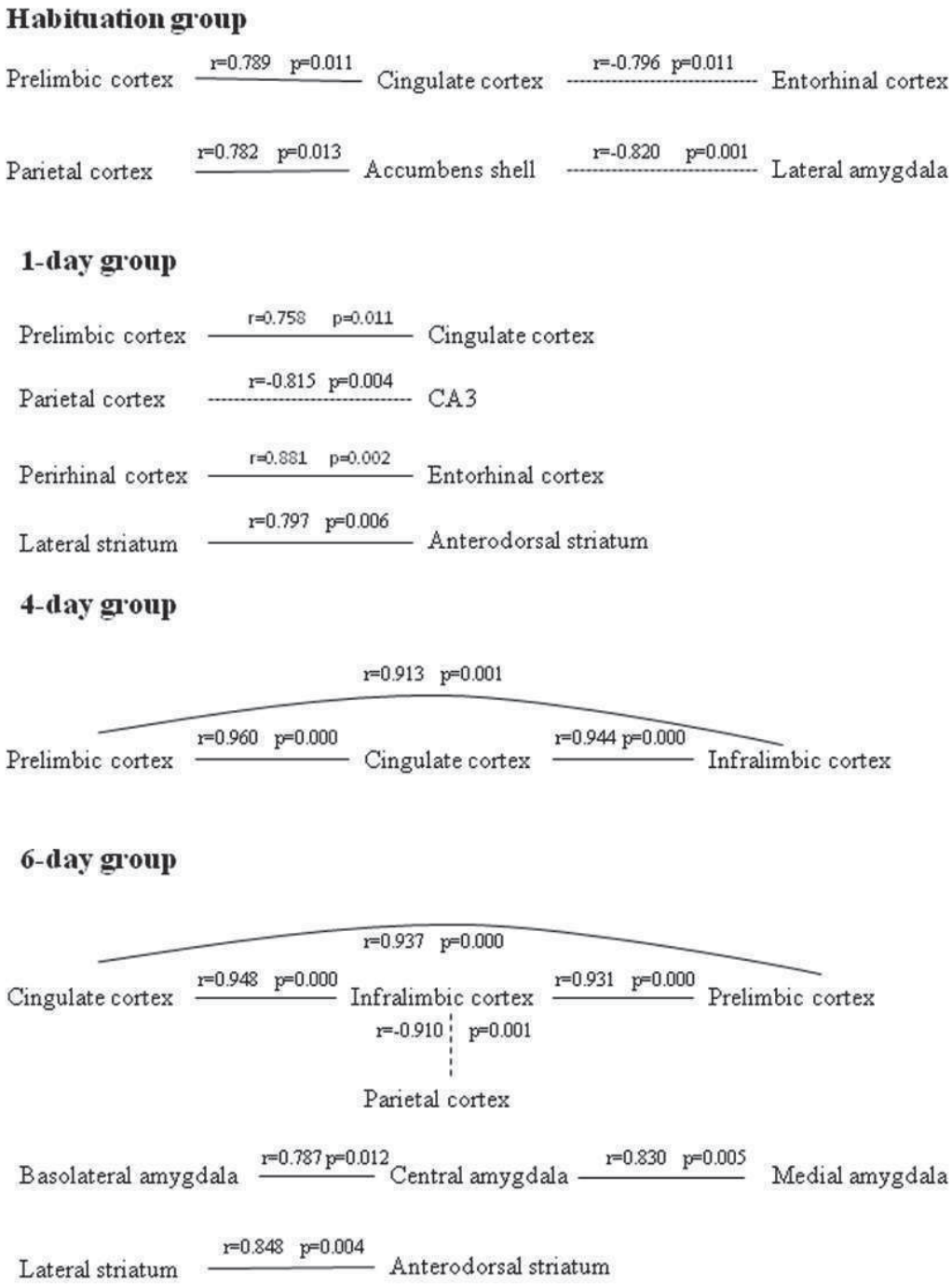

Figure 3: Schematic diagram showing the significant interregional correlations of CO activity calculated in the studies groups (habituation, 1-day group, 4-day group, 6 day group). Solid and dotted lines represent respectively highly positive and negative pair-wise Pearson´s correlations ($r>0.8$, $P<0.02$)

TABLE1

VISUAL DISCRIMINATION LEARNING	CONTROL	HABITUATION	1-DAY	4-DAY	6-DAY
CORTEX					
PRELIMBIC CORTEX	25.9±0.7	33.8±0.8†*	32.6±1.0†*	30.1±1.3*	27.5±0.8
CINGULATE CORTEX	27.1±0.7	34.3±0.8†*	32.3±1.0*	32.6±1.3*	27.9±0.9
INFRALIMBIC CORTEX	25.7±0.5	32.3±0.8†*	31.4±1.3†*	30.5±1.3*	26.1±1.7
PARIETAL CORTEX	28.2±0.8	33.4±1.3*†	32.0±1.0	32.9±0.6*	28.3±1.4
PERIRRHINAL CORTEX	18.5±0.4	28.2±1.4*†	24.6±1.1*	26.3±1.0*†	20.9±1.2
ENTORRINAL CORTEX	17.3±0.6	25.3±1.3*†[1]	21.3±0.9	23.6±0.8*†	17.6±0.9
TELENCEPHALIC STRUCTURES					
ACCUMBENS CORE	32.6±1.1	37.1±1.0	37.1±1.5	35.8±0.8	33.4±1.4
ACCUMBENS SHELL	36.4±1.1	41.9±1.3*	40.8±1.4	40.0±0.7	37.5±1.0
BASOLATERAL AMYGDALA	27.8±0.5	33.2±0.8*†[4]	32.9±0.6*†	32.4±0.7*†	27.8 ±0.6
CENTRAL AMYGDALA	19.1±0.4	26.1±0.8*†	26.9±0.7*†	26.4±0.8*†	21.3±0.5
MEDIAL AMYGDALA	21.5±0.9	24.9±0.8†	25.7±1.0†*	24.5±0.7†	19.3±0.6
LATERAL AMYGDALA	19.3±0.6	26.0±0.8*†	26.4±0.8†*	25.4±0.8†*	19.9±0.4
DORSAL HIPPOCAMPUS: CA1	20.3±0.7	25.6±0.7*†	27.3±1.3*†	26.0±0.6*†	20.4±0.6
DORSAL HIPPOCAMPUS: CA3	17.9±0.5	23.8±0.8*†	25.5±0.9*†	25.0±0.4*†	18.5±0.7
DORSAL HIPPOCAMPUS: DENTATE GYRUS	32.1±1.2	35.0±1.2	39.1±1.3*†	37.9±0.6*†	33.1±1.2
ANTERODORSAL STRIATUM	28.1±1.0	33.6±1.2*†	31.9±1.0	31.4±1.1	28.1±1.2
ANTEROMEDIAL STRIATUM	29.8±0.9	35.7±1.1*†	34.7±1.2†	33.8±1.3†	28.1±1.0
ANTEROLATERAL STRIATUM	30.4±1.1	36.1±1.6*†	34.4±0.9†	33.6±1.1†	28.2±1.3

Table 1: Cytochrome oxidase activity units (mean ± S.E.M.; µmol cytochrome c oxidized/min/g tissue) measured in the selected brain regions. *$p<0.05$ as compared to the naive control group. † $p<0.05$ as compared to the 6-day group. [1] $p<0.05$ as compared to the 1-day group.

III

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Research Report

Cortico-limbic–striatal contribution after response and reversal learning: A metabolic mapping study

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ABSTRACT

Learning of arbitrary stimulus–response associations is an adaptive behavior essential for species survival in an ever-changing environment. Particular subdivisions of the striatum have been shown to be critical for both motor–response learning and reversal learning. However, recent evidence suggests that different cortical and subcortical brain regions may be involved in response learning, a kind of learning more complex than previously thought. In fact, many brain regions subserving response learning seem to be also related to reversal learning, traditionally ascribed to the prefrontal cortex. The present study examined the role of different subdivisions of the rat prefrontal cortex, striatum, amygdala and the ventral tegmental area on both response and reversal learning evaluated in the water T-maze. Increased neuronal metabolic activity, as measured by cytochrome oxidase (CO) histochemistry, was found in most brain regions after training rats in a response learning task as compared to yoked controls. Reversal learning was associated with a return to baseline CO activity levels except for the orbitofrontal cortex and the ventral tegmental area. Analysis of functional connectivity among brain regions showed significant correlations in CO activity between particular cortical and striatal subdivisions in the reversal learning group. These findings suggest that the interaction of specific frontal and subcortical regions is required for reversal but not for response learning. However, our findings support the involvement of a cortico-limbic–striatal circuit in both types of learning.

1. Introduction

Learning theorists postulate that the hippocampal system and components of the basal ganglia (mostly the dorsal striatum) are parts of independent memory systems that mediate place and response learning, respectively (Packard, 2009). In addition, extensive research indicates that not only the striatum plays a major role in motor–response or habit learning but also it has been associated with response reversal learning and the general ability to flexibly shift response patterns after a change in environmental conditions also known as 'behavioral flexibility' (Ragozzino, 2007; Palencia and Ragozzino, 2005; Tzavos et al., 2004). Reversal learning can be understood as a simple form of behavioral flexibility because it implies adaptation to a changing environment in which subjects must reverse a previously established stimulus–reward association within a particular set of stimuli (Floresco et al., 2009; Ragozzino, 2007). These findings are consistent with the results obtained in rodent studies demonstrating that lesions or reversible inactivation of the striatum impair performance in reversal learning tasks (Ragozzino and Choi, 2004; Ragozzino et al., 2002).

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Moreover, several studies report that the dorsal striatum plays a critical role in response learning tasks given that lesions of this region are associated with performance deficits in these tasks (Pistell et al., 2009; DeCoteau and Kesner, 2000; Brasted et al., 1997). Since the dorsal striatum receives a major input from the prefrontal cortex, particular subdivisions of this region have been also associated with response and reversal learning (Floresco et al., 2008; McAlonan and Brown, 2003; Ragozzino et al., 1999). Accordingly, there is evidence suggesting that corticostriatal circuits play a critical role in learning when conditions require a shift in response patterns to solve a task (Block et al., 2007; McDonald et al., 2008; Ragozzino and Choi, 2004; Ragozzino et al., 2002). However, several studies suggest that reversal learning is also mediated by a number of subcortical brain regions anatomically related to the prefrontal cortex besides the striatum, like several amygdala nuclei, and the mesocorticolimbic dopaminergic pathway originating in the ventral tegmental area (Floresco et al., 2009; Hampton et al., 2007).

It is difficult to understand the contribution of the striatum to response and reversal learning due to its functional heterogeneity since particular striatal subregions may differentially contribute to several cognitive functions (Brasted et al., 1999; DeCoteau and Kesner, 2000; Devan et al., 1999). Furthermore, behavioral studies support functional heterogeneity among striatal subregions (Featherstone and McDonald, 2004; McDonald and Hong, 2004; Ragozzino et al., 2009; Yin et al., 2004). The proposed functional dissociation of the dorsal striatum is consistent with the differences in the anatomical connections present in the lateral and medial subdivisions of the dorsal striatum.

In comparison to the role of the striatum in reversal learning, growing evidence suggests that the prefrontal cortex in rats supports learning when conditions require inhibition of a previously relevant behavioral response and when acquiring a new one (Birrell and Brown, 2000; Block et al., 2007; Floresco et al., 2006, 2008 Ragozzino, 2007; Nair et al., 2001; Ragozzino et al., 1999). Specifically, the orbitofrontal cortex seems to be necessary for reversal learning (Young and Shapiro, 2009; Boulougouris et al., 2007; Ghods-Sharifi et al., 2007). Conversely, manipulations of medial prefrontal cortex in rats have equivocal effects on reversal learning (Boulougouris et al., 2007; Chudasama and Robbins, 2003; Joel et al., 1997; Ragozzino and Rozman, 2007; Ragozzino et al., 1999, 2003). Taken together, these findings suggest that prefrontal cortex subdivisions contribute differentially to reversal learning. On the other hand, recent studies suggest an involvement of the ventral tegmental area in reversal learning tasks or basic reinforcement learning (Kehagia et al., 2010; Takahashi et al., 2009) although the role of this dopaminergic brain region on response or reversal learning is still unknown.

The present study aimed to evaluate the role of different anterior striatal subregions, prefrontal cortex subregions (cingulate, infralimbic, prelimbic and orbitofrontal regions) and the ventral tegmental nucleus after training in response and reversal learning tasks. In addition, we examined the contribution of the amygdala (basolateral, lateral, central and medial nuclei) to these tasks due to its pivotal role on emotional aspects of learning and direct anatomical relationship with the medial prefrontal cortex and the striatal regions. Quantitative cytochrome oxidase (CO) histochemistry was used as a metabolic brain mapping technique because it can be used as a reliable index of neuronal oxidative metabolism. CO histochemistry has a high anatomical resolution and it provides a measure of steady or sustained changes in oxidative metabolism associated with brain function (Wong-Riley, 1989). CO histochemistry has been successfully used in previous studies to map changes in brain oxidative metabolism involved in several learning tasks in rats (Conejo et al., 2010; González-Pardo et al., 2008; Hu et al., 2006; Villarreal et al., 2002).

2. Results

2.1. Behavioral results

The results regarding the number of correct arm choices across training days made by the response group are shown in Fig. 1. Both the response and reversal groups reached the learning criterion on day 1. The animals in the reversal group made only 1 or 2 errors out of 12 trials on the last training day (reversal phase).

2.2. CO activity

Significant differences in CO activity among groups were found in the anterior cingulate cortex ($H(2,27)=13.9$, $p<0.001$), prelimbic cortex ($H(2,27)=14.5$, $p<0.001$), infralimbic cortex ($F(2,26)=27.2$, $p\leq0.001$), orbitofrontal cortex ($F(2,22)=15.8$, $p<0.001$), anterodorsal striatum ($F(2,27)=5.8$, $p=0.008$), anterolateral striatum ($F(2,27)=12.6$, $p=0.001$), lateral amygdala ($F(2,26)=3.6$, $p=0.045$) and ventral tegmental area ($F=(2,24)=18.9$, $p<0.001$). Table 1 shows the mean CO activity values measured in the selected brain regions of both experimental and yoked control groups.

2.3. Interregional within-group correlations of CO activity

Significant interregional correlations of CO activity were found in both the response and reversal groups. A high cross-

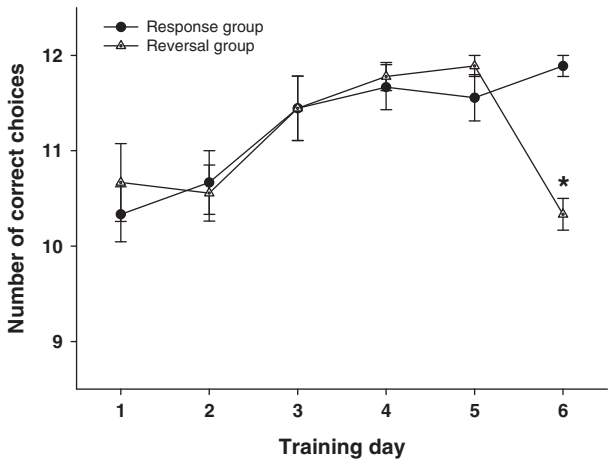


Fig. 1 – Number of correct choices across training days in the response and the reversal group. In the last one, the sixth day corresponds with the platform change to the contralateral arm. *$p<0.05$ as compared to the response group.

Table 1 – Cytochrome oxidase activity units measured in the selected brain regions mean ± SEM.

Brain region	n	Yoked control	n	Response group	n	Reversal group
Motor cortex	10	26.2±1.0	9	**33.1±0.5** *	9	27.2±2.0
Anterior cingulate cortex	10	25.6±1.1	8	**35.1±0.6** *	9	25.9±1.6
Prelimbic cortex	10	24.9±0.9	8	**34.3±0.7** *	9	24.5±1.5
Infralimbic cortex	10	23.2±0.9	8	**33.3±0.9** *	9	24.3±1.2
Orbital cortex	7	23.2±1.00	9	**30.9±0.5** #	7	**31.4±1.8** #
Anteromedial striatum	10	31.0±1.3	10	35.2±1.5	9	30.3±1.6
Anterodorsal striatum	10	28.7±1.9	10	32.3±1.2	9	**27.5±1.1** †
Anterolateral striatum	10	31.1±1.2	10	**37.5±1.7** *	9	30.4±0.9
Lateral amygdala	10	20.1±1.1	9	**23.6±0.8** #	9	22.3±0.7
Basolateral amygdala	10	28.1±1.2	9	30.7±0.8	9	28.4±1.2
Central amygdala	10	22.4±0.8	9	24.2±0.8	9	23.1±0.9
Medial amygdala	10	20.4±1.0	9	22.7±0.7	9	22.2±1.0
Ventral tegmental area	10	12.0±0.7	7	**17.2±1.1** #	8	**18.5±0.7** #

* $p<0.05$ as compared to both yoked control and reversal group.
† $p<0.05$ as compared to the response group.
# $p<0.05$ as compared to the yoked control group.

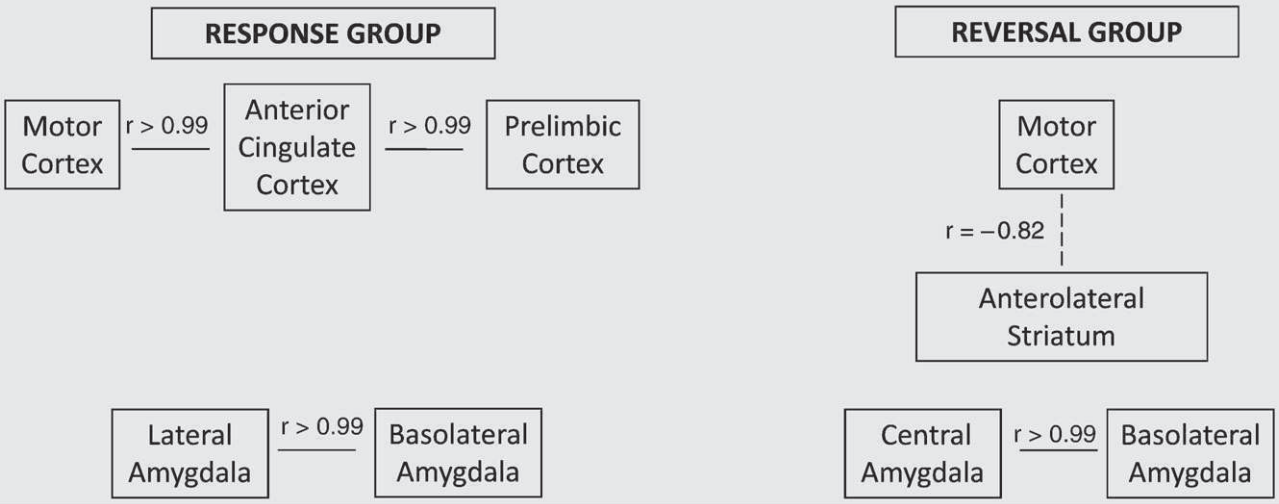
correlation between motor, anterior cingulate and prelimbic cortical areas ($r>0.99$, $p<0.001$) as well as between the basolateral and lateral amygdala ($r=0.99$, $p<0.001$) were found in the response group. On the other hand, interregional correlations in the reversal group were found between the motor cortex and the anterolateral striatum ($r=-0.82$, $p<0.01$) and between the basolateral and central amygdala ($r=0.99$, $p<0.001$). See Table 2 for detailed correlation matrix data.

3. Discussion

Our results show increased CO activity in the response learning group as compared to the yoked control in the prefrontal regions analyzed (anterior cingulate, prelimbic, infralimbic and orbitofrontal), the anterolateral striatum, the lateral amygdala and the ventral tegmental area. The reversal group showed significant changes as compared to the yoked control group in the orbitofrontal cortex and the ventral tegmental area. In addition, this group showed a return to baseline levels of CO activity in the anterior cingulate cortex, prelimbic and infralimbic cortical regions and the anterodorsal and anterolateral striatum.

The high metabolic capacity found in the prefrontal cortex and the anterolateral striatum of animals trained in the response learning task suggests an involvement of both brain regions in this kind of learning. Our results agree with previous studies suggesting that the frontostriatal system is required for basic reinforcement learning (Kehagia et al., 2010; Mizumori et al., 2009). Learning basically depends on efficiently using feedback from the environment about the outcome of behavior. In our case, feedback is represented by an expected rewarding stimulus (an escape platform) that enables the animal to quickly avoid the mild aversive situation when performing a particular behavior. During response learning, rats are reinforced for making a specific response (here, a body turn to the right or left arm of the maze).

Table 2 – Schematic diagram showing the significant interregional correlations of CO activity calculated in both response (left) and reversal (right) groups. Solid and dotted lines represent respectively highly positive and negative pair-wise Pearson's correlations ($r>0.8$, $p<0.02$).



Both the prefrontal cortex and the striatum are activated when a reward is expected and after its delivery (Schultz, 1997). In addition, studies with primates demonstrated that neurons of the prefrontal cortex and the striatum show sustained activity—even between trials—when successfully performed a simple associative learning task (Histed et al., 2009). As previously commented, CO activity reflects sustained changes in neuronal metabolic capacity linked to behavior. In this regard, our research group has previously reported that the use of a response learning strategy in a water maze task is associated with a significant increase of CO activity in the dorsal striatum (Miranda et al., 2006). Moreover, Pavlovian conditioning—a basic form of associative learning—has been reported to increase CO activity in several frontal regions like those evaluated here (Bruchey and Gonzalez-Lima, 2008). Pavlovian conditioning and response learning can be related because they are procedural learning types involving stimulus–response associations. Therefore, increases in CO activity found in frontal and striatal regions of the response learning group could be related with their role on reinforced motor or habit learning. Our results suggest that the dorsolateral striatum in particular is relevant for response learning. These results are in agreement with previous studies that reported an important role of this structure in habit formation (Balleine et al., 2007; Yin and Knowlton, 2004). Moreover, the striatum receives inputs from the prefrontal cortex, which in turn is also interconnected with the lateral amygdala (McDonald, 1991). Accordingly, we found a significant increase in CO activity of the lateral amygdala in the response learning group. Therefore, we could consider that a network including the prefrontal cortex, dorsal striatum and the amygdala may be related with reinforced motor–response learning.

Furthermore, it has been speculated that changes in functional coupling among different brain regions can be detected without necessarily involving increases or decreases of brain regional activity. Accordingly, we have found high correlations among the premotor, anterior cingulate and prelimbic cortices and between the basolateral and lateral amygdala nuclei in the response learning group. These results highlight the relevance of interactions among frontal cortical regions in response learning. In particular, activation of the medial prefrontal cortex and the anterior cingulate cortex is important to link actions to the reinforcement value of its outcome (Rushworth et al., 2004). Alternatively, our results regarding increases in CO activity and functional coupling among frontal regions would support current theories about the participation of the prefrontal cortex in memory consolidation and recall (Leon et al., 2010; Frankland and Bontempi, 2005, 2006). In agreement with our results, increased CO activity in prefrontal cortical regions has been reported after associative learning and spatial learning in rats (Bruchey and Gonzalez-Lima, 2008; Conejo et al., 2010, 2007).

On the other hand, the functional interaction between amygdala nuclei could be associated with emotional aspects required for associative learning. Our results show also that the amygdala nuclei are important for both response learning and reversal learning. In addition, cross-correlations between the basolateral amygdala and the lateral or central amygdala nuclei were found in the response and reversal learning groups respectively. The basolateral amygdala has been related with the modulation of emotional arousal associated with memory consolidation processes (McGaugh, 2002). Emotional arousal can influence the relative use of multiple memory systems in a manner that favors the use of striatal-dependent habit memory (Packard, 2009). Therefore, the functional coupling of amygdala nuclei highlights their role on emotional modulation of response learning. In this regard, the amygdala together with the prelimbic cortex and the anterior cingulate cortex seem to be important for goal-directed behavior that involves learning a response–outcome contingency (Cardinal et al., 2002).

In addition, the ventral tegmental area was activated in the response and reversal learning groups, a result that supports an involvement of the mesolimbic dopamine system in response and reversal learning. Dopamine release in the prefrontal cortex and the striatum by neurons of the ventral tegmental area has been related with the anticipation of reward in goal-directed behaviors (Kehagia, et al., 2010; Grace et al., 2007; Fields et al., 2007).

Training in a reversal learning task was associated with a return to baseline CO activity similar to the yoked control group in most of the brain regions evaluated. However, the orbitofrontal cortex and the ventral tegmental area still showed high CO activity levels similar to the response learning group. Accordingly, the orbitofrontal cortex seems to be relevant for switching response strategies as previously suggested by several authors (Bissonette et al., 2008; Boulougouris et al., 2007; Ragozzino, 2007). This result is also in agreement with studies in humans using PET or fMRI reporting a critical involvement of the orbitofrontal cortex and the caudate nucleus in reversal learning (Ghahremani et al., 2010; Young and Shapiro, 2009; Xue et al., 2008; Rogers et al., 2000). Moreover, simultaneous activations of the orbitofrontal cortex and the ventral tegmental areas similar to those found here have been involved in learning from unexpected outcomes (Takahashi et al., 2009). Extinction of a previously rewarded behavior is associated with significant changes in fluorodeoxyglucose uptake in the orbitofrontal cortex and the ventral tegmental area (Nair et al., 2001; Nair and Gonzalez-Lima, 1999). Therefore, the orbitofrontal cortex together with the ventral tegmental area could be key components of a brain circuit involved in reversal learning.

In addition, a significant CO activity correlation between the anterolateral striatum and the motor cortex was found after training in a reversal learning task. This result indicates that this particular subdivision of the striatum, and not the whole dorsal striatum, could be specifically related with reversal learning. Recent studies suggest that a circuit between the orbitofrontal cortex and the dorsomedial striatum may form a basic neural network underlying behavioral inhibition in rats (Eagle and Baunez, 2010). However, most studies reporting involvement of the dorsomedial striatum in reversal learning are based on lesion methods. It should be taken into consideration that metabolic mapping techniques like CO histochemistry allow the study of changes in regional brain activity in intact subjects and also allow to accurately map regional changes in brain activity. Since reversal learning basically involves inhibition of a previously reinforced behavior, it is reasonable to think that changes in the metabolic capacity of the orbitofrontal cortex and the anterolateral striatum could be also related with behavioral inhibition.

In summary, our results suggest that training in a response learning task requires the participation of many brain regions including mainly the prefrontal cortex, the anterolateral striatum, the basolateral amygdala and the ventral tegmental area. These brain regions have been associated generally with reinforced motor or habit learning. On the other hand, reversal learning could be linked to the activation of the orbitofrontal cortex and the ventral tegmental area, as well as the return to baseline levels of brain regions formerly involved in response learning. In conclusion, a common network comprising cortical, limbic and striatal regions would be related with response and reversal learning.

4. Experimental procedures

4.1. *Animals*

A total of 30 male Wistar rats weighing between 150 and 250 g were used in this experiment. The animals were obtained from the University of Oviedo central vivarium (Oviedo, Asturias, Spain) and were randomly housed in groups of five under standard conditions (12-h light/dark cycle with lights on from 08:00 to 20:00 h) at constant room temperature of 21±2 °C with *ad libitum* access to food and water. All experiments were done in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Spanish legislation (R.D. 1201/2005). All efforts were made to minimize the number of animals used and their suffering.

4.2. *Apparatus*

Rats were trained in a water T-maze made of black fiber glass filled with tap water (23±1 °C). The main alley (100×20×40 cm) was connected to two side arms (right and left) measuring 45×20×40 cm. A black square escape platform made of Plexiglas (15×18 cm) was placed at the end of each arm 2 cm beneath the water surface. The maze was located in a dark room illuminated by red lights without visual cues that could allow the animals to guide their response. Each trial was recorded and swim paths of the animals were analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

4.3. *Behavioral procedure*

In order to discard possible motor and sensory deficits, animals were tested in a neurological assessment battery. 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 (according to Bures et al., 1976). No animals were discarded due to abnormal neurological responses. Rats were handled daily during 5 days. The spatial memory task was performed between 09:30 and 13:00 h. During the habituation day, rats of the 'response learning' group (n=10) and the 'reversal' group (n=10) were gently immersed in the water T-maze for 1 min, without any escape platform available. The training phase started the next day. The escape platform was located in the same position across training days. The position of the escape platform changed to arm opposite to that chosen for the first time during the habituation day in order to favor alternation. Each animal in all experimental groups received a single 12-trial session per day during 6 days. In each trial, rats were allowed to swim to locate the platform or they were placed on it after 60 s, where they remained for 15 s before returning them to the cage for 30 s. In the reversal group, the platform was moved to the opposite arm on the sixth day, so that the total number of trials was the same as compared to the response group. The learning criterion used for both experimental groups was eight or more correct choices out of 12 trials.

A free-swimming group (yoked control) was composed of rats that were placed in the maze the same number of times and days as compared to the experimental groups but without escape platform available. The yoked control group swam during an amount of time equivalent to the mean daily escape latencies recorded for the experimental groups.

4.4. *Tissue preparation*

Ninety minutes after finishing the behavioral procedure, the animals were decapitated. Their brains were quickly removed, frozen rapidly in isopentane at –70 °C (Sigma-Aldrich, Madrid, Spain) and stored at –40 °C to preserve the brain tissue and enzyme activity. Next, 30 μm-thick coronal sections were obtained from the brain tissue using a cryostat microtome (Microm International GmbH, HM 505 E, Heidelberg, Germany). These sections were mounted on slides and stored at –40 °C until processing with quantitative CO histochemistry. Some sections from a few subjects could not be used as a result of tissue processing, although the final number of sections available for histochemistry was enough in all cases.

4.5. *CO histochemistry*

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 (1994). To control staining variability across different baths, sets of tissue homogenate standards from Wistar rat brain were cut at different thicknesses (10, 30, 50 and 70 μm) and included with each bath of slides. In brief, 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% (wt./vol.) sucrose and 5 ml dimethyl-sulfoxide. Once 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), 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 for 30 min at room temperature with 10% (wt./vol.) sucrose and 4% (vol./vol.) formalin. The slides were dehydrated, cleared with xylene for 10 min 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) composed of a high precision illuminator, a digital

camera and a computer with specific image analysis software. A total of twelve measurements were taken per region. These measures were averaged to obtain one mean per region for each animal and were expressed as arbitrary units of optical density (OD) in the prefrontal cortex (anterior cingulate, prelimbic and infralimbic areas), striatum (anterodorsal, anteromedial and anterolateral regions) and amygdala (lateral, basolateral, medial and central nucleus). The selected brains regions anatomically were defined according to the Paxinos and Watson's (1997) atlas. Fig. 2 shows coronal sections of CO stained sections.

4.6. *Statistical analysis*

The CO activity measured as relative OD values of each region was evaluated by one-way ANOVA using experimental group (response, reversal and free-swimming group) as the independent

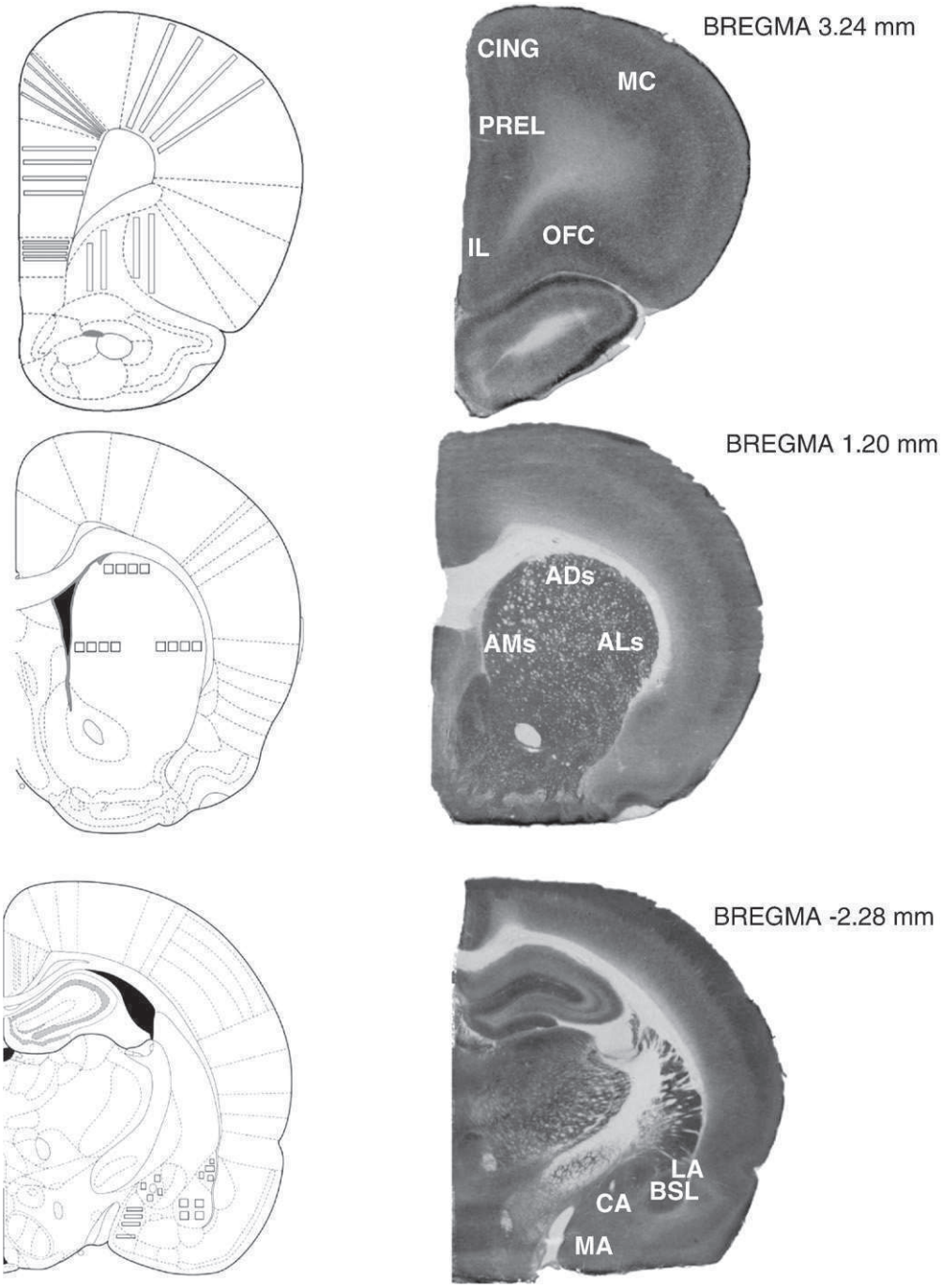


Fig. 2 – Microphotographs showing coronal CO stained sections and schematic representations of the selected brain regions (squares and rectangles indicate the sampled areas). A. Prefrontal cortex. MC: motor cortex, CING: anterior cingulate cortex, PREL: prelimbic cortex, IL: infralimbic cortex and OFC: orbitofrontal cortex. B. Dorsal striatum. ADs: anterodorsal striatum, AMs: anteromedial striatum, ALs: anterolateral striatum. C. Amygdala nuclei. LA: lateral amygdala, BLA: basolateral amygdala, CA: central amygdala and MA: medial amygdala.

variable. 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 $p<0.05$. Tukey's HSD test was used as a post hoc test to assess differences between means when the analysis of variance (ANOVA) revealed significant differences between groups. When the normality test failed, Kruskal–Wallis tests were used. In order to evaluate changes in functional connectivity among brain regions, regional CO activity data were analyzed in terms of pair-wise correlations within 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 all structures measured for each animal. This was done to reduce variation in the intensity of the CO staining not resulting from the experimental manipulation. In addition, in order to avoid errors due to an excessive number of significant correlations using small sample sizes we used a 'jackknife' procedure (Shao and Tu, 1995) 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 ($p<0.02$) across all possible combinations.

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A role for dorsal and ventral hippocampus in response learning

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ABSTRACT

The hippocampus and the striatum have been traditionally considered as part of different and independent memory systems despite growing evidence supporting that both brain regions may even compete for behavioral control in particular learning tasks. In this regard, it has been reported that the hippocampus could be necessary for the use of idiothetic cues in several types of spatial learning tasks. Accordingly, the ventral striatum receives strong anatomical projections from the hippocampus, suggesting a participation of both regions in goal-directed behavior. Our work examined the role of the dorsal and ventral hippocampus on a response learning task. Cytochrome c oxidase (C.O.) quantitative histochemistry was used as an index of brain oxidative metabolism. In addition, determination of C.O. subunit I levels in the hippocampus by western blot analysis was performed to assess the contribution of this subunit to overall C.O. activity. Increased brain oxidative metabolism was found in most of the studied hippocampal subregions when experimental group was compared with a swim control group. However, no differences were found in the amount of C.O. subunit I expressed in the hippocampus by western blot analysis. Our results support that both the dorsal and ventral hippocampus are associated with the use of response strategies during response learning.

1. Introduction

Traditionally, the hippocampal system and components of the basal ganglia (mainly the dorsal striatum) are considered as important neural substrates underlying independent memory systems that mediate place and response learning respectively (Packard, 2009). In this regard, the hippocampus seems to be involved in the declarative memory system (Squire and Zola-Morgan, 1991) whereas the dorsal striatum is considered as a key brain region for the procedural, implicit or habit memory system (Packard et al., 1989). Accordingly, previous studies of our research group demonstrated the participation of the dorsal striatum in response learning (Fidalgo et al., 2011) where the animal uses idiothetic cues (based on vestibular or motor/proprioceptive information) to solve it. Animals use response strategies when the environmental cues alone are not enough for spatial orientation.

Although conventionally it is assumed that only the striatum seems to be necessary for habit memory, recent research suggests that the hippocampus could be also involved. In this regard, lesion studies reported the development of retrograde amnesia in habit learning tasks after hippocampal lesions (Sutherland et al., 2001; Driscoll et al., 2005; Epp et al., 2008). Hence, rats with hippocampal lesions showed impaired performance in a water maze task involving the use of idiothetic cues run in the dark (Zheng et al., 2003). Moreover, there is evidence supporting that the hippocampus and the striatum may even compete for behavioral control in several learning tasks requiring particular memory systems (White and McDonald, 2002). In particular, since the ventral striatum receives strong anatomical projections from the hippocampus, both brain regions have been related with goal-directed or motivated behavior in general (Pennartz et al., 2011).

Although it is well known that some hippocampal memory functions in humans are lateralized, the significance of functional cerebral asymmetries in rodents is still not clear. It has been suggested that genetic factors, sex hormones and the environment may participate in the development of cerebral asymmetry (Toga and Thompson, 2003). Moreover, there is some evidence suggesting that hippocampal function might be lateralized in rats during spatial memory acquisition or retrieval (Klur et al., 2009). In this regard, it has been recently reported the interhemispheric transfer of information acquired during learning tasks in rats using C.O. histochemisty combined with temporal inactivation techniques (Cimadevilla et al., 2011).

Therefore, the aim of this study was to evaluate the participation of the hippocampus in habit memory using cytochrome c oxidase (C.O.) as an index of brain oxidative metabolism. In addition,

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activity of both hemispheres will be analyzed to detect possible asymmetries in hippocampal function. In particular, brain tissue has a continuous dependence on oxidative metabolism. Due the essential role of the C.O. in energy metabolism, C.O. activity has been extensively used as an indirect measurement of the functional level of brain activity. Cytochrome c oxidase (C.O.; EC 1.9.3.1) is an enzyme of the mitochondrial electron transport chain involved in the generation of ATP by the process of oxidative phosphorylation. More specifically, this enzyme catalyzes the oxidation of cytochrome c, thereby reducing oxygen into water. Mammalian C.O. is made of 13 subunits with a 1:1 stoichiometry. Its largest three catalytic subunits I, II and III are encoded in the mitochondrial genome and are highly conserved while the rest of the subunits (IV, Va, b, VIa, b, c, VIIa, b, c and VIII) are of nuclear origin and manifest interspecies and inter-organ variability (Wong-Riley, 1989). In this context, C.O. activity has been used in studies of learning and memory by us and others in a variety of animal species (Agin et al., 2001; Bruchey and Gonzalez-Lima, 2008; Conejo et al., 2010). In contrast to other functional brain mapping techniques like 2-DG (Bontempi et al., 1999), PET or fMRI (mostly used in humans) (Bäckman et al., 2011; Clatworthy et al., 2009) C.O. activity reflects a more sustained or stable state of energy metabolism (metabolic capacity). The subunit I is part of the catalytic center of the protein, so might play a more important role in perceiving energy requirements than other nuclear encoded subunits. A recent paper performed in chicks revealed a relationship between the amounts of C.O. subunit I and learning abilities (Solomonia et al., 2011). Therefore, additional determination of C.O. subunit I levels in the hippocampus by western blot analysis was performed to assess the contribution of this subunit to overall C.O. activity. This is the first study to our knowledge about the contribution of C.O. subunits to learning and memory in mammals.

2. Experimental procedure

2.1. Animals

A total of 35 male Wistar rats weighing between 150 and 250 g were used in this experiment. The animals were obtained from the University of Oviedo central vivarium (Oviedo Asturias Spain) and were randomly housed in groups of five under standard conditions (12-h light/dark cycle with lights on from 08:00 to 20:00 h) at constant room temperature of $21 \pm 2\,^{\circ}C$ with ad libitum access to food and water. All experiments were done in accordance with the European Communities 2010/63/UE and the Spanish legislation (RD 1201/2005). All efforts were made to minimize the number of animals used and their suffering.

2.2. Apparatus

Rats were trained in a water T-maze made of black fiber-glass filled with tap water ($23 \pm 1\,^{\circ}C$). The main alley (100 cm × 20 cm × 40 cm) was connected to two side arms (right and left) measuring 45 cm × 20 cm × 40 cm. A black square escape platform made of Plexiglas (15 cm × 18 cm) was placed at the end of each arm 2 cm beneath the water surface. The maze was located in a dark room illuminated by red lights without visual cues that could allow the animals to guide their response. Each trial was recorded and swim paths of the animals were analyzed later using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

2.3. Behavioral procedure

The animals were randomly divided into two groups: experimental ($n = 28$) and control ($n = 17$). After finishing the behavioral procedures, approximately half of the animals in each group ($n = 10$ and $n = 9$ from experimental and control groups, respectively) were also used for C.O. histochemistry and the concentration of C.O. subunit I was measured with western blot analysis in the rest of animals ($n = 8$ from each group).

Rats of the experimental group were handled daily during 5 days. On the habituation day, animals were gently immersed in the water T-maze for 1 min without any escape platform available. The training phase started the next day. The escape platform was located in the same position across training days. The position of the escape platform changed to the arm opposite to that chosen for the first time during the habituation day in order to favor alternation. Each animal received a single 12-trial session per day during 6 days. In each trial, rats were allowed to swim to locate the platform or they were placed on it after 60 s, where they remained for 15 s before returning them to the cage for 30 s. The learning criterion used was eight or more correct choices out of 12 trials. The control group was placed in the maze the same number of times and days as compared to the experimental group but without escape platform available. Rats in the control group swam during an amount of time equivalent to the mean daily escape latencies recorded for the experimental group.

2.4. Cytochrome oxidase hystochemistry

2.4.1. Tissue preparation

After completion of the behavioral procedures all animals were decapitated. Their brains were quickly removed frozen rapidly in isopentane at $-70\,^{\circ}C$ (Sigma–Aldrich, Madrid, Spain) and stored at $-40\,^{\circ}C$ to preserve the brain tissue and enzyme activity. Next 30 μm-thick coronal sections were obtained from the brain tissue using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). These sections were mounted on slides and stored at $-40\,^{\circ}C$ until processing with quantitative C.O. histochemistry. Some sections from a few subjects could not be used as a result of tissue processing although the final number of sections available for histochemistry was enough in all cases.

2.4.2. C.O. histochemistry

We used a modified version of the method originally described by Wong-Riley based on the quantitative C.O. histochemical method developed by Gonzalez-Lima and Jones (1994). To control staining variability across different baths sets of tissue homogenate standards from Wistar rat brain of known C.O. activity determined spectrophotometrically were cut at different thicknesses (10, 30, 50 and 70 μm) and included with each bath of slides. In brief 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. Once the sections had been rinsed in phosphate buffer (pH 7.6; 0.1 M) they were incubated at $37\,^{\circ}C$ 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 for 30 min at room temperature with 10% (w/v) sucrose and 4% (v/v) formalin. The slides were dehydrated, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). C.O. 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 a computer with specific image analysis software. A total of twelve measurements of relative optical density were taken per region. In order to establish comparisons and

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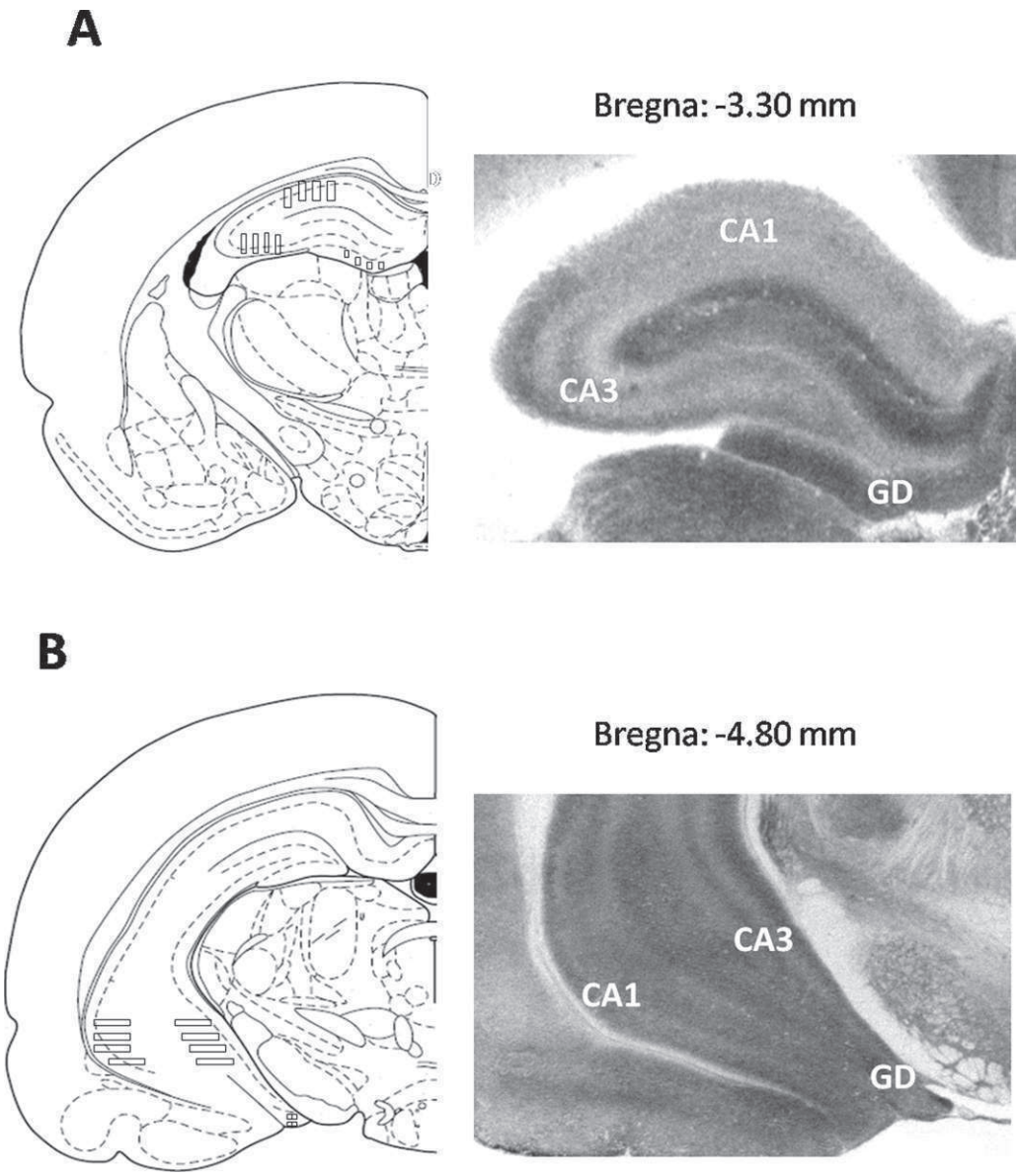

Fig. 1. Microphotographs showing coronal CO stained sections (right) and schematic representations (left) of the selected brain regions (squares and rectangles indicate the sampled areas). (A) Dorsal hippocampus and (B) ventral hippocampus.

consider possible staining variations between brain sections from different staining baths, measurements were also taken from C.O.-stained brain homogenate standards. Regression curves between section thickness and known C.O. activity as previously measured by spectrophotometric assay in each set of standards were calculated for each incubation bath. Finally, average relative optical density measured in each brain region was converted into C.O. 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 areas CA1, CA3 and dentate gyrus of the dorsal and ventral hippocampus (Fig. 1). The selected brains regions anatomically were defined according to Paxinos and Watson (1997).

2.5. Western blot

Brain tissue preserved in ice was homogenized in ice-cold lysis buffer. The lysis buffer contained 50 mM Tris–HCl, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA, 2 mM sodium pyrophosphate, 10 μg/ml leupeptine, 4 μg/μl aprotinine, 1 mM sodium orthovanadate, 30 mM NaF, 4 mM p-nitrophenol phosphate and 1 mM PMSF. After tissue homogenization in the buffer, it was sonicated and stored at −40 °C.

Homogenate aliquots were collected in Laemmli buffer for western blot analysis after determination of protein concentration using a Bradford protein assay kit (Pierce, Rockford, IL, USA). Later, aliquots were heat-denatured and 50 μg of protein were run on 12% SDS-PAGE electrophoresis gel. Proteins were then transferred to PVDF membranes (Millipore Ibérica, Madrid, Spain). Membranes were blocked with TBS/Tween-20 supplemented with 5% (w/v) non-fat milk for 1 h at room temperature, and incubated overnight at 4 °C with a polyclonal antibody against CO-I subunit (COX1/C-20) (1:200, sc-23982, Santa Cruz Biotechnology, USA), an anti-goat secondary antibody (1:6000, A8919, Sigma–Aldrich, Madrid, Spain) for 1 h at room temperature and developed with enhanced chemiluminescence reagents (GE Healthcare, Madrid, Spain). Beta-actin was used as loading control for electrophoresis and labeled using a monoclonal anti-beta-actin antibody (1:2000, A-5441, Sigma, Madrid, Spain) (see Fig. 2).

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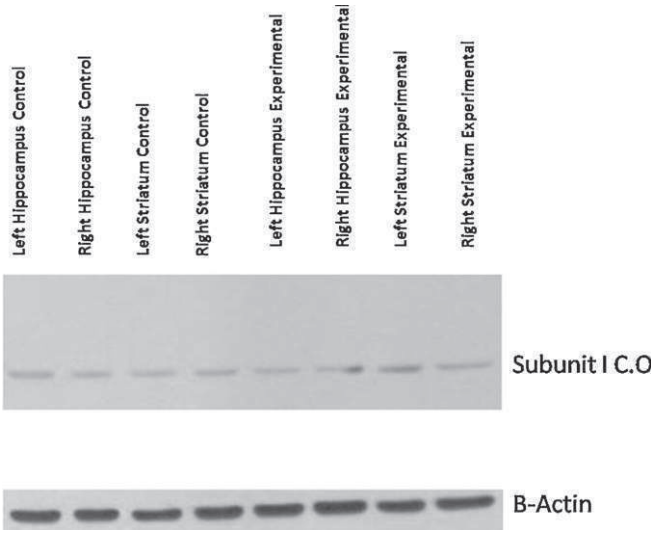

Fig. 2. Representative images of immunoblots showing the contents of CO subunit I and β-Actin in the different brain regions selected by western blot analysis.

2.6. Statistical analysis

Overall group differences in CO activity between left and right hippocampus were evaluated by Student's *t*-test. For multiple group comparisons, two-way ANOVAs were used with group (experimental, control) and hemisphere (left, right) as factors. Tukey's tests were applied for post hoc analysis of significant differences. Results were considered statistically significant when $p < 0.05$.

In order to analyze C.O. subunit I protein levels, intensities of inmunoreactive bands were measured by densitometric analysis using the computer-assisted image analysis system described above. Data were normalized by dividing the optical density value corresponding to the C.O. subunit I band between the intensity obtained in the β-actin bands. Group differences in the amount of C.O. subunit I in the hippocampus were evaluated by Student's *t*-tests. Data were analyzed by SigmaStat 3.2 software (Systat Software, Chicago, USA) and were expressed as mean ± SEM.

3. Results

3.1. Behavioral results

The results regarding the number of correct arm choices across training days made by the experimental group are shown in Fig. 3. The animals reached the learning criterion on day 1 and showed continuing improvement with learning days.

3.2. C.O. activity

Significant differences in C.O. activity between groups were found in the right and left hippocampus ($t(16) = 3.17$; $p = 0.006$ and $t(16) = 2.92$; $p = 0.01$, respectively) (see Table 1A). In addition, there was a statistically significant effect of group in the C.O. activity when it was analyzed using a two-way ANOVA with group and hemisphere as factors. There was a statistically significant effect of group in CA1 ($F = 7.24$; $p < 0.011$, two-way ANOVA), CA3 ($F = 24.89$; $p < 0.001$, two-way ANOVA), ventral CA1 ($F = 8.89$; $p < 0.006$, two-way ANOVA), ventral CA3 ($F = 12.74$; $p < 0.001$, two-way ANOVA) and ventral dentate gyrus ($F = 28.02$; $p < 0.001$, two-way ANOVA). No significant differences have been found in the dorsal dentate gyrus ($F = 1.34$; $p = 0.25$, two-way ANOVA). However, no significant effects of hemisphere factor were found in the brain regions measured and group by hemisphere interaction was also nonsignificant (see Table 1C).

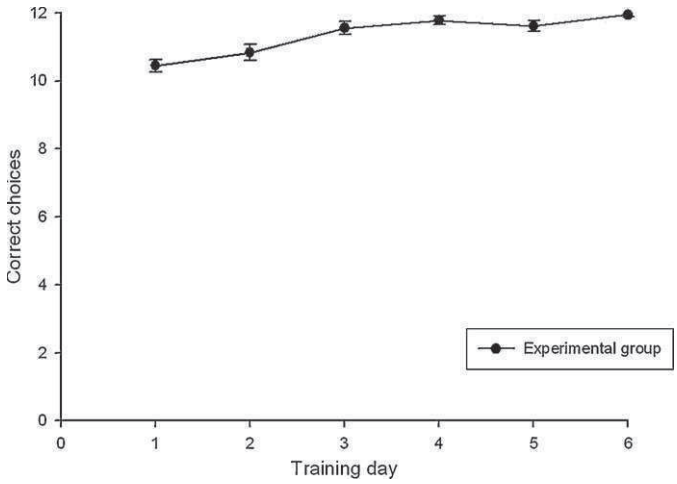

Fig. 3. Number of correct choices across training days in the experimental group. All animals reached the learning criterion on the first training day.

3.3. Western blot

No group differences have been found in the western blot analysis in left ($t(17) = 0.76$; $p = 0.43$) and right ($t(16) = 0.80$; $p = 0.43$) hippocampus (see Table 1B).

4. Discussion

Our results show a differential contribution of particular hippocampal areas to response learning. Increased C.O. activity was found in both the right and left hippocampus in rats mastering a response task as compared with a swim control group. Although lesions studies have proposed that the striatum is the structure involved in habit or response learning (Mizumori et al., 2009),

Table 1
(A) Cytochrome oxidase activity units (mean ± SEM; μmol cytochrome c oxidized/min/g tissue) measured in left and right hippocampus, (B) normalized data resulting from dividing the measurement obtained in the C.O. subunit I between the intensity obtained in the β-actin bands in the selected brain regions, and (C) cytochrome oxidase activity units in the selected brain regions. Two-way ANOVA showed that there was no difference between hemispheres, or interference between hemispheres and groups. However, significant differences between the groups have been shown.

Structure	Experimental group	Control group
(A) C.O. activity		
Left Hippocampus	35.7 ± 1.3*	29.4 ± 0.91
Right Hippocampus	27.3 ± 1.0*	23.7 ± 0.55
(B) Western blot analysis		
Left Hippocampus	0.82 ± 0.03	0.81 ± 0.03
Right Hippocampus	0.87 ± 0.03	0.83 ± 0.03

Structure	Experimental group		Control group	
	Left	Right	Left	Right
(C) C.O. activity in selected brain regions				
CA1#	22.6 ± 0.6	22.9 ± 0.8	21.0 ± 0.9	20.3 ± 0.7
CA3#	21.6 ± 0.5	21.4 ± 0.6	19.3 ± 0.7	17.9 ± 0.5
DG	33.9 ± 0.7	33.4 ± 1.3	33.3 ± 1.1	32.1 ± 1.2
vCA1#	29.0 ± 1.3	29.5 ± 1.4	29.5 ± 1.4	25.5 ± 1.0
vCA3#	30.6 ± 1.3	29.6 ± 1.4	29.6 ± 1.4	26.4 ± 0.8
vDG#	27.2 ± 1.2	27.0 ± 2.0	27.0 ± 2.0	20.5 ± 0.4

# $p < 0.05$ as compared experimental to the control groups.
* $p < 0.05$ as compared experimental to the control group.

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it has been also considered that both hippocampus and striatum should be necessary in goal-directed navigation. This hypothesis is in agreement with our results since we found an increase in hippocampal C.O. activity after response learning. In addition, we have previously reported that the striatum is necessary in the performance of this kind of tasks (Fidalgo et al., 2011).

Electrophysiological data suggest that the hippocampus is involved in path integration or dead reckoning, the ability to use proprioceptive and vestibular information to keep track of changes in orientation and position without reference to external or allothetic cues (Taube, 1998; Jeffery and O'Keefe, 1999; McNaughton et al., 2006). An experiment using rats with lesions affecting the entire hippocampus in a radial maze under light and dark conditions strongly supports the crucial role of the hippocampus in the use of idiothetic or self-movement cues (Allen et al., 2007).

In particular, the dorsal hippocampus seems not to be only specifically involved in spatial learning but also in response learning because excitotoxic lesions of this region impair performance in a nonmatching-to-place task in a T-maze in the dark (Potvin et al., 2007). The hippocampus is part of a brain circuit involved in spatial orientation because it has cells that discharge as a function of the animal's head direction on the horizontal plane known as "head direction cells" and it also has efferent and afferent connections with the subiculum that projects in turn to all hippocampal fields and the dentate gyrus (Van Groen and Wyss, 1990). The hippocampus contains also "place cells" that fire selectively only in particular locations of the environment depending on vestibular inputs (Stackman et al., 2002). Therefore, it could be considered that the hippocampus may integrate signals from head direction cells and place cells necessary for path integration (Leutgeb et al., 2000). According to this, the hippocampus continually integrates sensory, movement and motivational information involved in response learning. However, no significant group differences were found in the dorsal dentate gyrus. In agreement with our results, although the dentate gyrus is critically involved in spatial behavior, it is mostly associated with the use of place strategies but not response strategies (Xavier and Costa, 2009).

In addition, increased C.O. activity was found all of the ventral hippocampal regions analyzed in the response learning group as compared to the control group. The ventral hippocampus is a brain region mostly linked to anxiety-related behaviors (Bannerman et al., 2004; Engin and Treit, 2007) and it has anatomical connections with the amygdala and the hypothalamus (Witter and Amaral, 2004). However, in our opinion, these results may not be related with anxiety because the control group was also submitted to similar experimental conditions during an equivalent time that could be also considered as a similar situation since no escape platform was available.

Alternatively, it seems more likely that the ventral hippocampus in memory processes. It has been suggested that spatial memory is generally encoded by a widely distributed hippocampal network (Moser and Moser, 1998) and that critical modifications could be distributed along the septotemporal axis. Several studies in rodents evaluating 2-deoxyglucose uptake or immediate early gene expression have shown activations of the ventral hippocampus during retrieval of recently acquired spatial memory tasks (Gusev et al., 2005; Maviel et al., 2004; Bontempi et al., 1999). Recently, it has been reported that temporal inactivation of the ventral hippocampus impairs spatial memory retrieval in rats (Loureiro et al., 2011). In this regard, our results support the participation of the ventral hippocampus in a response task, at least when the task has been probably acquired with the entire hippocampus.

Although some hippocampal memory functions in humans are lateralized, we found no differences between left and right hippocampus when we compared C.O. activity between experimental and control groups. One possible explanation for this discrepancy would be based on inter-species variability and differences in the type of memory task evaluated, since most studies in rodents reporting lateralization in hippocampal function are based on spatial memory tasks (Klur et al., 2009).

Finally, we found no significant differences in C.O. subunit I contents in neither the right nor the left hippocampus. Western blot analysis has been previously used to study the regulation of C.O. after KCl and tetrodotoxine treatment (Liang et al., 2006) caffeine administration (Jones et al., 2008) or ischemia (Racay et al., 2009). However, western blot analysis of C.O. subunit I has been applied in a single study evaluating memory associated with visual imprinting in chicks (Solomonia et al., 2011). The authors report in this study increased contents of C.O subunits I and II after imprinting, detected in the left intermediate medial mesopallium, a region specifically involved in visual imprinting in chicks. These results seem difficult to compare with ours, since differences between species, type of memory task and the brain region measured would not allow for appropriate comparisons. In our opinion, it is possible that the response learning process did not require significant changes in protein amount of C.O. subunit I. In addition, western blot analysis did not reveal which C.O. subunits were parts of functional C.O. holoenzyme or not. In this regard, it has been reported that decreased C.O. activity in neurons is related with decreased mRNA contents of C.O. mitochondrial subunits after treatment with tetrodotoxin (Hevner and Wong-Riley, 1993). However, subtle C.O. increases after learning do not necessarily imply increased C.O. subunit I contents, since C.O. holoenzyme assembly of existing nuclear and mitochondrial subunits after increased energy demands would be possibly required.

5. Conclusions

Our results support that both the dorsal and ventral hippocampus are associated with response learning although functional lateralization of hippocampal C.O. activity was not observed.

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V

Effect of lighting conditions on brain network complexity associated with response learning

Fidalgo C, Conejo NM, González-Pardo H and Arias JL

Abstract

Many studies report the involvement of the hippocampus and the striatum in response learning. However, the precise role of these structures together with anatomically related brain regions on response learning is still unclear. The aim of this study was to examine the contribution of different brain networks during response learning in a water T-maze under two different lighting conditions (light versus dark). A total of 40 male Wistar rats weighing between 150-250 g were used in this experiment. Animals swam in a water T-maze in a single trial during the habituation day and the following day they were trained in a response learning task. The lighting conditions had no effect on performance in this task and all subjects reached the learning criterion of 80% correct arm choices. Quantitative cytochrome oxidase (CO) histochemistry was used as a metabolic brain mapping technique since it can be considered as a reliable index of brain oxidative metabolism. Our results show that the ventral hippocampus and the parietal cortex are associated with the acquisition of a response learning task regardless of lighting conditions. In addition, when the task is run in the dark, a more widespread recruitment of structures involving cortical, limbic and striatal regions was found. Lastly, the hypothesis about independent and competing multiple memory systems for spatial learning is not supported by our findings.

INTRODUCTION

It is commonly held that brain regions functionally interact in large-scale brain networks involved in particular aspects of learning and memory. In addition, it could be possible that the recruitment of different brain regions in a common network occurs under particular learning conditions. In this regard, it has been established that a core functional network comprising cortical, limbic and striatal regions would be related with response or habit learning, a basic form of associative learning based on stimulus-response associations (Fidalgo et al., 2011). In this context, the striatum has been regarded as a key structure in response learning (Yin and Knowlton, 2006; Packard and Knowlton, 2002). In addition, lesions of the striatum are associated with performance deficits in these tasks (Pistell et al., 2009; DeCouteau and Kesner, 2000). Since the striatum a major input from the prefrontal cortex, this region has also been associated with response learning (Floresco et al., 2008, McAlonan and Brown 2003; Ragozzino et al., 1999).

Although the hippocampus has been traditionally related with the integration of spatial relationships between distal visual stimuli, this region together with other associated regions is also involved in the integration of self-motion cues, also known as path integration or dead reckoning (Sharp et al., 1995; McNaughton et al., 2006). Accordingly, rats with hippocampal lesions showed impaired performance in a water T-maze task run in the dark that involves the use of idiothetic or self-motion cues (Zheng et al., 2003). According to this, lesion studies reported the development of retrograde amnesia in habit learning tasks after hippocampal lesions (Sutherland et al., 2001; Driscoll et al., 2005; Epp et al., 2008). However, the dorsal part of the hippocampus has been more consistently related with the internal representation of the space because of the presence of the place cells that fire in particular locations of the environment (Jung

et al., 1994) although it has been recently reported that temporal inactivation of the ventral hippocampus also impairs spatial memory retrieval in rats (Loureiro et al., 2011).

Taken together, previous studies reported the involvement of the hippocampus and the striatum in response learning. In addition, growing evidence suggest that the hippocampus and the striatum are both recruited at least for particular spatial tasks requiring the use of allocentric or environmental cue-based strategies (Miyoshi et al., 2012). However, the role of the hippocampus and the striatum together with anatomically related brain regions on response learning processes is still unclear. The aim of this study was to evaluate the contribution of different brain regions during response learning in a water T-maze under two different lighting conditions (light versus dark). In this regard, our study investigated whether: 1) there is any effect of the lighting conditions on the mastery of a response learning task, and 2) if these conditions have any effects on the brain networks involved in the response learning task.

We used quantitative CO histochemistry as a metabolic brain mapping technique because it has a high anatomical resolution and it provides a measure of steady or sustained changes in oxidative metabolism associated with brain function (Wong-Riley, 1989). CO histochemistry has been successfully used in previous studies to map changes in brain oxidative metabolism involved in several learning tasks in rats (González-Pardo et al., 2012; Hu et al., 2006; Villarreal et al., 2002). In addition, the CO method also can be used to investigate the functional interactions between brain regions. In this regard, it has been previously demonstrated that brain regions that are functionally coupled show dynamical and coordinated changes in their metabolic capacity, expressed as changes in the strength of correlation in CO activity between regions (Puga et al., 2007; Conejo et al., 2010; Fidalgo et al., 2012).

EXPERIMENTAL PROCEDURE

Animals

A total of 40 male Wistar rats weighing between 150-250 g were used in this experiment. The animals were obtained from the University of Oviedo central vivarium (Oviedo Asturias Spain) and were randomly housed in groups of five under standard conditions (12-h light/dark cycle with lights on from 08:00–20:00 h) at constant room temperature of 21±2 °C with *ad libitum* access to food and water. The animals were randomly divided into two groups: experimental (n=20) and control (n=20). During behavioral procedures, half of the animals in each group (n=10 and n=10 from experimental and control groups, respectively) run the task under light or dark conditions respectively. All experiments were done in accordance with the European Communities 2010/63/UE and the Spanish legislation (RD 1201/2005). All efforts were made to minimize the number of animals used and their suffering.

Apparatus

Rats were trained in a water T-maze made of black fibre-glass filled with tap water (23±1 ºC). The main alley (100 × 20 × 40 cm) was connected to two side arms (right and left) measuring 45 × 20 × 40 cm. A black square escape platform made of Plexiglas (15 × 18 cm) was placed at the end of each arm 2 cm beneath the water surface. Two experiments were done by changing lighting conditions. Under dark conditions, the maze was located in a dark room illuminated by red lights. On the other hand, when the animals where trained under light conditions, the maze was surrounded by dark curtains and no visual cues were available.

Behavioral procedure

In order to discard possible motor and sensory deficits animals were tested in a neurological assessment battery. The neurological tests used include the following tests: abduction response of hindlimbs, grasping reflex, extension and flexion reflexes, hearing and vestibular responses, head shaking, pupillary reflex, negative geotactic response and righting reflex (according to Bures et al., 1976). No animals were discarded due to abnormal neurological responses. The spatial memory task was performed between 09:30 and 13:00 h. After daily handling during five days, animals were placed in a water T-maze for one-trial habituation. During the habituation day, rats were gently immersed in the water T-maze for 1 min without any escape platform available and then trained for response learning during the next day. Each animal received a single 12-trial session. The submerged platform was located in the same arm during the whole training period. To be sure that the animals were using the correct strategy, the last trial was run in a T-maze placed the other way around the one used previously. The learning criterion used for both experimental groups was eight or more correct choices out of 12 trials.

Two free-swimming groups (one for each experimental group) were composed of rats that were placed in the maze the same number of times and under the same lighting conditions as compared to the experimental groups but without escape platform available. Their daily swimming time was equivalent to the mean daily escape latencies recorded for the experimental groups.

Tissue preparation

After finishing the behavioral procedure the animals were decapitated. Their

brains were quickly removed frozen rapidly in isopentane at -70 ºC (Sigma–Aldrich Madrid Spain) and stored at -40 ºC to preserve the brain tissue and enzyme activity. Next 30 µm-thick coronal sections were obtained from the brain tissue using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany), and they were mounted on slides and stored at -40 ºC until processing with quantitative CO histochemistry. Some sections from a few subjects could not be used as a result of tissue processing although the final number of sections available for histochemistry was enough in all cases.

CO histochemistry

We used a modified version of the method originally described by Wong-Riley (1989) based on the quantitative CO histochemical method developed by Gonzalez-Lima and Cada (1994). To control staining variability across different baths sets of tissue homogenate standards from Wistar rat brain of known CO activity determined spectrophotometrically were cut at different thicknesses (10, 30, 50 and 70 µm) and included with each bath of slides. In brief slides were fixed for 5 min with a 0.5% glutaraldehyde solution rinsed three times in phosphate buffer and pre-incubated 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. Once 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 for 30 min at room temperature with 10% (w/v) sucrose and 4% (v/v) formalin. 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) composed of a high precision illuminator a digital camera and a computer with specific image analysis software. A total of twelve measurements of relative optical density were taken per region. In order to establish comparisons and consider possible staining variations between brain sections from different staining baths, measurements were also taken from CO-stained brain homogenate standards. Regression curves between section thickness and previously known CO activity as assessed spectrophotometrically were calculated for each set of standards included in the incubation baths. Finally, 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 23ºC) using the calculated regression curve in each homogenate standard. The selected brains regions anatomically were defined according to the Paxinos and Watson´s (1997) atlas. The regions of interest and the distance in mm of the regions counted from bregma was: +3.20 mm for the infralimbic cortex, prelimbic cortex, the cingulate cortex; +1.70 for parietal cortex, accumbens core, accumbens shell, medial septum, lateral septum, anterodorsal stritum and anteromedial striatum; -3.12 mm for the CA1, CA3 and the dentate gyrus subfields of the dorsal hippocampus and for the basolateral, lateral and central amygdale; -4.80 mm for lateral dorsal geniculat nucleus, ventral hippocampus (CA1, CA3 and dentate gyrus) and -6.30 mm for the primary visual cortex.

Statistical analysis

Data were analyzed by SigmaStat 3.2 software (Systat Software, Chicago, USA). Group differences in the number of correct arm choices were analyzed by a Mann-Whitney Rank sum test because data were not normally distributed. Differences in mean CO activity between the different groups for each brain region were analyzed using two-way ANOVA with main effects of group (experimental or control) and light condition (light or darkness). Post-hoc Tukey´s tests were performed in case of significant group×light condition interaction. A probability level of at least $P \leq 0.02$ was used as the criterion for statistical significance.

In order to evaluate changes in functional connectivity among brain regions regional CO activity data were analyzed in terms of pair-wise correlations within 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 all structures measured for each animal. This was done to reduce variation in the intensity of the CO staining not resulting from the experimental manipulation. In addition, in order to avoid errors due to an excessive number of significant correlations using small sample sizes we used a 'jackknife' procedure (Shao and Dongsheng, 1995) based on the calculation of all possible pairwise correlations resulting from removing one subject each time and taking into consideration only those correlations that remain significant ($p<0.02$) across all possible combinations.

RESULTS

Behavioral results

Analysis of the number of correct arm choices (Fig. 1) across training days made by both experimental groups showed no differences between groups (t=89.5; p= 0.1). All the animals reached the learning criterion on day 1.

Main brain CO activity

Mean regional CO activity measured in the different experimental groups is summarized in Table 1. Significant main effects of lighting conditions were found in nucleus accumbens shell ($F_{1,29}$=59.4; p<0.001) CA3 dorsal hippocampal area ($F_{1,31}$=11.8; p=0.002), lateral dorsal geniculate nucleus ($F_{1,30}$=29.2; p<0.001), ventral CA1 area ($F_{1,30}$=18.8; p<0.001), visual cortex ($F_{1,30}$=24.7; p<0.001) and anterodorsal striatum ($F_{1,31}$=14.1; p<0.001).

In addition, significant main effects of group were also found in nucleus accumbens shell ($F_{1,29}$=39.0; p<0.001), CA3 dorsal ($F_{1,31}$=15.5; p<0.001), central amygdale ($F_{1,30}$=15.6; p<0.001) and anterodorsal striatum ($F_{1,31}$=13.9; p<0.001).

Finally, highly significant group×light conditions interactions (p<0.02) were found in the following four brain regions: in prelimbic ($F_{1,31}$=30.14; p<0.001), infralimbic ($F_{1,31}$=25.14; p<0.001), cingulate ($F_{1,31}$=43.0; p<0.001) and parietal cortex ($F_{1,30}$=11.380; p<0.002). In addition, interactions between both factors have been observed in anteromedial striatum ($F_{1,31}$=21.4; p<0.001), accumbens core ($F_{1,30}$=8.0; p=0.008), dorsal CA1 ($F_{1,31}$=6.8; p=0.014), dentate gyrus ($F_{1,31}$=12.2; p<0.001), ventral CA3 ($F_{1,31}$=16.24; p<0.001) and ventral dentate gyrus ($F_{1,31}$=11.85; p=0.002).

Further analysis of the significant interactions by post hoc tests (Table 1) showed a significant effect of group under dark conditions in prelimbic cortex (p<0.001),

infralimbic cortex (p<0.001), cingulate cortex (p<0.001) parietal cortex (p<0.001) accumbens core (p<0.001), dorsal CA1 (p<0.001), dorsal CA3 (p<0.001), dorsal dentate gyrus (p<0.001), central amygdala nucleus (p<0.001) and medial amygdala nucleus (p<0.001) and dorsal striatum (p<0.001). In all cases, brain activity was significantly higher in experimental group compared to the free-swimming control group.

Moreover, significant main effects were observed after post hoc analysis between L-experimental and L-control group in the parietal cortex (p=0.016) the ventral CA3 (p=0.001) and the ventral dentate gyrus (p<0.001). Increased CO activity of the experimental group as compared to the control group was found in the parietal cortex and the ventral CA3 area. However, CO activity was significantly decreased in experimental group compared to the control one in the ventral dentate gyrus.

Interregional within-group correlations in CO activity

Interregional correlations in CO activity of the L-response group were found between ventral CA1 and CA3 hippocampus. On the other hand, the D-response group presents the same correlation between ventral CA1 and CA3 hippocampus but with additional correlations with ventral dentate gyrus and CA3. In addition, a correlation between anteromedial and anterodorsal striatum has been observed in this group. (See Figure 2).

DISCUSSION

The level of performance of rats in the response learning task was measured by the number of correct arm choices, and the learning criterion was set to 8 correct trials over 12. According to this learning criterion, all subjects in both experimental groups (L-

response and D-response groups) mastered the task after the first day of training. Moreover, we have previously observed that once the animals reach the learning criterion on the first day, a similar performance is maintained during the following training days (Fidalgo et al., 2011) a result indicating that animals successfully learned the task. On the other hand, when rats were tested under light conditions, the maze was surrounded by dark curtains and no distant visual cues were available. Since visual, olfactory and auditory cues were not relevant to solve the task, and the animals chose the correct arm when they were placed in the rotated T-maze, we assumed that they were using self-motion cues or a response strategy.

The analysis of CO activity after training in the response learning task showed a differential involvement of particular brain regions depending on lighting conditions. Although a learned sequence of fixed motor responses seems to be the strategy used to solve the task, the stimuli available under light and dark conditions are not the same. This is probably the reason why different brain regions are involved under light or dark conditions. In this regard, significant differences between L- response group and the yoked control group were found in parietal cortex and ventral hippocampus (CA3 and dentate gyrus) under light conditions. Our data also show an involvement of the parietal cortex in response learning in both dark and light conditions. Moreover, rats with parietal lesions present deficits in a task that requires the use of path integration under light conditions (Save and Poucet, 2009). has been reported that lesioned rats could not learn a response task under dark conditions (Save and Moghaddam, 1996). Accordingly, the parietal cortex sends projections to cortical areas such as prefrontal cortex (Musil and Olson 1988) and subcortical regions like the striatum (Kamishina et al., 2008) and it is functionally related to the hippocampus being part of a functional network (Save and Poucet, 2009). The parietal cortex could be thus related with response learning, as

related with its function in route planning towards a particular goal during spatial navigation (Calton and Taube, 2009).

In addition, the ventral hippocampus showed significant group differences in CO activity. In this context, it has been reported that several brain functions could be distributed through the hippocampus (Bannerman et al., 2004). Moreover, olfactory, proprioceptive and emotional cues could be processed by the ventral hippocampus (Moser and Moser, 1998; Pennartz, 2011). In our opinion, the increase of the activity in the ventral hippocampus may not be related with anxiety. In this regard, we found an involvement of the ventral hippocampus in both experimental groups as compared with the yoked controls that were submitted to similar experimental conditions during an equivalent period of time. Moreover, several studies in rodents evaluating 2-deoxyglucose uptake or immediate early gene expression reported activations of the ventral hippocampus during retrieval of recently acquired spatial memory tasks (Gusev et al., 2005; Maviel et al., 2004; Bontempi et al., 1999). In addition, temporal inactivation of the ventral hippocampus produces impairments in spatial memory retrieval in rats (Loureiro et al., 2011). Accordingly, functional correlation between ventral CA1 and ventral CA3 areas was only observed under light conditions, a result suggesting that CO activity of the ventral hippocampus could be related with mastery of the response learning task.

On the other hand, we observed under dark conditions a recruitment of many cerebral structures including the parietal cortex and the ventral hippocampus (CA1) that we have previously discussed. Furthermore, CO activity in accumbens core, prefrontal cortex, dorsal (CA1, CA3 and dentate gyrus), central amygdala, and anterodorsal and

anteromedial striatum was significant higher in the D-group compared to the yoked control.

Moreover, we found an involvement of the dorsal and ventral hippocampus during response learning under dark conditions. In this regard, Allen et al. (2007) showed that rats with lesions affecting the entire hippocampus are unable to learn a task where animals have to remember visited locations on a radial maze under dark conditions. It has been previously reported that the dorsal hippocampus may participate in spatiotemporal contexts defined by environmental and egocentric cues (McNaughton et al., 2006). The latter region together with the cerebellum also receives indirect visual input from the visual cortex (Karpova et al., 2010). Therefore, the results we obtained show that the lateral dorsal geniculate nucleus and the visual cortex show significant differential CO activity depending on lighting conditions, although training on the response learning task had no effect on CO activity of the latter regions. In addition, another study reported increased firing of neurons in dentate gyrus and CA1 areas of the dorsal hippocampus during a spatial navigation task under dark conditions (Gothard et al., 2001). In our opinion, both the learning process and the light conditions may be related with increased CO activity of the dorsal hippocampus.

It has been also reported that the nucleus accumbens constitute a system that integrates inputs from the amygdala, prefrontal cortex and hippocampus to generate motivational signals, also including the dorsal striatum. The dorsal striatum mediates stimulus-response learning, but also the nucleus accumbens nucleus might combine inputs from different brain regions to facilitate goal-directed behaviors (Pennartz et al., 2011). The ventral hippocampus together with the amygdala and the nucleus accumbens core could play an important role on the control of goal-directed behaviours, involving

their direct anatomical connections and dopamine content as previously suggested (Grace et al., 2007). Furthermore, we have previously reported a participation of prefrontal cortex, dorsal striatum and amygdala in a similar task performed under the same lighting conditions (Fidalgo et al., 2011).

According to our findings, both dorsal and ventral hippocampus are necessary to learn the task when it was run in the dark. In addition, interregional correlations of CO activity were found in the D-response group between dorsal and ventral hippocampus. Furthermore, a correlation involving subregions of the striatum has been also observed. In this regard, it has been recently demonstrated that both the dorsal hippocampus and the dorsal striatum are required to learn a cue-version (involving a stimulus-response learning) of the water maze (Miyoshi et al., 2012). Therefore, our results do not support the concept of independent multiple memory systems for spatial and stimulus-response learning (Lee et al., 2008; White and McDonald, 2002; Packard and McGaugh, 1992). In this regard, there is now scientific evidence supporting a general role of the hippocampus on episodic memory, involving the use of both allocentric and egocentric strategies to solve spatial learning tasks in rodents (Rondi-Reig et al., 2006) and humans (Iglói et al., 2010).

In summary, response learning would rely on a neural system comprising parietal cortex and hippocampus. In addition, when the task is run in the dark, a more complex network involving cortical, limbic and striatal regions is required for response learning.

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Table 1

	D-exp group	D-control group	L-exp group	L-control group
Cingulate cortex	32.5±0.6*	22.4±0.4	29.8±0.8	31.5±1.7
Prelimbic cortex	30.8±0.9*	21.0±0.7	28.5±1.0	29.8±1.6
Infralimbic cortex	30.0±0.8*	21.3±0.6	29.9±1.1	31.0±1.6
Parietal cortex	32.0±0.8*	23.6±0.7	33.3±0.8^{+}	30.3±0.9
Visual cortex[a]	29.9±1.1	30.5±0.7	26.0±0.7	25.7±0.5
N. accumbens core	36.5±1.5*	28.1±0.4	39.1±1.3	37.5±1.3
N. accumbens shell[ab]	39.7±1.3	31.6±0.5	46.2±1.2	41.2±1.1
Dorsal CA1 area	28.5±0.9*	22.8±1.0	23.8±0.7	22.7±0.7
Dorsal CA3 area[ab]	27.8±0.7*	22.5±1.0	22.9±0.8	21.4±0.8
Dorsal dentate gyrus	40.1±1.1*	32.4±0.8	33.3±1.1	32.5±0.7
Ventral CA1 area[a]	32.8±1.5*	32.1±1.5	29.2±0.4	30.1±0.7
Ventral CA3 area	33.6±1.5	31.4±2.2	30.6±0.4^{+}	24.8±0.9
Ventral dentate gyrus	29.6±1.3	28.7±1.6	24.9±1.9^{+}	32.2±0.8
Lateral amygdala n.	27.2±1.3	24.4±1.0	25.4±0.9	22.9±1.3
Basolateral amygdala n.	33.1±1.5	30.4±1.2	31.3±1.3	28.3±1.0
Central amygdala n. [b]	28.2±0.8*	23.6±1.0	25.8±0.6	23.2±1.3
Lateral geniculate n. [a]	32.3±1.0	30.9±1.1	27.7±0.8	24.0±1.1
Anterodorsal striatum[ab]	32.7±1.1*	26.4±0.6	34.9±1.6	32.8±1.1
Anteromedial striatum	35.5±0.9*	25.8±0.4	35.7±1.1	33.5±0.7

Table 1: Cytochrome oxidase activity units (mean ± S.E.M.; μmol cytochrome c oxidized/min/g tissue) measured in the selected brain regions. [a] represents significant main effects of lighting conditions. [b] represents significant main effects of group conditions. *****$p<0.02$ when experimental group is compared to the swim control group under dark conditions. $^{+}p<0.02$ when experimental group is compared to the swim control group under light conditions.

Figure 1

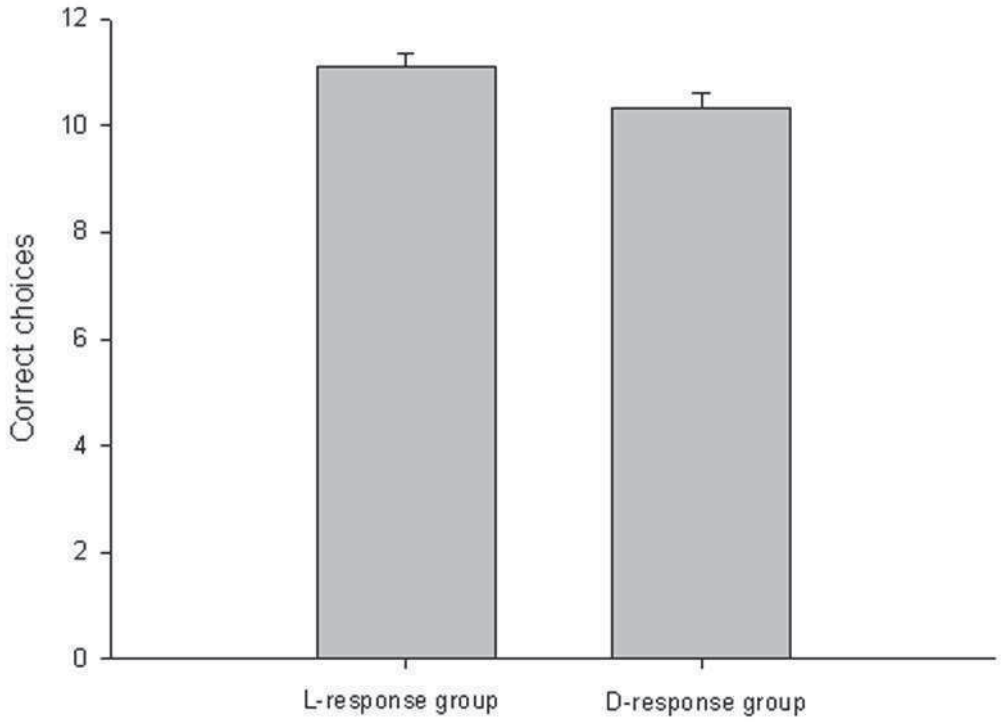

Figure 1: Number of correct choices across training days in the experimental group. All animals reached the learning criterion on the first training day.

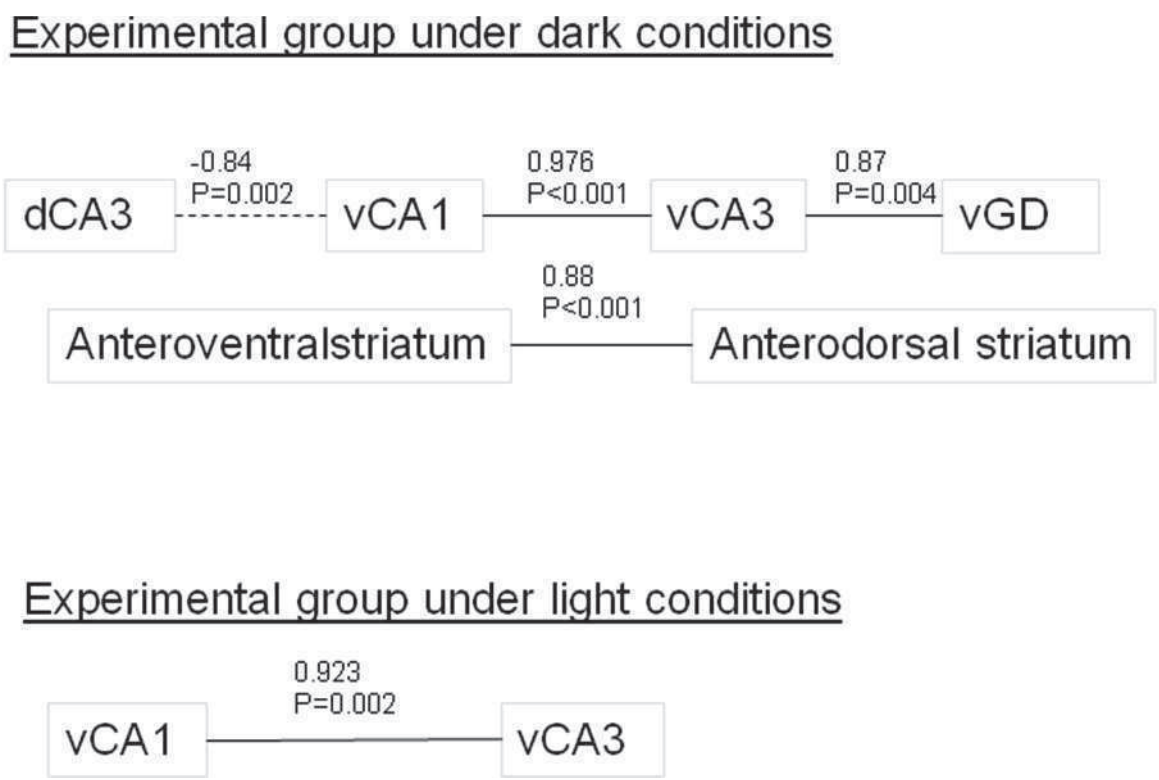

Figure 2: Schematic diagram showing significant interregional correlations of CO activity calculated in the experimental groups. Solid and dotted lines represent respectively highly positive and negative pair-wise Pearson's correlations (r>0.8, P<0.02).

UNIVERSIDAD DE OVIEDO
Departamento de Psicología

INFORME DEL FACTOR DE IMPACTO DE LAS PUBLICACIONES PRESENTADAS

La Tesis doctoral presentada por la Licenciada Camino Álvarez Fidalgo titulada: "Redes neuronales de la conducta espacial: uso de estrategias egocéntrica y de guía", se presenta como un compendio de publicaciones, cuyo factor de impacto es:

I. Fidalgo C, Conejo NM, González-Pardo H, Arias JL. Functional interaction between the dorsal hippocampus and the striatum in visual discrimination learning. 2012. **Journal of Neuroscience Research**. 90(3):715-20. DOI: 10.1002/jnr.22774. **Factor de impacto: 2.958**

II. Fidalgo C, Conejo NM, Gonzalez-Pardo H, Arias JL. Dynamic functional brain networks involved in simple visual discrimination learning. (manuscrito). En revisión en la revista **Neuroscience**. **Factor de impacto: 3.215**

III. Fidalgo C, Conejo NM, González-Pardo H, Arias JL. Cortico-limbic-striatal contribution after response and reversal learning: a metabolic mapping study. 2011. **Brain Research.** 12; 1368:143-50. **Factor de impacto: 2.623**

IV. Fidalgo C, Conejo NM, González-Pardo H, Lazo PS, Arias JL. A role for dorsal and ventral hippocampus in response learning. **Neuroscience Research**. 2012 DOI: 10.1016/j.neures.2012.03.011. **Factor de impacto: 2.096**

V. Fidalgo C, Conejo NM, Gonzalez-Pardo H, Arias JL. Effect of lighting conditions on brain network complexity associated with response learning. (Manuscrito). En revisión en la revista **Brain Structure and Function**. **Factor de impacto: 4.982**

En Oviedo a 3 de Mayo de 2012

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