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Programa de doctorado: PSICOLOGÍA

MECANISMOS CEREBRALES DE LA MEMORIA DE RECONOCIMIENTO GUSTATIVA EN ROEDORES: INTERACCIÓN ENTRE CIRCUITOS COGNITIVOS Y AFECTIVOS

ALEJANDRO BORJA GRAU PERALES

Tesis doctoral.

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"My goal is to disclose how the brain gains its smartness from the organized complexity of its constituents"

György Buszáki.

ÍNDICE

Resumen

La presente Tesis Doctoral está formada por 9 capítulos agrupados en los que 5 de ellos se corresponden con las publicaciones que componen el cuerpo del trabajo de investigación.

El primer capítulo consiste en una introducción a la memoria gustativa, centrándose en la atenuación de neofobia como modelo de memoria gustativa segura con especial interés en la descripción de los circuitos cerebrales y mecanismos neurobiológicos que la sustentan. Se describe el sistema dopaminérgico mesocortocolímbico y se revisan los conocimentos actuales sobre su relación con la memoria gustativa. Por último, se presenta una introducción a los cambios que se producen en la memoria gustativa a lo largo del ciclo vital, especialmente en referencia al envejecimiento no patológico.

En el segundo capítulo, se plantea la investigación en función de los conocimientos actuales en torno a la participación del sistema dopaminérgico mesocorticolímibico e hipocampal en la atenuación de neofobia gustativa y su modulación por el contexto. Se incluye una lista detallada de objetivos de investigación que han dirigido la presente tesis doctoral.

A continuación, se presentan los cinco capítulos correspondientes a cada una de las publicaciones científicas que componen el cuerpo de

investigación de la Tesis Doctoral. Tres de ellos se encuentran publicados en revistas JCR Q1 y Q2 (capítulos 3, 4 y 6), mientras que los dos restantes actualmente han sido enviados para su publicación y se encuentran en proceso de revisión:

En el Experimento 1 (Capítulo 3) se exploró el papel que desempeña el sistema olfativo en la atenuación de neofobia gustativa. Se evaluó la actividad de la corteza piriforme, que conforma la corteza olfativa secundaria en el cerebro de los roedores, mediante la identificación inmunohistoquímica de la proteína c-Fos. Para ello, se emplearon grupos de ratas macho adultas expuestos a un sabor novedoso de vinagre una, dos o seis veces. La región rostral de la corteza piriforme posterior mostró un incremento de actividad durante la sexta exposición al sabor de vinagre, indicando su implicación en el proceso de familiarización del sabor. Además, se exploró el papel del envejecimiento no patológico, encontrándose una hiperexcitabilidad de esta región pero no de la región anterior sensorial en ratas macho de 24 meses de edad. Ello sugiere dificultad en el *reconocimiento* del sabor como familiar, lo que concuerda con un retraso de la atenuación de neofobia en el caso de ratas envejecidas.

En el Experimento 2 (Capítulo 4) se exploró el papel que desempeña el circuito dopaminérgico mesocorticolímbico en la atenuación de neofobia gustativa. Para ello, se utilizó la misma aproximación que en la serie anterior, cuantificando la proteína c-Fos en el núcleo accumbens de ratas

macho adultas expuestas a un sabor novedoso una, dos o seis veces. Se encontró un incremento en el número de células c-Fos positivas en la región shell del núcleo accumbens de animales expuestos al sabor novedoso por segunda vez, coincidiendo con el incremento en consumo que define operacionalmente la atenuación de neofobia gustativa. También se exploró el papel del envejecimiento no patológico, encontrándose una disrupción del patrón de actividad c-Fos, cuyo incremento se pospone hasta la sexta exposición al sabor en ratas de 24 meses de edad.

En la Serie Experimental 3 (Capítulo 5) se profundizó en el análisis de la participación del núcleo accumbens en la atenuación de neofobia gustativa a través de una serie de experimentos que combinan el análisis de la conducta con técnicas de intervención farmacológica (depleción catecolaminérgica, administración de agonistas y antagonistas de receptores D1 de dopamina) y farmacogenética mediante DREADDs que permiten el control de la actividad de vías anatómicas selectivas. Los resultados indicaron una relación inhibitoria entre la actividad de la amígdala basolateral y la región shell del núcleo accumbens. Se observó que la activación dopaminérgica del núcleo accumbens shell a través de los receptores dopaminérgicos de tipo D1 es necesaria para que se produzca un incremento en el consumo en la segunda exposición al sabor, es decir, atenuación de neofobia. Dicha actividad inhibe la actividad de la amígdala basolateral relacionada fundamentalmente con la respuesta neofóbica al

sabor. Así, en ausencia de la inhibición de la amígdala, se mantiene la respuesta neofóbica. Por el contrario, la activación de la amígdala basolateral se asocia al desarrollo de una aversión condicionada al sabor, de manera que los animales evitan el consumo del sabor en sucesivos encuentros.

En el Experimento 4 (Capítulo 6) se investigó la relación entre la atenuación de la neofobia gustativa y su modulación por el contexto en el que se produce la formación de la memoria gustativa segura. Los resultados mostraron que la atenuación de neofobia gustativa es dependiente del contexto no espacial auditivo empleando como contexto el sonido ambiente, de manera que un cambio de contexto interfiere con la atenuación de la neofobia. Este fenómeno requiere la integridad de la región CA1 del hipocampo, ya que las lesiones excitotóxicas mediante NMDA en dicha región eliminan la modulación contextual de la atenuación de neofobia gustativa.

En la Serie Experimental 5 (Capítulo 7) se continuó el estudio de los mecanismos cerebrales de la dependencia contextual de atenuación de la neofobia al sabor. La intervención farmacológica del sistema hipocampal y farmacogenética de las conexiones entre el hipocampo y el núcleo accumbens indicó que la modulación contextual de la atenuación de neofobia se produce mediante la actividad de circuitos dopaminérgicos en la región de CA1 ventral del hipocampo mediados por receptores D1,

siendo responsable de dicha actividad la vía accumbens-hipocampo en las primeras fases de familiarización con el sabor pero no cuando el proceso de atenuación de la neofobia ya ha tenido lugar.

Por último, el Capítulo 8 incluye una discusión general de los resultados obtenidos en cada una de las cinco publicaciones correspondientes a cada serie experimental y en el Capítulo 9 se enumeran las principales conclusiones de forma breve y concisa.

Capítulo 1

Introducción

1.1. La memoria gustativa.

La memoria gustativa es un buen modelo para estudiar los circuitos neurales y mecanismos moleculares relacionados con aprendizaje y memoria. Ser capaces de recordar y reconocer un sabor tiene gran relevancia desde el punto de vista biológico, puesto que esta información permite aprender sobre la naturaleza de lo ingerido y sus consecuencias sobre el organismo. Ello determina la aceptación o el rechazo de estas sustancias en encuentros futuros y explica en buena parte la selección de la dieta. En efecto, la presentación de sustancias con sabor novedoso desencadena una respuesta inicial de rechazo que se denomina neofobia al sabor. El rechazo o la aceptación del sabor una vez que se convierte en familiar dependen de las consecuencias viscerales que se asocien con él.

Así, tras la ingestión de un sabor novedoso se genera una memoria de dicho sabor que parece procesarse y almacenarse en paralelo en diferentes regiones cerebrales (Federico Bermúdez-Rattoni, 2004). La memoria gustativa generada posee al menos dos componentes, dependiendo de las consecuencias viscerales posteriores que sean atribuidas al sabor. De esta forma, si el sabor es seguido de consecuencias viscerales negativas, será clasificado como aversivo y se evitará su consumo en futuros encuentros. Este tipo de aprendizaje es denominado aversión gustativa condicionada y puede ser inducido experimentalmente en el laboratorio. Se considera un tipo de condicionamiento clásico en el

que se asocian un estímulo condicionado (EC) y un estímulo incondicionado (EI), siendo el EC el sabor y el EI malestar gástrico, que generalmente es inducido en roedores mediante una inyección intraperitoneal de Cloruro de Litio (LiCl). Sin embargo, en el caso de que el sabor novedoso no vaya seguido de consecuencias viscerales aversivas, se genera una memoria gustativa segura, de manera que cuando el animal vuelva a encontrar dicho sabor en el futuro, pueda ser reconocido como seguro e incremente su consumo, como resultado de la atenuación de la neofobia gustativa. Ambos tipos de memoria gustativa –aversiva y segura- han demostrado ser procesadas por mecanismos cerebrales compartidos, aunque algunos aspectos parecen ser específicos para cada una de ellas.

Adicionalmente, la asociación del sabor con consecuencias apetitivas, bien de naturaleza hedónica o nutritiva, induce la formación de memorias apetitivas que conducen al desarrollo de preferencias gustativas condicionadas. En el primer caso, el denominado aprendizaje sabor-sabor, un sabor neutro es emparejado con un sabor muy agradable al paladar (altamente preferido), mientras que en el segundo, denominado aprendizaje sabor-nutriente, el sabor neutro es emparejado con un nutriente, tal como glucosa empleando animales privados de comida. Se trata en ambos casos modelos de aprendizaje de preferencias gustativas, en los que se desarrolla un consumo elevado de una sustancia sápida que ha sido asociada con consecuencias reforzantes (Sclafani, Touzani, & Bodnar, 2011).

1.2. Memoria gustativa y envejecimiento:

A pesar de que los cambios en la ejecución de tareas de aprendizaje y memoria que se producen durante el envejecimiento no patológico son descritos fundamentalmente en términos de decaimiento funcional, esto no parece aplicable a la memoria de sabores, la cual parece permanecer preservada – o incluso potenciada – en el caso de ratas envejecidas (Gámiz & Gallo, 2011). Ello contrasta con los problemas de identificación y reconocimiento de olores asociados a edades avanzadas y que representan los signos más tempranos de trastornos neurodegenerativos tales como enfermedad de Parkinson y Alzheimer(Aliani et al., 2013). El decaimiento progresivo tradicionalmente asociado al envejecimiento se ha relacionado con cambios en la organización funcional y la conectividad de neuronas en el sistema hippocampal (Manrique, Morón, Ballesteros, Guerrero, & Gallo, 2007). Así, Dardou et al. (2008)compararon la ejecución entre ratas jóvenes y envejecidas en una serie de tareas de memoria que se focalizaban en diferentes aspectos incluyendo una tarea de reconocimiento de objetos, una tarea de discriminación de olores, una tarea espacial de búsqueda de recompensa en hoyos (hoaming board spatial task) y una tarea de potenciación de la aversión al olor por el sabor. Encontraron que las ratas envejecidas mostraban peor ejecución en comparación con las jóvenes sólo en las tareas espaciales, sugiriendo que las memorias no dependientes del

hipocampo pueden mantenerse relativamente intactas durante el envejecimiento.

Efectivamente, las ratas envejecidas continúan mostrando una respuesta neofóbica ante la presentación de un sabor novedoso. Se ha señalado que la respuesta neofóbica en ratas envejecidas puede estar incluso aumentada, especialmente cuando se han sufrido experiencias aversivas previas con otros sabores. De hecho, tanto ratas adultas como envejecidas con experiencias previas de sabores seguros muestran una reducción similar en la respuesta neofóbica ante un sabor desconocido. Sin embargo, las ratas de edad avanzada son mucho más sensibles que las adultas a los efectos de haber sido sometidas a aversiones gustativas condicionadas previas mostrando respuestas neofóbicas aumentadas(Morón & Gallo, 2007a).

En el paradigma de la aversión condicionada de sabores, los roedores envejecidos muestran una respuesta aversiva muy intensa al sabor condicionado, además de soportar un intervalo mayor que los jóvenes entre el EC y el EI durante la adquisición,así como exhibir una extinción más lenta de la respuesta condicionada al EC. Se han propuesto múltiples explicaciones basadas en el enlentecimiento en la actividad metabólica, el procesamiento alterado de la información gustativa y la importancia de experiencia previa con aprendizaje de sabores (Gámiz & Gallo, 2011;

Manrique, Gámiz, Morón, Ballesteros, & Gallo, 2009; Manrique, Morón, et al., 2009; Manrique et al., 2007).

La atenuación de neofobia también parece verse afectada por el envejecimiento no patológico, de manera que las ratas envejecidas requieren más exposiciones que ratas adultas más jóvenes(Gómez-Chacón, Morillas, & Gallo, 2015). La interpretación de este retraso en la atenuación de neofobia a edades avanzadas es controvertida. Se ha planteado como un déficit de memoria, es decir, incapacidad de recordar el sabor previamente encontrado y sus consecuencias seguras, o bien como el resultado de una respuesta neofóbica exacerbada que requiere más presentaciones seguras para habituarse. Sin embargo, esta última explicación no puede aplicarse en los casos en los que se emplean animales naÏve sin experiencia previa con otros sabores ya que los resultados previos indican que la edad, en ausencia de experiencias previas con sabores, no modifica la respuesta neofóbica (Gámiz and Gallo, 2011; Morón and Gallo, 2007).

En conjunto, la evidencia sugiere que las experiencias de aprendizaje de sabores son procesadas de manera diferente a lo largo de la vida. Ello es congruente con la relevancia del aprendizaje gustativo para la regulación de la dieta y el hecho de que condiciones fisiológicas y necesidades nutricionales sufran modificaciones asociadas a la edad. Sin embargo, aún se conoce poco sobre los mecanismos neurales subyacentes relacionados con memoria de sabores en ratas envejecidas. En este sentido, únicamente

se ha descrito un patrón de actividad diferente en la corteza perirrinal durante la habituación de la neofobia gustativa en ratas adultas y envejecidas empleando determinación inmunohistoquímica de la proteína c-Fos (Gómez-Chacón et al., 2015). Sin embargo, no existen datos sobre los cambios que pueden estar ocurriendo en otras estructuras del mismo circuito.

1.3. Circuitos cerebrales de la memoria gustativa.

La aversión gustativa condicionada depende de un circuito neural muy bien estudiado (**Figura 1A**) [para una revisión Yamamoto et al., 1994; Bures et al., 1998]. La formación de la asociación requiere la participación del núcleo parabraquial troncoencefálico (Ivanova & Bures, 1990) y su interacción con estructuras diencefálicas, tal como la amígdala basolateral, y corticales, tal como la corteza insular gustativa (Gallo & Bures, 1991). El mantenimiento de la memoria gustativa aversiva parece depender tanto de la corteza insular como de la amígdala entre otras áreas (Yamamoto, 1993).

Por otra parte, la evidencia actual indica que la función del hipocampo es esencial en fenómenos complejos de aprendizaje de sabores como puede ser el fenómeno de bloqueo (Gallo & Cándido, 1995; Moron, Ballesteros, Candido, & Gallo, 2002), y en la información acerca del contexto en el que se produce el aprendizaje (Gallo and Cándido, 1995a, 1995b; Gallo et al., 1999). Las lesiones electrolíticas del hipocampo dorsal

impiden tanto el aprendizaje aversivo asociado al contexto como el efecto del bloqueo del contexto en el aprendizaje aversivo (Aguado, Hall, Harrington, & Symonds, 1998). La dependencia del contexto de la extinción de la aversión condicionada también se ve afectada por lesiones electrolíticas del hipocampo dorsal (Fujiwara et al., 2012), así como la propia extinción *per se*(Garcia-Delatorre, Rodríguez-Ortiz, Balderas, & Bermúdez-Rattoni, 2010), que se considera dependiente de contexto. La dependencia del contexto temporal de la inhibición latente empleando aversión condicionada también es hipocampo-dependiente(Manrique, Gámiz, et al., 2009; Molero et al., 2005).

En cuanto a la formación de memoria gustativa segura, parece requerir la participación de un circuito neural que incluye junto ala amígdala basolateral y la corteza insular,areasdel lóbulo temporal, tales como la corteza perirrinal y el hipocampo. La amígdala basolateral ha sido relacionada tanto con la neofobia como con su atenuación. Su lesión impide la atenuación de neofobia al sabor y modifica la actividad de la corteza perirrinal relacionada con la familiaridad del sabor (Gómez-Chacón, Gámiz, & Gallo, 2012). Del mismo modo, la lesión excitotóxica de la corteza insular impide la atenuación de neofobia. Su actividad, medida a través de la proteína c-Fos, también varía con la familiaridad del sabor (Bahar, Dudai, & Ahissar, 2004; Bermudez-Rattoni, 2014; Lin, Roman, Arthurs, & Reilly, 2012; Miranda, Ferreira, Ramírez-Lugo, &

Bermúdez-Rattoni, 2003; Moraga-Amaro, Cortés-Rojas, Simon, & Stehberg, 2014).

Dentro de este circuito merece especial atención la corteza perirrinal que muestra una mayor actividad de la proteína c-Fos ante la presentación de sabores novedosos que ante familiares (Gómez-Chacón et al., 2015; Gutiérrez, De la Cruz, Rodriguez-Ortiz, & Bermudez-Rattoni, 2004). Tanto su inactivación temporal mediante anisomicina(De la Cruz, Rodriguez-Ortiz, Balderas, & Bermudez-Rattoni, 2008) como la lesión neurotóxica permanente(Morillas, Gómez-Chacón, & Gallo, 2017) inducen déficits similares en la atenuación de neofobia y en la memoria de reconocimiento de objetos. Ello apoya la existencia de circuitos neurales temporales compartidos entre la memoria de reconocimiento de distintas modalidades sensoriales (Morillas et al., 2017; Warburton et al., 2010; 2015).

Por su parte, el hipocampoposee múltiples conexiones anatómicas y funcionales bi-direccionales con la amígdala, la corteza insular y la corteza perirrinal, además del estriado ventral que incluye el núcleo accumbens (Balderas, Morin, Rodriguez-Ortiz, &Bermudez-Rattoni, 2012; Garcia-Delatorre et al., 2010; Miranda et al., 2003). En el caso de la memoria gustativa segura, la evidencia es contradictoria y escasa sobre su participación en la atenuación de la neofobia (De la Cruz et al., 2008). Sin embargo, permanece inexplorada la posibilidad de que el hipocampo cumpla un papel importante en su modulación contextual. Efectivamente,

existe evidencia que muestra una modulación contextual de la atenuación de neofobia, consistente en el enlentecimiento de la atenuación de neofobia cuando un sabor se presenta en un contexto físico novedoso (una jaula nueva) en comparación con un contexto físico familiar, tal como su jaula hogar(De la Casa, Cárcel, Ruiz-Salas, Vicente, & Mena, 2018).La única evidencia sobre sus mecanismos neurales apuntan al sistema dopaminérgico, pues dicha modulación desaparece cuando se administra un antagonista de receptores dopaminérgicos D1 (SCH-23390) (De la Casa & Díaz, 2013; Quintero et al., 2011).

Dado que el fenómeno de atenuación de la neofobia implica un cambio hedónico por el cual el sabor familiar se convierte en seguro y aceptable, incrementando su ingestión, parece relevante investigar la posible participación de circuitos cerebrales de recompensa compartidos con otros fenómenos de aprendizaje gustativo apetitivo, tal como las preferencias condicionadas al sabor. Como es bien conocido, los circuitos de neurotransmisión del sistema de recompensa dependen del sistema dopaminérgico mesocorticolímbico (**Figura 1B**) en el que el núcleo accumbens juega un papel esencial en relación con preferencias gustativas aprendidas(Pedroza-Llinás, Ramírez-Lugo, Guzmán-Ramos, Zavala-Vega, & Bermúdez-Rattoni, 2009; Ramírez-Lugo, Núñez-Jaramillo, & Bermúdez-Rattoni, 2007). El sistema dopaminérgico mesocorticolímbico comparte,además, conexiones anatómicas y funcionales con estructuras del

circuito de procesamiento de sabores lo que facilita el procesamiento de cambios inducidos por las consecuencias seguras o apetitivas de la ingestión (**Figura 1**C).

1.4. Núcleo accumbens y procesamiento del valor hedónico del sabor.

El núcleo accumbens (NAcb) es una estructura que forma parte del estriado ventral localizada en la parte rostral y medial del prosencéfalo que en algunas nomenclaturas es incluido en la denominada amígdala extendida. Recibe aferencias dopaminérgicas del circuito meso-límbico originado en el área tegmental ventral (ATV) y representa un componente fundamental del denominado sistema de recompensa o reforzamiento. En él se pueden encontrar dos subregiones: el núcleo accumbens core (NAcb-Core) y el núcleo accumbens shell (NAcb-Shell), subregiones que pueden disociarse anatómica y funcionalmente. El NAcb está ampliamente implicado en el desarrollo de conductas adictivas, en el control de la ingesta, así como en el control de aprendizajes motivacionales innatos y aprendidos (Bassareo & Di Chiara, 1999; Bassareo, De Luca, & Di Chiara, 2002; Yamamoto, 2006).

El NAcb además parece estar implicado en el control del consumo en función del sabor de los comestibles. Así, estudios muestran que la lesión del área tegmental ventral (ATV), la principal fuente de dopamina del sistema meso-cortico-límbico y cuyas aferencias alcanzan

fundamentalmente al NAcb, redujo el consumo de sacarosa, siendo el sabor dulce innatamente preferido, frente a sabores salados, amargos y ácidos en concentraciones no preferidas (cloruro sódico, clorhidrato de quinina y ácido hidroclórico). Ello pone de manifiesto el posible papel del circuito dopaminérgico en el procesamiento del valor hedónico (palatabilidad) de los sabores (Shimura, Kamada, & Yamamoto, 2002). Más tarde, Yamamoto(2006) señaló la implicación del NAcb en el procesamiento de la palatabilidad, ya que la administración intracerebral de agonistas dopaminérgicos en este núcleo producía un incremento en el consumo de sabores menos agradables al paladar.

En esta línea, estudios que registran niveles de dopamina mediante microdiálisis, muestran un incremento significativo en la liberación de dopamina tanto en el NAcb como en la corteza prefrontal (CPF) de ratas expuestas a un alimento con un sabor muy palatable (altamente preferido). Esta respuesta desaparecía en el NAcb tras una segunda exposición a dicho sabor, mientras que se mantenía elevada en la CPF (Bassareo & Di Chiara, 1997). De esta manera, se propone que el núcleo accumbens podría participar en el cambio del valor hedónico específico de un sabor, a través de conexiones bidireccionales con otros núcleos cerebrales, fundamentalmente la amígdala basolateral (BLA), corteza insular y otros núcleos de la amígdala extendida.

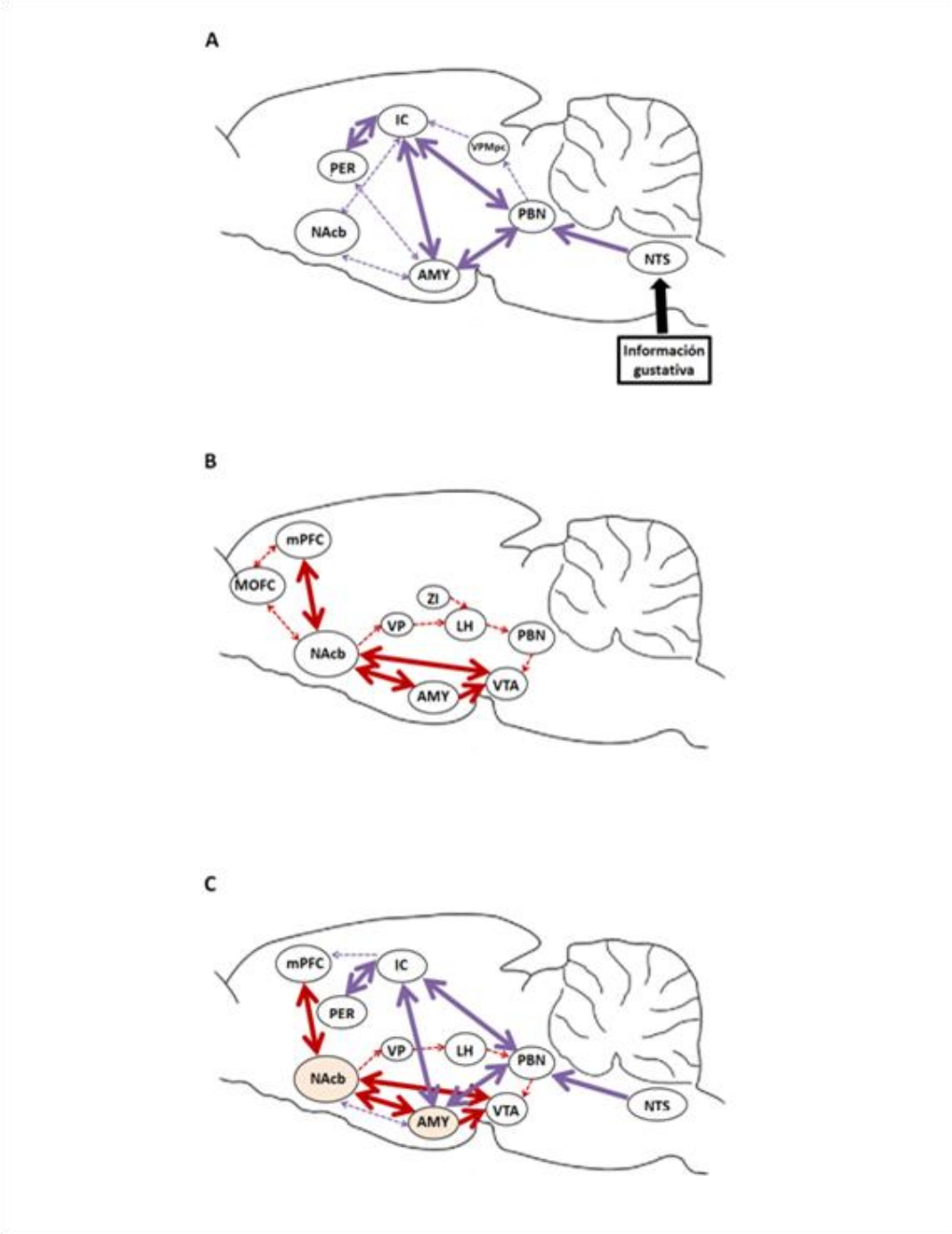

Figura 1. Representación esquematizada de las principales vías anatómicas implicadas en el procesamiento del sabor y la recompensa, así como su interacción. **A)**Conexiones del sistema gustativo en el cerebro de la rata. La información gustativa periférica alcanza el tronco cerebral a través del núcleo del tracto solitario (NTS), desde donde se envía al núcleo parabraquial (PBN). PBN proyecta a distintos núcleos de la amígdala (AMY) y a la corteza insular (CI) directamente o a través de su relevo talámico en el núcleo ventroposteromedial - pars compacta (VPMpc). Desde la IC, existen proyecciones eferentes sobre otras áreas corticales de asociación, destacando la corteza perirrinal (PER). Tanto AMY como IC mantienen conexiones anatómicas recíprocas entre sí y con el núcleo accumbens (NAcb). **B)** Conexiones

del circuito dopaminérgicomesocorticolímbico en el cerebro de la rata. La principal fuente de dopamina se corresponde con el área tegmental ventral (VTA) que surte de este neurotransmisor al NAcb. Desde allí, las proyecciones dopaminérgicas alcanzan la corteza orbitofrontal medial (MOFC) y mPFC así como núcleos subcorticales como AMY. El NAcb además proyecta hacia el pálido ventral (VP), núcleo que junto con la zona incerta (ZI) regulan la liberación de dopamina en el hipotálamo lateral (LH). LH proyecta información al PBN que regula la liberación de dopamina en el VTA, a modo de feedback negativo del circuito.**C)** Interacciones entre ambos circuitos, destacando zonas relevantes para la memoria gustativa como AMY, PBN y NAcb.

1.5. Núcleo accumbens y formación de la memoria gustativa.

Los distintos tipos de memoria gustativa descritos (aversiva, segura o apetitiva) inducen alteraciones en el consumo de los sabores debido a cambios en su palatabilidad. Por ello, no es descartable que el sistema de recompensa esté implicado en cada uno de ellos. Uno de los aspectos más estudiados ha sido la participación selectiva de las subregiones del núcleo accumbens tanto en sabores seguros como aversivos. En conjunto, los hallazgos sugieren que ambas subregiones (NAcb-Core y NAcb-Shell) estarían implicadas en la formación de una memoria gustativa aversiva, pero solamente la región del shell (NAcb-Shell) se relacionaría con la formación de un sabor seguro (**Tabla 1** en **ANEXO)**.

Así, se ha estudiado el papel de los neurotransmisores glutamato y acetilcolina en el NAcb utilizando una tarea de atenuación de neofobia y una tarea de aversión condicionada al sabor, respectivamente. En ambas tareas, administraron AP-5 (dl-2-amino-5 Phosphonopentanoate, antagonista selectivo de receptores de N-methyl-d-aspartate, NMDA) y

escopolamina (antagonista no selectivo de receptores colinérgicos muscarínicos) antes de la primera exposición a un sabor novedoso (sacarina). Tanto la inyección de escopolamina como de AP-5 impidieron la adquisición de una aversión condicionada al sabor cuando fueron administradas en ambas regiones (NAcb-Core y NAcb-Shell). Sin embargo, la atenuación de la neofobia gustativa sólo se vio interrumpida por la administración de escopolamina (pero no AP-5) administrada en el núcleo accumbens shell, mientras no obtuvo efecto en el core (Ramírez-Lugo, Zavala-Vega, & Bermúdez-Rattoni, 2006). Asimismo, se ha demostrado que la inducción de actividad glutamatérgica mediante NMDA aplicada tras la primera exposición a un sabor novedoso impidió tanto la recuperación de una aversión condicionada al sabor como la atenuación de neofobia al sabor (Núñez-Jaramillo, Rangel-Hernández, Burgueño-Zúñiga, & Miranda, 2012). Adicionalmente, se ha estudiado el efecto de la inactivación reversible del NAcb-Shell mediante la administración intracerebral de anisomicina, resultando en una interrupción de la atenuación de la neofobia al sabor (Pedroza-Llinás et al., 2009).

Otros sistemas de neuromodulación participan en la adquisición de ambos tipos de memoria de sabores. En este sentido, la administración de propanolol (antagonista de β-adrenorreceptores) en el núcleo accumbens core y shell interfirió con la atenuación de neofobia a una solución de sacarina y con la adquisición de una aversión condicionada cuando la

sacarina fue emparejada con una administración de LiCl. Sin embargo, su administración en el NAcb-Core únicamente produjo efecto sobre la aversión condicionada. Además, también demostraron que la administración de RU28362 (un agonista de receptores de glucocorticoides de tipo GR) en el NAcb produjo los efectos opuestos (facilitación tanto de la aversión condicionada como de la atenuación de neofobia), indicando una gran relevancia del sistema noradrenérgico en la consolidación de memoria de sabor aversiva y segura en el núcleo accumbens (Wichmann, Fornari, & Roozendaal, 2012).

1.6. Dopamina y memoria gustativa.

De acuerdo con la relevancia del circuito dopaminérgico mesocorticolímbico en el procesamiento del valor hedónico de los sabores, la formación tanto de aversiones como de preferencias gustativas parece estar estrechamente relacionada con la neurotransmisión en este circuito. En cambio, su posible participación en la atenuación de neofobia gustativa permanece inexplorada.

En primer lugar, la implicación de la neurotransmisión dopaminérgica en NAcb-Core ha mostrado ser relevante para la adquisición de la aversión gustativa condicionada. Fenu et al.(2001)expusieron ratas a una solución novedosa de sacarosa (15%) seguida por la administración de LiCl. Durante la dilación entre la exposición al sabor y la inyección de LiCl

inyectaron sistémicamente e intracerebralmente SCH-23390 y SCH-39166 (antagonistas selectivos de receptores D1 de dopamina) o vehículo. Los resultados indicaron que la administración de los antagonistas dopaminérgicos impidió la formación de una memoria gustativa aversiva. Esta ausencia de aversión condicionada fue especialmente evidente para el caso de administraciones intracerebrales realizadas en el NAcb-Core (Fenu et al., 2001).

Posteriormente, se replicaron los resultados anteriores en un experimento con administración intracerebral de anfetamina (como agonista dopaminérgico con gran afinidad por receptores de tipo D1) en el intervalo entre el sabor y el LiCl. Los resultados mostraron que la administración de anfetamina produjo facilitación de la adquisición de la aversión gustativa condicionada. Con este experimento,se demuestra que la actividad de los receptores dopaminérgicos D1 en el núcleo accumbens es necesaria para la adquisición de una memoria gustativa aversiva (Fenu & Di Chiara, 2003).

En segundo lugar, la dopamina y los receptores D1 especialmente, han obtenido una considerable atención en el estudio de la formación de la memoria gustativa apetitiva, bien sea a través de una asociación sabor-sabor o sabor-nutriente. Tanto en el aprendizaje sabor-sabor como sabor-nutriente la actividad de los receptores dopaminérgicos D1 parece jugar un papel crucial (Bodnar, 2018a; Sclafani et al., 2011). Estudios

farmacológicos han demostrado que la actividad de los receptores D1 implicados en el procesamiento de preferencias de sabores interacciona con la actividad glutamatérgica, a través de receptores de NMDA en el NAcb (Touzani, Bodnar, & Sclafani, 2008). Algo similar ocurre en otros núcleos incluidos en el sistema del reforzamiento, como son núcleos de la amígdala, fundamentalmente la basolateral y central (Touzani, Bodnar, & Sclafani, 2013) y el hipotálamo lateral (Amador et al., 2014; Touzani, Bodnar, & Sclafani, 2009) que participan directamente en el control de la ingesta de sabores muy palatables. La co-localización post-sináptica de receptores D1 y NMDA también se produce en zonas del estriado ventral, incluyendo el núcleo accumbens (Tarazi & Baldessarini, 1999).

Capítulo 2

Planteamiento de la investigación: objetivos.

Las tareas de memoria de reconocimiento de sabores suelen emplear solucionesgustativas con diversa contribución del componente olfativo, incluso cuando se emplean sabores básicos. Sin embargo, los circuitos cerebrales identificados hasta el momento solo incluyen áreas que forman parte del sistema sensorial gustativosin que se haya explorado la posible participación del sistema olfativo. Parece necesario emplear soluciones con sabores que posean componentes gustativos y olfativos con el fin de explorar esta posibilidad.

Por otro lado, el envejecimiento y los procesos patológicos asociados a la edad avanzada no producen un efecto unitario en todos los sistemas de memoria, sino que están asociados al deterioro selectivo de determinadas funciones mientras que otras permanecen preservadas e incluso potenciadas. Así, el reconocimiento de olores puede resultar afectado mientras que el de estímulos gustativos resulta preservado. La exploración de los circuitos cerebrales empleados a edades avanzadas en tareas de reconocimiento de sabores con claves olfativas se presenta como una herramienta útil para identificar la interacción entre sus componentes cognitivos y emocionales. Ello es especialmente relevante en lo que respecta a áreas cerebrales no previamente exploradas en relación con la atenuación de la neofobia, tales como la corteza piriforme, parte del sistema olfatorio y el núcleo accumbens, parte del sistema de recompensa.

Como se ha mencionado en la introducción, la aversión gustativa condicionada y el condicionamiento de preferencias gustativas se utilizan como paradigmas de aprendizaje en los que se empareja un sabor con consecuencias aversivas o apetitivas, respectivamente, mientras que en la atenuación de neofobia gustativa la ingestión de un sabor desconocido no va seguida de consecuencias. Sin embargo, la ausencia de consecuencias podría constituir en sí misma un reforzador positivo, de manera que un sabor que inicialmente es consumido con cautela, incrementa su valor hedónico, y como consecuencia, el consumo en sucesivas exposiciones a medida que se produce la atenuación de la respuesta neofóbica. Este planteamiento cobra mayor fuerza teniendo en cuenta las condiciones experimentales a las que suelen estar sometidos los animales de experimentación durante los procedimientos de atenuación de neofobia. Por cuestiones metodológicas, los animales suelen tener acceso restringido a la bebida, por lo que están sedientos y muy motivados a beber. Aunque los procedimientos incluyen sesiones de rehidratación que permiten a los animales poder tener acceso a suficiente bebida como para evitar la deshidratación, ello no impide que estén sedientos durante la sesión experimental. Por este motivo, a pesar de que el sabor no va emparejado explícitamente con ninguna consecuencia, el hecho de poder beber constituye un reforzador por sí mismo, por lo que hay que tener en cuenta posibles fenómenos de aprendizaje apetitivo subyacentes.

El neurotransmisor dopamina ha demostrado ser fundamental en el procesamiento de sabores y en la adquisición de aprendizaje gustativo tanto aversivo como apetitivo. Se ha estudiado en profundidad el papel del núcleo accumbens y su neurotransmisión a través de los receptores dopaminérgicos de tipo D1 en la adquisición de aversiones gustativas condicionadas, así como en la adquisición de preferencias gustativas. Sin embargo, son escasos los datos sobre la participación del núcleo accumbens en el proceso de atenuación de neofobia. Según nuestro conocimiento, tampoco existe apenas investigación sobre el papel que desempeña la dopamina y el sistema de reforzamiento en general en el desarrollo de la atenuación de neofobia gustativa. Por tanto, se hace necesaria nueva investigación sobre la interacción entre el sistema de recompensa y los componentes afectivo-cognitivos asociados a la función de la amígdala y la corteza perirrinal durante la formación de memorias gustativas seguras.

Por último, se ha identificado la participación del sistema hipocampal en la dependencia contextual de diversos tipos de memoria gustativa y se ha descrito dicha dependencia del contexto físico en el caso de la atenuación de la neofobia al sabor. Se requiere más información sobre la modulación de la memoria gustativa segura por parte de distintos tipos de contexto que no incluyan claves espaciales, el posible papel del hipocampo en el fenómeno y los mecanismos que posibilitan la interacción

del sistema hipocampal con el circuito neural responsable de la atenuación de la neofobia gustativa.

Con el fin de estudiar la interacción entre los componentes cognitivo, emocional y afectivo de la memoria de reconocimiento gustativa segura y su evolución con la edad, se plantean los siguientes objetivos:

1. Evaluar la relación entre la actividad de la corteza piriforme y la memoria de reconocimiento gustativa empleando una solución de sabor ácido con un fuerte componente olfativo mediante determinación inmunohistoquímica de la proteína c-Fos en ratas adultas y envejecidas.

2. Evaluar la relación entre la actividad del núcleo accumbens y la memoria de reconocimiento de sabores mediante determinación inmunohistoquímica de la proteína c-Fos en ratas adultas y envejecidas.

3. Explorar el papel de las proyecciones eferentes dopaminérgicas del núcleo accumbens sobre la amígdala en la formación de la memoria gustativa segura, así como la participación selectiva de los receptores dopaminérgicos D1, mediante una variedad de técnicas (determinación inmunohistoquímica de la proteína c-Fos, intervención farmacológica mediante agonistas y antagonistas,y técnicas farmacogenéticas empleando DREADDs).

4. Determinar la dependencia contextual de la atenuación de la neofobia gustativa empleando un cambio de contexto auditivo sin ningún

componente espacial e investigar el efecto de la lesión hipocampal sobre dicho fenómeno.

5.Explorar el papel de las proyecciones eferentes dopaminérgicas del núcleo accumbens sobre el hipocampo en la formación de la memoria gustativa segura, así como la participación selectiva de los receptores dopaminérgicos D1, mediante una variedad de técnicas (lesión selectiva de vías dopaminérgicas mediante 6-OHDA, intervención farmacológica mediante agonistas, y técnicas farmacogenéticas empleando DREADDs).

Capítulo 3

Experimento 1.*Flavor recognition memory related activity of the posterior piriform cortex in adult and aged rats.*

1. Abstract

The relationship between the piriform cortex and flavor recognition memory was investigated in adult and aged rats. By using c-Fos immunohistochemistry, we assessed the piriform cortex activity induced by flavor familiarity. The results indicated increased activity in the rostral region of the posterior piriform cortex elicited by the most familiar cider vinegar solution after six exposures. Aged rats exhibited overall increased activity in the posterior, but not the anterior piriform cortex, which was not related to flavor familiarity. This suggests that the posterior piriform cortex is related to flavor recognition memory and that aging modifies its activity pattern which might underlie their slower attenuation of flavor neophobia.

Key Words: aging, flavor, c-fos, attenuation of neophobia,
recognition memory, piriform cortex.

2. Introduction

The general tendency to avoid novel tastes called taste neophobia plays a very important role for survival. Safe taste recognition memory refers to the ability of assessing the familiarity of a previously ingested food or drink not followed by negative consequences (Federico Bermúdez-Rattoni, 2004). As the novel taste becomes familiar, it is classified as safe and its consumption increases, thus evidencing attenuation of the neophobic response. Taste is the critical cue for this type of memory but, due to the multimodal integration involved in ingestion, other sensory cues might become relevant. This is what happens with the olfactory cues in case of flavors since they combine taste and smell.

In spite of their independent sensory pathways, taste-odor interaction is evident in behavioral effects such as odor taste potentiated aversion (F. Bermúdez-Rattoni, Coburn, Fernández, Chávez, & Garcia, 1987; Rusiniak, Hankins, Garcia, & Brett, 1979; Rusiniak, Palmerino, & Garcia, 1982) which depends on the amygdala (Miranda, 2012).

Regarding taste recognition memory, the amygdala has been related to the neophobic response to both taste and flavored solutions (Gómez-Chacón et al., 2012; Lin et al., 2012) and the perirhinal cortex with the attenuation of flavor neophobia (Gómez-Chacón et al., 2015; Morillas et al., 2017). However, the specific role of the olfactory component of

flavor has not been studied yet. Other cortical areas that might be relevant for recognition memory if an odorant component is added to taste are those receiving projections from both gustatory and olfactory pathways such as some prefrontal cortex regions (Rolls, 2005; Verhagen & Engelen, 2006). In fact, the orbitofrontal cortex has received special attention in animals and humans (Small, Veldhuizen, & Green, 2013).

Although not previously related to flavor recognition memory, the piriform cortex (PirCx) might be proposed as relevant for taste-odor integration (Small et al., 2013) since it sends projections to both amygdala and orbitofrontal cortex among other brain areas (Ennis, Puche, Holy, & Shipley, 2014). PirCx is an anatomically and functionally complex region (Ekstrand et al., 2001; Haberly & Price, 1978) consisting of two main divisions with different functions: the anterior piriform cortex (aPirCx) and the posterior piriform cortex (pPirCx) being the boundary placed at the level of the anterior commissure (Dardou, Datiche, & Cattarelli, 2010).

On the one hand, the aPirCx is mainly related to the detection and discrimination of odors and purely the smell sensation. Accordingly, it receives inputs from the primary olfactory neurons and is also well connected with the orbitofrontal cortex (H. Eichenbaum, Clegg, & Feeley, 1983; Johnson, Illig, Behan, & Haberly, 2000; Roesch, Stalnaker, & Schoenbaum, 2007). On the other hand, many studies suggest that the

pPirCx is an area not only involved in odor information processing but it is also important for multisensory integration. In addition to neurons that respond selectively to odors, neurons that respond selectively to gustatory information and neurons that respond to both senses with different firing patterns have been described (Maier, Wachowiak, & Katz, 2012). The pPirCx is connected not only with the aPirCx but also with areas involved in taste and flavor recognition memory such as the amygdala, the perirhinal cortex and the orbitofrontal cortex. Electrophysiological studies have shown that the activity of pPirCx neurons is highly plastic during a reversal learning task. They exhibit both earlier and more pronounced phasic electrical activity to positive than to negative odor cues (Calu, Roesch, Stalnaker, & Schoenbaum, 2007). Also, Dardou et al. (Dardou et al., 2010) found a different pattern of expression of Egr1 protein, used as a marker of neural activity, between the aPirCx and the pPirCx in a typical task of odor taste potentiated aversion.

However, to our knowledge, there are no data on the potential relationship between the PirCx and safe flavor recognition memory. If the pPirCx processes flavor information and has higher order sensory plastic functions, then its activity might be sensitive to flavor familiarity. A feasible hypothesis is that increases in flavor familiarity during the attenuation of neophobia would induce changes in the activity of the pPirCx. This would support an involvement of the area in flavor

recognition memory. Moreover, if this were so, the activity of pPirCx related to flavor familiarity could be modified by aging since it has been reported slower attenuation of neophobia in aged than in adult rats together with a different pattern of perirhinal cortex activity (Gómez-Chacón et al., 2015). Hence, adult rats exhibited increased c-Fos activity in the perirhinal cortex after the exposure to a novel flavor meanwhile aged rats showed increased c-Fos activity after the exposure to the most familiar flavor, suggesting functional brain reorganization by aging leading to enhancement of the positive consequences of safe flavors. Accordingly, Dardou and Catarelli (Dardou et al., 2008) found an increased expression of Egr1in the pPirCx of senescent compared to adult rats after odor taste potentiated aversion, suggesting a potentiation of the negative consequences of odor stimuli in aged rats.

The main aim of this study was to investigate if the piriform cortex of rats exhibits activity changes over repeated exposures to a novel vinegar solution. In order to establish comparisons with previous data on other brain areas (Gómez-Chacón et al., 2015; Gómez-Chacón et al., 2012; Lin et al., 2012) we measured the number of c-Fos positive cells during the first, second and sixth flavor exposure. We also assessed the piriform cortex activity in aged rats during the attenuation of flavor neophobia.

3.Materials and method

3.1 Subjects

The brains of 21 adult (5-month-old) and 24 aged (24-month-old) male Wistar rats were used in this experiment. Housing, grouping and general management conditions have been described elsewhere since these are the same animals' brains previously used in Gómez-Chacon et al., (2015). This is in accordance with the reuse rule for reducing the number of animals in research.

In brief, depending on the day in which the animals were euthanized to remove their brains, each rat was assigned to one of the following groups: Novel (with one exposure to vinegar; adult: n=7; aged: n=8), F-I ("Familiar-I" groups with two exposures to vinegar: adult; n=7; aged: n=8) and F-II ("Familiar-II" groups with six exposures to vinegar; adult: n=7; aged: n=8). Four brains (Novel=1, F-I=2 and F-II=1) were discarded for the data analyses due to tissue damage during the immunohistochemical procedure. All procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (17-02-15-195).

3.2 Behavioral Procedure

All the subjects were subjected to the same behavioral procedure, which consisted of daily 15-minute drinking sessions in which consumption was recorded. Water intake during the morning drinking period was recorded for five days during the acclimation period to the deprivation schedule. Once the water intake baseline (BL) was stabilized all rats had access to a 3% (vol/vol) cider vinegar solution instead of water during the morning daily drinking session. Consumption (ml) was recorded after each session (see Gómez-Chacón et al.2015).

3.2.1 Immunohistochemical Procedure

All the animals were euthanized 90 minutes after the drinking the novel cider vinegar solution on Day 1 for the first time (Novel), the already familiar solution on Day 2 (F-I) or the most familiar solution on Day 6 (F-II). The immunohistochemical procedure has been described elsewhere (Gómez-Chacón et al., 2015). In brief, deeply anesthetized rats were transcardially perfused. The brains were removed and coronal sections were cut at 20µm in a cryostat (Leica CM1900). Tissue sections were then rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4), incubated for 15 minutes with 3% hydrogen peroxide, rinsed again and incubated in a solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 30

minutes. Slices were transferred to a c-Fos primary antibody (1:10,000; Cal-biochem) for 48 h at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Biotinylated goat anti-rabbit IgG, 1:500; Calbiochem) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, 0.4% Triton X-100 and PBS. The sections were rinsed, then processed using the ABC-kit (Vector Laboratories, Burlingame, CA), and the reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA). Finally they were rinsed, mounted on gelatine-bubbed slides, rehydrated with ethanol and xylenes and finally they were cover-slipped.

3.2.2 Data Acquisition

In order to quantify the number of c-Fos positive cells, coronal sections of the brain containing the anterior and posterior PirCx were identified using *Neurolucida Software.* Two images of both hemispheres were captured using a light microscope (Olympus BX41) at 20X magnification. The coronal sections were separated into three regions following a rostro-caudal division according to the different areas that were adjacent to the PirCx. Images of the rostral, central and caudal regions of aPirCx were captured approximately at +3mm, +2mm and +1mm,

respectively, relative to Bregma according to Paxinos and Watson (2009). Images of the rostral, central and caudal regions of the pPirCx were captured at -2.5mm, -3.00mm and -3.5mm relative to bregma according to Paxinos and Watson (2009). See **Figure 1**.

The number of c-Fos positive cells was counted using the Image J Software (National Institute of Mental Health) automatically. For each image threshold objects having specific area (20-150) and circularity (0.25-1.00) values matching those c-Fos positive nuclei were automatically counted by the software. In order to equalize all images and cancel out background noise, they were previously converted into 8-bit type image and the background was lightened (50.0 pixels). Mean values were calculated for both hemispheres.

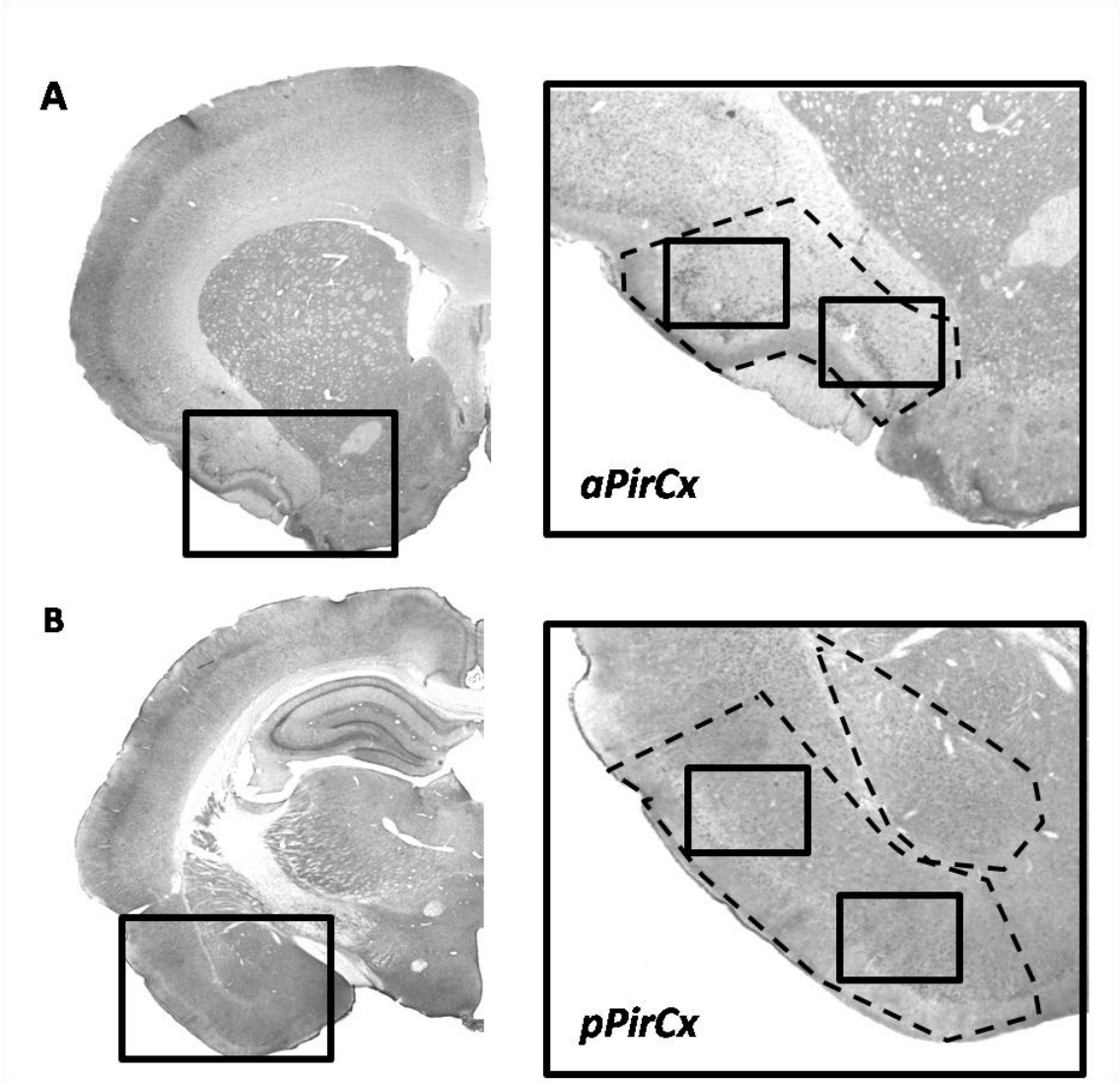

Figure 1. Representation of the location of the images containing the aPirCx (**A**) and pPirCx (**B**) captured with the light microscope at 20X magnification.

4. Results

4.1 Behavioral results

A two-way ANOVA analysis (*Familiarity* X *Age*) of the water amount drank during the last baseline day yielded a significant effect of *Age* [F(1, 38)=5.22; *p*=.027] but not *Familiarity* [F(2, 38)=0.06; *p*=.941] or

the interaction *Age* X *Familiarity* [F(2, 38)=0.6; *p*=.55]. Aged rats drank more water (Mean: 11.02 ± 0.71 ml) than adult rats (Mean: 13.29 ± 0.68 ml). This can be attributed to the higher weight of the aged rats. However, no significant differences between the different age groups in the amount of vinegar solution drank were found during the novel flavor exposure. A two-way ANOVA analysis (*Familiarity* X *Age*) of the vinegar solution drank on Vin1did not yield significant effects of *Age* [F(1, 38)=0.002; *p*=0.96], *Familiarity* [F(2, 38)=0.093; *p*=.91] or the interaction *Age* X *Familiarity* [F(2, 38)=0.36; *p*=0.76]. Moreover, during the second exposure to the vinegar solution the effect of Age was opposite to that found in the baseline since aged rats (Mean: 7.40 ± 0.64 ml) drank lower amounts than adult rats (Mean: 9.42 ± 0.68 ml). A two-way ANOVA analysis (*Familiarity* X *Age*) of the vinegar solution drank in Vin 2 yielded a significant effects of *Age* [F(1, 26)=4.68; *p*=.04] but not *Familiarity* [F(1, 26)=0.003; *p*=.96] or the interaction *Age* X *Familiarity* [F(1, 26)=0.275; *p*=.61]. Therefore, adult rats but not aged rats showed attenuation of neophobia after the first exposure to vinegar as intake of vinegar on day 2 increased in comparison with day 1 in adult but not in aged rats.

The behavioral results of Familiar-II groups have been previously described (B. Gómez-Chacón et al., 2015). In brief, both adult and aged groups exhibited the neophobic response to vinegar. Attenuation of neophobia was evident in both age groups as vinegar intake increased over

repeated exposures until there were no differences along the last days. However, it was slower in the aged group than in the adult group. While adult rats did not exhibit increased vinegar consumption from Day 2 after two vinegar exposures, aged rats required one more vinegar exposures since there were no differences with Vin6 from Day 3and no differences were found between Days 1 and 2 (**Table 1**). The slower attenuation of neophobia in the aged group is evident also including the baseline water in the analysis. One-way ANOVA analysis of the F-II adult group consumption along the drinking sessions indicated lower vinegar than baseline water intake only in Vin1 (p=.022). A similar analysis of the FII aged group showed lower vinegar intake in comparison with water consumption during the last baseline day in Vin1 (p=.011), Vin2 (p=.02), Vin3 (p=.011) and Vin4 (p=.011).

4.2 Immunohistochemical results

4.2.1 Anterior Piriform Cortex

A mixed repeated measures ANOVA (*Region* X *Familiarity* X *Age*) was used to compare differences in c-Fos positive cells in the aPirCx between adult and aged rats. This ANOVA revealed a significant effect of the main factor *Region* [$F(2,43)=4.19$;p=.021] and no other effect or interaction was significant (all p>.1). Further post-hoc analyses of the main

effect using Bonferroni tests revealed a higher number of c-Fos positive cells in the most rostral region of the aPirCx compared to the other two regions (**Figure 2B**).

4.2.2 Posterior Piriform Cortex.

A repeated-measures ANOVA (*Region* X *Familiarity* X *Age*) was applied to compare differences in c-Fos positive cells in the pPirCx between adult and aged rats. This ANOVA revealed significant effects of the main factor *Age* [$F(1,34)=35.56$; $p<.001$] and the interaction *Region* X *Familiarity* [$F(4,34)=5.16$; $p<.003$]. Further post-hoc analyses of the main effect *Age* using Bonferroni tests revealed an overall increased number of c-Fos positive cells in the aged rats compared to adult rats regardless the familiarity or the region of the posterior piriform cortex studied (**Figure 2A**).

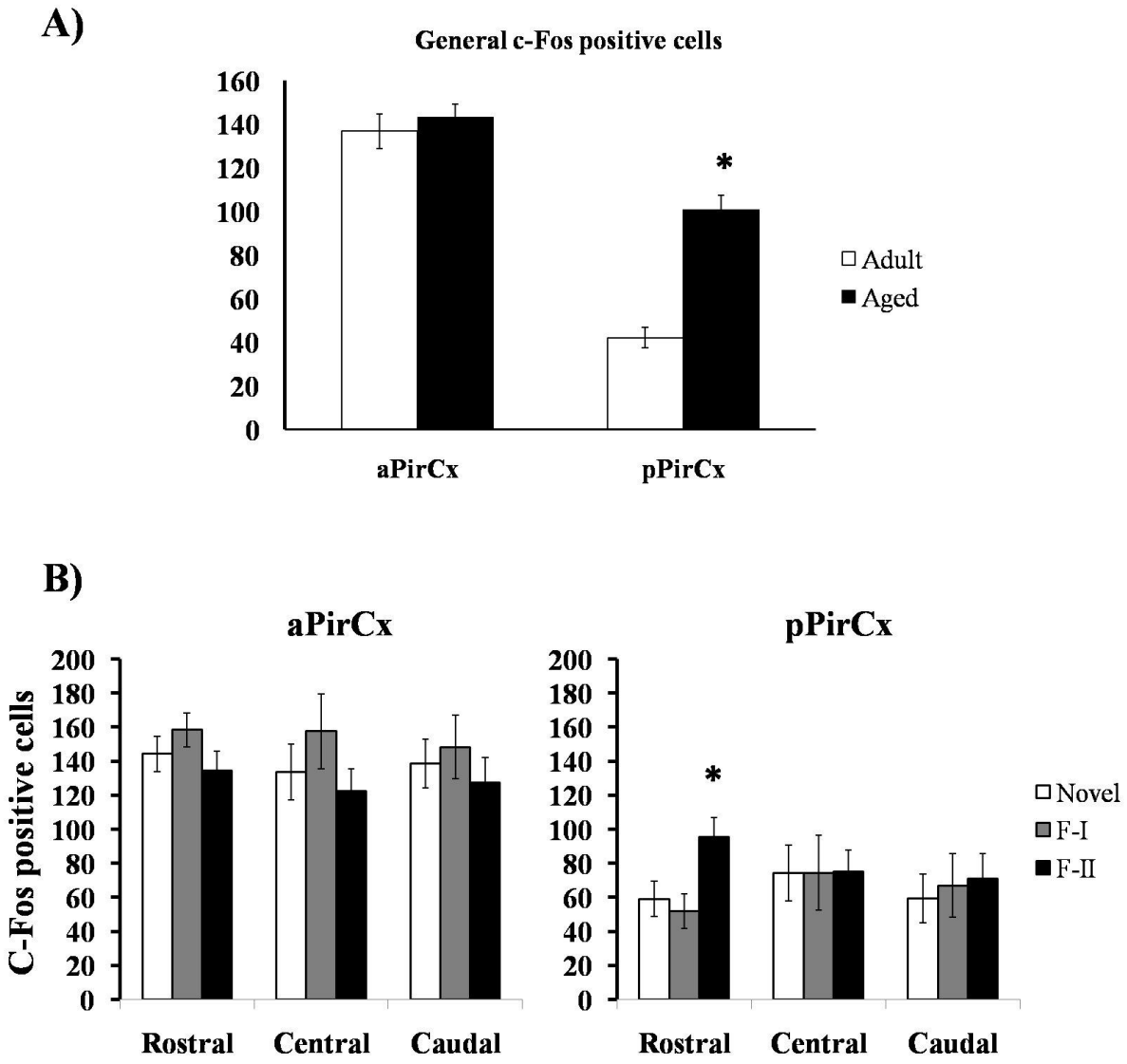

Figure 2. Panel **A** represents number of c-Fos positive cells ±SEM in both regions of the Piriform Cortex. The symbol * represents statistically significant difference (p<.05) compared to the adult group. Panel **B** represents the number of c-Fos positive cells in the three sub-regions of the aPirCx and the pPirCx of both adult and aged rats. The symbol * represents statistically significant differences (p<.05) compared to the Novel and F-I groups.

In order to understand the second order interaction *Region* X *Familiarity*, a one-way ANOVA was performed for each of the three Regions. The analysis performed for the rostral Region revealed a significant effect of *Familiarity* [$F(2,34)=7.79$; p<.002]. Further post-hoc analyses using Bonferroni tests revealed a higher number of c-Fos

positive cells in the F-II group compared to the Novel Group (p=.016) and the F-I Group (p=.003) and no differences in c-Fos positive cells were found between Novel and F-I Groups (p=1). Analyses performed for the central and caudal Regions of the pPirCx did not reveal any significant effect of Familiarity (all p>.9 and p>.8 respectively). **Figure 3** represents the results of the adult and aged groups.

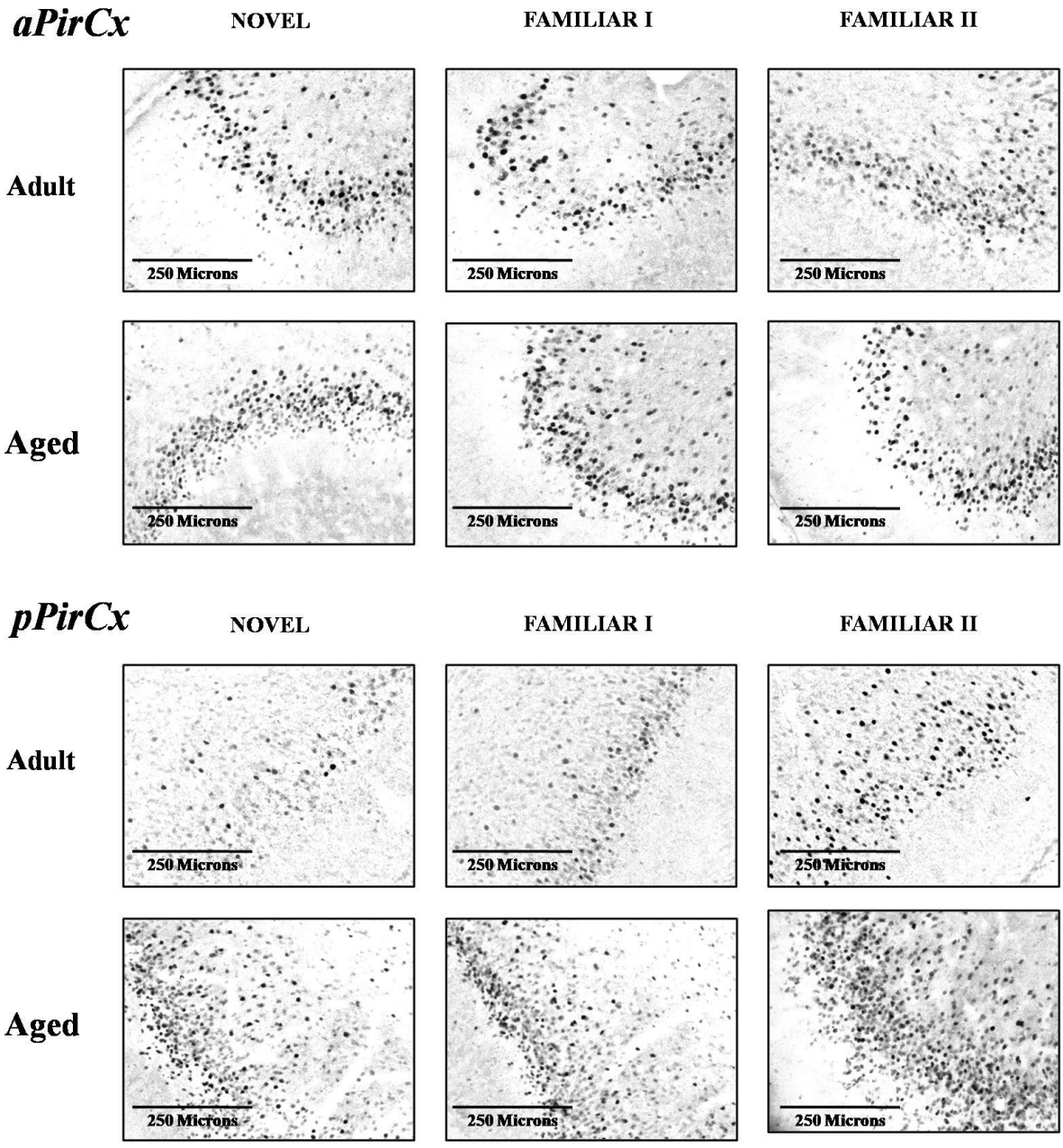

Figure 3. Panel **A** shows representative microphotographs at 20X magnification of the Rostral aPirCx for the three familiarity groups (Novel, Familiar I and Familiar II) of adult and aged rats. Panel **B** shows

representative microphotographs at 20X magnification of the Rostral pPirCx for the three familiarity groups (Novel, Familiar I and Familiar II) of adult and aged rats.

Additionally, another one-way ANOVA *Region* X *Familiarity* was performed for each familiarity group separately. There were no significant effects in the Novel and F-I groups. There was a significant effect of *Region* in the F-II group [$F(2,25)=14.4$; $p<.001$]. Post-hoc analyses by Bonferroni tests revealed an increased number of c-Fos positive cells in the rostral region compared to the central ($p=.002$) and caudal ($p=.001$) regions.

Thus, the relevant significant differences can be attributed to a higher number of c-Fos positive cells in the pPirCx after drinking the familiar vinegar solution during the sixth exposure in the F-II group. This increase was found in the most rostral region of the pPirCx. We also found an overall increased number of c-Fos positive cells in the pPirCx of aged rats compared to adult rats, regardless the number of exposures to the flavor.

5. Discussion

The main finding reported in the present study is that the activity of the pPirCx is selectively modified during repeated flavor exposures. This is to our knowledge the first evidence reporting a potential role of the PirCx in flavor memory. Immunohistochemistry data revealed selective activity

changes in the rostral pPirCx related to the familiarity of the flavor while no changes were found within the aPirCx. The number of c-Fos positive cells in this portion of the pPirCx was higher in the F-II group of adult rats than in Novel and F-I groups. Thus, the activity of this region increased significantly when the rats drank the most familiar vinegar solution after six exposures. In addition, there were no differences between Novel and F-I groups in the number of c-Fos positive cells.

The results are in accordance with previous reports relating pPirCx but not aPirCx with learning and memory processes. The fact that the increased number of c-Fos positive cells reaches significance after sixth exposures when the flavor familiarity is consolidated suggests a potential role of pPirCx in long-term flavor recognition memory. Alternative explanations in terms of increased activity induced by drinking-related sensory, motor and motivational processes cannot be ruled out but they do not seem feasible. First, the increase in the number of c-Fos positive cells is not parallel to the consumption pattern of the flavored solution that shows a significant increase at the second exposure (F-I group). Second, the animals could smell the odor during the entire 15 min drinking sessions including the periods in which they did not drink so that the odor exposure did not differ among the groups. Third, if higher intake or longer odor exposure were the reasons for the increased PirCx activity it would be evident in all the regions and the differences appeared only in rostral pPirCx. A similar

argument applies for changes in the motor and motivational state. There are no data to support a selective role of pPirCx in these processes.

An overall increased expression of c-Fos was found in the pPirCx of aged rats indicating a hyper-activation regardless the number of vinegar exposures. Such over-expression of c-Fos was not found in aPirCx, which is the most pure sensory area of the secondary olfactory cortex. In fact, it has been reported that the aPirCx receiving olfactory information from the tufted and mitral cells of the Olfactory Bulb, does not exhibit learning-related activity changes. Roesch et al. (2007) found no changes in the firing patterns of the aPirCx neurons after a reversal learning task. In contrast, Calu et al. (2007) reported that the electrical activity of pPirCx neurons was highly plastic during a reversal learning task. This evidence together with the anatomical connections of the pPirCx with areas involved in taste learning, such as basolateral amygdala and perirhinal cortex, as well as the present data suggest that the aPirCx might be involved in smell sensation while the pPirCx would be more likely involved in flavor learning being more sensitive to the effect of aging on memory.

Our results are in accordance with previous reports of increased protein expression in the pPirCx of aged rats (Dardou et al., 2010). This over-expression could be reflecting compensatory mechanisms to deal with aging-related memory difficulties. In this line, Foster et al. (2012) found

excitatory changes in glutamatergic neurons of the cortex proposed as early markers of cognitive decline. This could be consistent with the delayed attenuation of flavor neophobia exhibited by aged animals. The evidence indicating that aged and adult rats did not differ in the neophobic response in spite of exhibiting pPirCx over-activation further supports a selective involvement of this region in the attenuation of neophobia, but not in neophobia itself. The fact that flavor neophobia was present in both age groups is in accordance with previous findings on the effect of aging in taste memory. In contrast to the deleterious effect of aging in other learning and memory tasks, taste learning is largely preserved at advanced ages (for reviews see (Gámiz & Gallo, 2011). Taste neophobia might exhibit age-related changes (Manrique et al., 2007), but they seem to depend on previous life experiences more than on aging itself (Morón & Gallo, 2007b).

Also, as it has been previously reported (Gómez-Chacón et al., 2015), aged rats exhibited slower attenuation of neophobia than adult rats. It cannot be ruled out that during the familiarization process different learning effects such as habituation, appetitive learning, habit learning or aversion counterconditioning had taken place. In any case, the increase in vinegar consumption with repeated exposures indicates attenuation of neophobia.

The finding relating the pPirCx with the attenuation of flavor neophobia is particularly relevant because it has been demonstrated to be a multisensory integration area that could be receiving afferents related to taste memory. In fact, the rostral portion of pPirCx borders with the insular cortex. Areas involved in other types of memory, such as perirhinal cortex, entorhinal cortex, and amygdala are also bordering with other regions of the pPirCx. Thus, we consider that the selective increased activity of the rostral pPirCx could be related with a role of this region in taste memory. Consistently, electrophysiological findings have demonstrated responsiveness of pPirCx neurons to taste stimulation (Maier et al., 2012). Interestingly, the recording electrodes were located at -1,4 mm posterior to bregma, i.e., a region of the PirCx bordering with the insular cortex. Also, reciprocal connectivity between insular cortex and PirCx could be responsible for taste-odor interactions relevant for memory (Maier et al., 2012; Small et al., 2013).

In summary, it is conceivable that pPirCx might be included together with amygdala, insular cortex, perirhinal cortex and orbitofrontal cortex as part of a flavor memory brain circuit. Further research on the specific role of the piriform cortex in recognition memory could help to understand dysfunctional restriction of dietary intake described in eating disorders often affecting to the older population (Grau-Perales et al., 2018a) and it

will contribute to advancements in diagnosis assessment, behavioral intervention and nutritional management.

6. Acknowledgments.

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Capítulo 4

Experimento 2. ***Differential activity pattern of c-fos in the nucleus accumbens between adult and aged rats during flavor recognition memory.***

1. Abstract

Previous studies have addressed the role of the nucleus accumbens core (NAcbC) and shell (NAcb-Shell) in taste aversion learning and in the processing of taste palatability which is affected by aging. However, little is known about its implication in safe taste memory and the aging impact. To explore the role of the NAcb in flavor neophobia and its attenuation during aging, we applied c-Fos immunohistochemistry as an index of neural activity of the NAcbC and NAcb-Shell. Twenty one adult (5-month-old) and 24 aged (24-month-old) male Wistar rats were exposed to a 3% cider vinegar solution for 1, 2 or 6 consecutive days (*n*=7 adult and *n*=8 aged rats per group). Aged rats exhibited slower attenuation of flavor neophobia than adult rats. Adult rats showed increased NAcb-Shell c-Fos activity on day 2 compared to days 1 and 6, while this increase was delayed to day 6 in aged rats. There were no differences in the number of NAcbC c-Fos positive cells. This suggests that changes in the activity of neural circuits of palatability processing during normal aging could contribute to the slower attenuation of flavor neophobia in aged rats.

Key Words: Accumbens; Aging; Attenuation of Neophobia; Flavor; c-Fos; Recognition Memory; Taste.

2. Introduction

The ingestion of novel flavors is a model of recognition memory useful to study the neural and molecular mechanisms of memory (Federico Bermúdez-Rattoni, 2004). After having ingested a novel substance, a taste memory of its flavor is generated and stored among several brain regions in parallel (Bermudez-Rattoni, 2014; De la Cruz et al., 2008). If the flavor is not followed by negative consequences, a safe taste memory is generated and it allows the recognition of the flavor as safe. Therefore the animal increases consumption of the flavor over repeated exposures, showing an attenuation of neophobia (Federico Bermúdez-Rattoni, 2004).

Different brain areas have been related with the formation of the safe taste memory, such as the hippocampus (Balderas, Morin, Rodriguez-Ortiz, & Bermudez-Rattoni, 2012; Grau-Perales, Levy, Fenton & Gallo, 2019b), the insular cortex (Bahar, Dudai, & Ahissar, 2004; Bermudez-Rattoni, 2014; Lin, Roman, Arthurs, & Reilly, 2012; Miranda, Ferreira, Ramírez-Lugo, & Bermúdez-Rattoni, 2003; Moraga-Amaro, Cortés-Rojas, Simon, & Stehberg, 2014), the basolateral amygdala (Gómez-Chacón, Gámiz, & Gallo, 2012), the piriform cortex(Grau-Perales et al., 2019a), the perirhinal cortex (Gómez-Chacón, Morillas, & Gallo, 2015; Gutiérrez, De la Cruz, Rodriguez-Ortiz, & Bermudez-Rattoni, 2004; Morillas, Gómez-Chacón, & Gallo, 2017) and also the nucleus accumbens (Pedroza-Llinás et al., 2009; Ramírez-Lugo et al., 2007).

The nucleus accumbens (NAcb), a well known component of the reward system, has been involved in different processes related to addiction and feeding (Bassareo & Di Chiara, 1999; Bassareo, De Luca, & Di Chiara, 2002; Yamamoto, 2006) as well as in the hedonic shifting of flavors (Shimura, Kamada, and Yamamoto, 2002; Yamamoto, 2006). Regarding the implication of the NAcb in the formation of taste memories, pharmacological studies have shown that both the nucleus accumbens core (NAcbC) and shell (NAcb-Shell) are involved in aversive taste responses (Fenu et al., 2001), but only NAcb-Shell seems to be involved in the case of safe taste memory formation (Pedroza-Llinás et al., 2009; Ramírez-Lugo et al., 2007).

Even though changes in performance of learning and memory tasks related to aging have been described in terms of functionality decay, this is not applicable to taste memory, which in fact, seems to be spared -or even enhanced- in the case of aged rats (Gámiz & Gallo, 2011; Gallo, 2018). In fact, the progressive memory decay traditionally related to aging has been selectively associated to spatial memory (Dardou, Dariche & Catarelli, 2008) and hippocampal-dependent tasks. (Manrique, Morón, Ballesteros, Guerrero, & Gallo, 2007; Manrique, Morón, Ballesteros, Guerrero, Fenton & Gallo, 2009). However, aged rats continue to show neophobia when a novel flavor is encountered (Morón & Gallo, 2007a), although the exhibit a slower attenuation of neophobia than younger rats (Gómez-Chacón et al.,

2015). This evidences that taste learning proceeds differently throughout life. However, little is known about the neural mechanisms underlying taste recognition memory in aged rats. It has been described a different pattern of c-Fos expression in some brain areas that belong to the taste recognition memory circuit in the case of aged rats, such as the perirhinal cortex (Gómez-Chacón et al., 2015) as well as the posterior piriform cortex (Grau-Perales, et al., 2019a). The aged brains exhibited a higher number of c-Fos positive cells in these areas than the adult brains after the exposure to a highly familiar rather than a novel flavor.

In order to explore the impact of normal aging in the function of nucleus accumbens related to flavor neophobia and its attenuation, we applied c-Fos immunohistochemistry as an index of neural activity of the nucleus accumbens core and shell of adult and aged male Wistar rats which were exposed to a novel flavor solution for one, two or six sessions. Therefore, a 2 x 3 (age x familiarity) design was applied and the number of c-Fos positive cells was measured in NAcbC and NAcb-Shell separately.

Given the proposed role of NAcb-Shell but not NAcbC in the formation of safe taste memories, an increased number of c-Fos positive cells in NAcb-Shell during the second exposure session rather than the first and the sixth exposure might be expected in adult rats. In addition, as slower attenuation of flavor neophobia revealing slower taste memory formation has been found in aged rats, it can be hypothesized a delay of

such increase in the aged NAcb-Shell that would be evident later, during the sixth but not the second exposure to the flavor.

3. Materials and methods

2.1. Subjects

Twenty-one adult (5-month-old) and twenty-four aged (24-month-old) male Wistar rats were used in this experiment. Housing, grouping and general management conditions have been described elsewhere since these are the same animals' brains previously used in Gómez-Chacón et al. (2015). This is in accordance with the reuse rule for reducing the number of animals in research.

All the experimental procedures were performed during the light cycle at the same time each morning in the home cage. Rats were given *ad libitum* food and water until the experiment started, when access to water was restricted. All procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (17-02-15-195), and were in accordance with the European Communities Council Directive 86/609/EEC.

3.2. Behavioral Procedure

The procedure of attenuation of neophobia has been described elsewhere (Gómez-Chacón et al., 2015). Briefly, the behavioral procedure consisted on 15-minute daily drinking sessions. Water intake during the morning drinking period was recorded for five days during the acclimation period in order to get the animals used to the deprivation schedule. Once the water intake was stabilized as baseline consumption (BL) all rats had access to a novel 3% cider vinegar solution during the morning 15-minute drinking session. The amount ingested was recorded by weighing the tubes containing the solution before and after each drinking session.

All the adult and aged rats were randomly assigned to the following familiarity groups: 15 rats were euthanized after drinking the vinegar solution on day 1 (*Novel: n=7 Adult; n=8 Aged*); 15 rats were euthanized after drinking the vinegar solution on day 2 (*Familiar-1: n=7 Adult*; *n=8 Aged*); and the remaining 15 rats were euthanized after drinking the vinegar solution on day 6 (*Familiar-2: n=7 Adult*; *n=8 Aged*).

3.3. Immunohistochemistry.

All the animals were euthanized 90 minutes after the corresponding drinking session depending on the c-Fos immunohistochemical group they were assigned. They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by

4% paraformaldehyde 90 minutes after drinking the vinegar solution the first (Novel group), second (Familiar-1) and sixth day (Familiar-2). The brains were removed and placed in a 4% paraformaldehyde solution for 4 h at 4ºC before being transferred to a 30% sucrose solution until they sank for cryoprotection. Coronal sections were cut at 20µm in a cryostat (Leica CM1900). Some of the brains from each groups of age were randomly selected so that some sections were stained with cresyl violet for general morphological study while immunohistochemistry for c-Fos was applied to the rest of sections.

Free floating tissue sections were rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4), incubated for 15 minutes with 3% hydrogen peroxide, rinsed again and incubated in a solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 30 minutes. Slices were transferred to a c-Fos primary antibody (1:10,000; Calbiochem) for 48 h at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Biotinylated goat anti-rabbit IgG, 1:500; Calbiochem) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, 0.4% Triton X-100 and PBS. The sections were rinsed, then processed using the ABC-kit (Vector Laboratories, Burlingame, CA), and the reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA).

Finally they were rinsed, mounted on gelatine-bubbed slides, rehydrated with ethanol and xylenes and finally they were cover-slipped.

3.4. Data Acquisition

Images of both hemispheres from brain slices containing the nucleus accumbens core and shell (NAcbC and NAcb-Shell) were captured using a light microscope (Olympus BX41). Slices containing the NAcbC and NAcb-Shell were identified using the *Stereo Investigator* Software (mbf Bioscience) from two coronal sections of the NAcb located at the level of the apparition of the lateral ventricles, approximately at +2,52 and +2,28mm relative to Bregma (Section 1 and Section 2 respectively) according to Paxinos and Watson (2009). Within each section 5 microphotographs at 40X magnification were captured for the NAcbC and the NAcb-Shell according to a dorso-ventral-medio-lateral axis in order to cover the entire extension of both nuclei. The microphotographs were labeled with the number of its position within each nucleus (ranging from 1 to 5) (See **Figure 1A**).

The number of c-Fos positive cells was obtained using the *Image J* Software (National Institute of Mental Health). For each microphotograph threshold objects (black circular dots over the white background) having specific size (35-150 μm^2) and circularity (0.35-1.00) values matching those c-Fos positive nuclei were automatically identified by the software as

c-Fos positive cells. In order to equalize all the microphotographs and cancel out possible background noise, they were previously converted into 8-bit type image and the background was lightened (150.0 pixels). Representative microphotographs of the different experimental groups are shown in **Figure 1B**.

Due to the immunohistochemical procedure used when the brain slices were mounted in the slides, it was impossible to determine to which hemisphere belonged. For this reason, in order to randomize a possible confounding effect, for each section the mean number of c-Fos positive cells was calculated in every microphotograph for both hemispheres.

4. Results.

To assess the correct location of the microphotographs, the perimeter (µm) and area (μm^2) of every region selected as NAcbC and NAcb-Shell was measured. A global mixed ANOVA that included all the groups of animals and the two nuclei only revealed an effect of size [$F(1, 33)=2219.4$; $p<.0001$] and nucleus [$F(1, 33)=9,32$; $p<.01$] that corresponded to a bigger size of the NAcbC relative to the NAcb-Shell. No other effect or interaction was found. This allowed us to ensure that all the microphotographs were captured in the correct location as all the regions selected for every group had the same perimeter (NAcbC perimeter = 4439,64 ± 20,22 µm; NAcb-Shell perimeter = 5251,78 ± 21,35 µm) and

area (NAcbC area = 1091601,13 ± 417 μm^2; NAcb-Shell area = 1023460,59 ± 368 μm^2).

4.1. Behavioral results

The behavioral results are available elsewhere (Gómez-Chacón et al., 2015; Grau-Perales et al., 2019a). In brief, both adult and aged groups exhibited a similar neophobic response to vinegar. However, the attenuation of neophobia was delayed in the aged group in comparison with the adult group. While adult rats required a single exposure to reach the asymptote by the second exposure to vinegar on Day 2, aged rats required two more exposures as there were no differences between the amounts of vinegar drank on Day 4 and the subsequent exposures on Days 5 and 6.

4.2. Attenuation of neophobia: c-Fos analyses.

The mean number of c-Fos positive cells (±SEM) for the three familiarity groups of both ages during the vinegar drinking sessions is shown in the **Figure 2** for both NAcbC and NAcb-Shell. A 2 x 3 bifactorial ANOVA (*Age* X *Familiarity*) was used to compare differences in c-Fos positive cells in the NAcbC and NAcb-Shell.

A 2 x 3 (*Age* X *Familiarity*) two-way ANOVA of the c-Fos positive cells number in the NAcbC did not reveal any significant effect (all *p´*s>.05) (See **Figure 2A**). The same two-way ANOVA 2 x 3 (*Age* X

Familiarity) of the c-Fos positive cells number in the NAcb-Shell revealed significant effects of *Familiarity* [F(2, 35)=4,021; $p<.05$] and the interaction *Age* X *Familiarity* [F(2,35)=4, 877; $p<.05$]. In order to understand this interaction, further one way ANOVAs were used for each group of age separately. The analysis performed for the adult rats revealed a significant effect of *Familiarity* [F(2,16)=5,59; $p<.05$] and further comparisons using Bonferroni-corrected tests revealed a statistically significant higher number of c-Fos positive cells in the Familiar-1 group compared to Novel ($p=.027$) and Familiar-2 ($p=.034$), but no differences between Novel and Familiar-2 ($p=1$). The analysis performed for the aged rats revealed a significant effect of *Familiarity* [F(2,18)=3,72; $p<.05$] and further comparisons using Bonferroni-corrected tests revealed a statistically significant higher number of c-Fos positive cells in the Familiar-2 group compared to Novel ($p=.04$) but not with Familiar-1 ($p=.2$). In brief, adult rats showed higher number of c-Fos positive cells in the NAcb-Shell after drinking the vinegar solution on day 2 compared to days 1 and 6, while aged rats showed higher c-Fos activity on day 6 compared to day 1. Consistently, there were significant differences between adult and aged groups in the number of c-Fos positive cells found in Familiar-1 (p=.012) and Familiar-2 (p=.03) groups in the NAcb-Shell, but not in the NAcbC (all $p´s > .3$). However, no differences related with taste familiarity were found in the NAcbC of adult and aged rats (See **Figure 2B**).

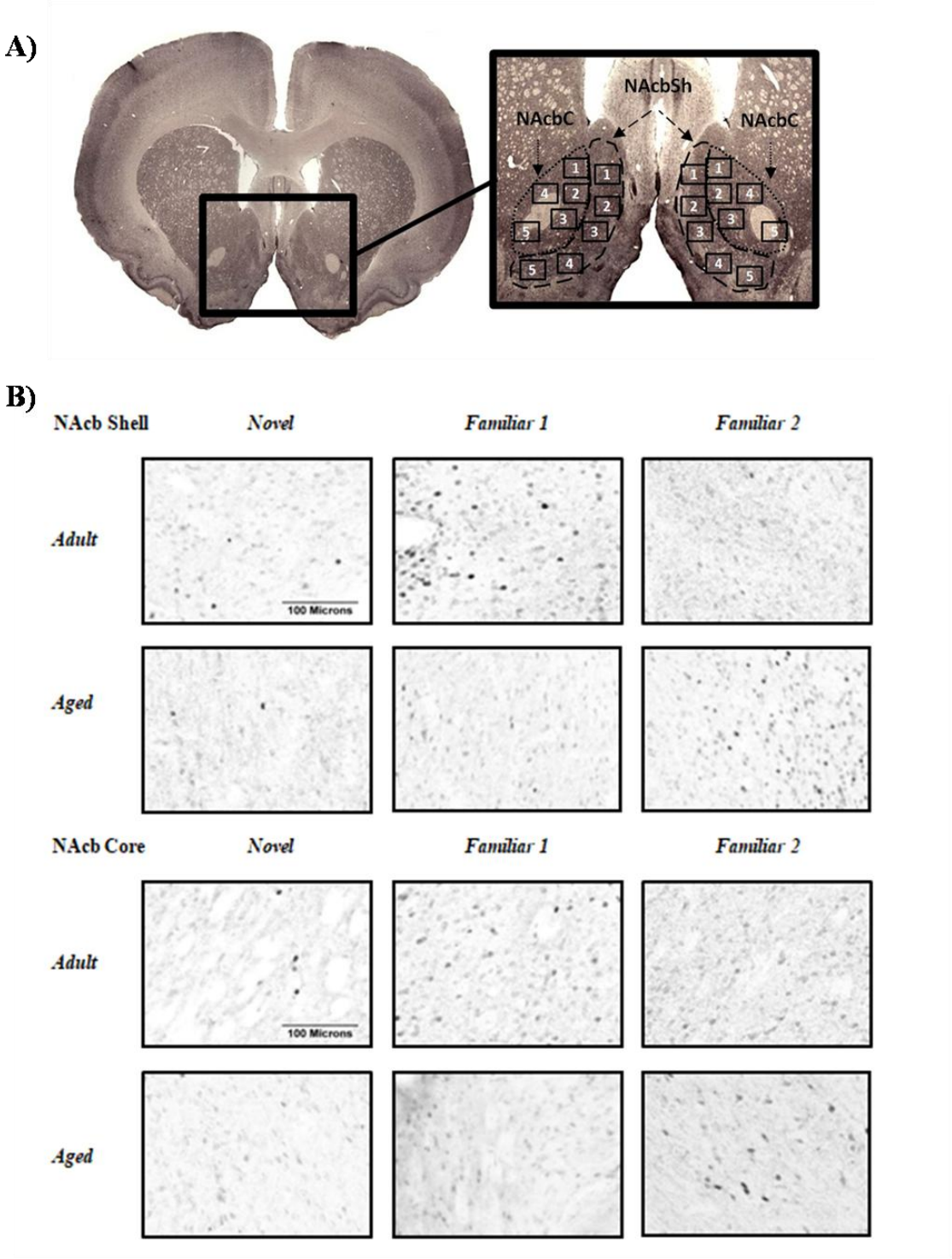

Figure 1:A) Schematics of the microphotographs (40X) of the nucleus accumbens core and Shell. All the microphotographs were captured following a dorso-ventral and medio-lateral axis and were labeled with numbers from 1 to 5 in order to cover the entire extension of both nuclei. **B)**Representative microphotographs obtained at 40X magnification of the medial part of the nucleus accumbens shell (top panel) and the medial part of the nucleus accumbens core (bottom panel).

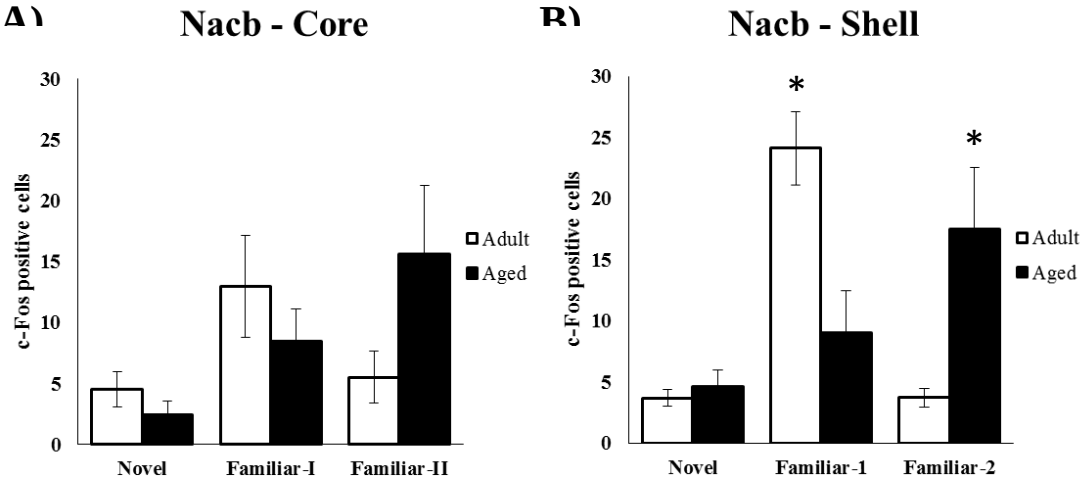

Figure 2: Mean (±SEM) of c-Fos positive cells in the five subregions of the nucleus accumbens core (A) and shell (B) for both groups of age. * symbol represents statistically significant differences (p<.05) compared to Novel group
of the same group of age

4.3. Cell quantification: Cresyl Violet staining.

Some of the brains were chosen for cell quantification semi-randomly, so 2 Cresyl Violet stained brains of each familiarity group (6 adult and 6 aged) were selected for each group of age. A two-way ANOVA 2 x 2 (*Age* X *Region*)was used to compare the number of Cresyl Violet stained cells between adult and aged rats in both NAcbC and NAcb-Shell. The analysis revealedno significant effect of any of the factors nor its interaction (all p´s > .3). The fact that no differences in the total number of stained cells were found in the NAcb-Shell allows us to exclude that the

differences found in the number of c-Fos positive cells between adult and aged groups could be due to the effect of age on neuron survival (**Figure 3**).

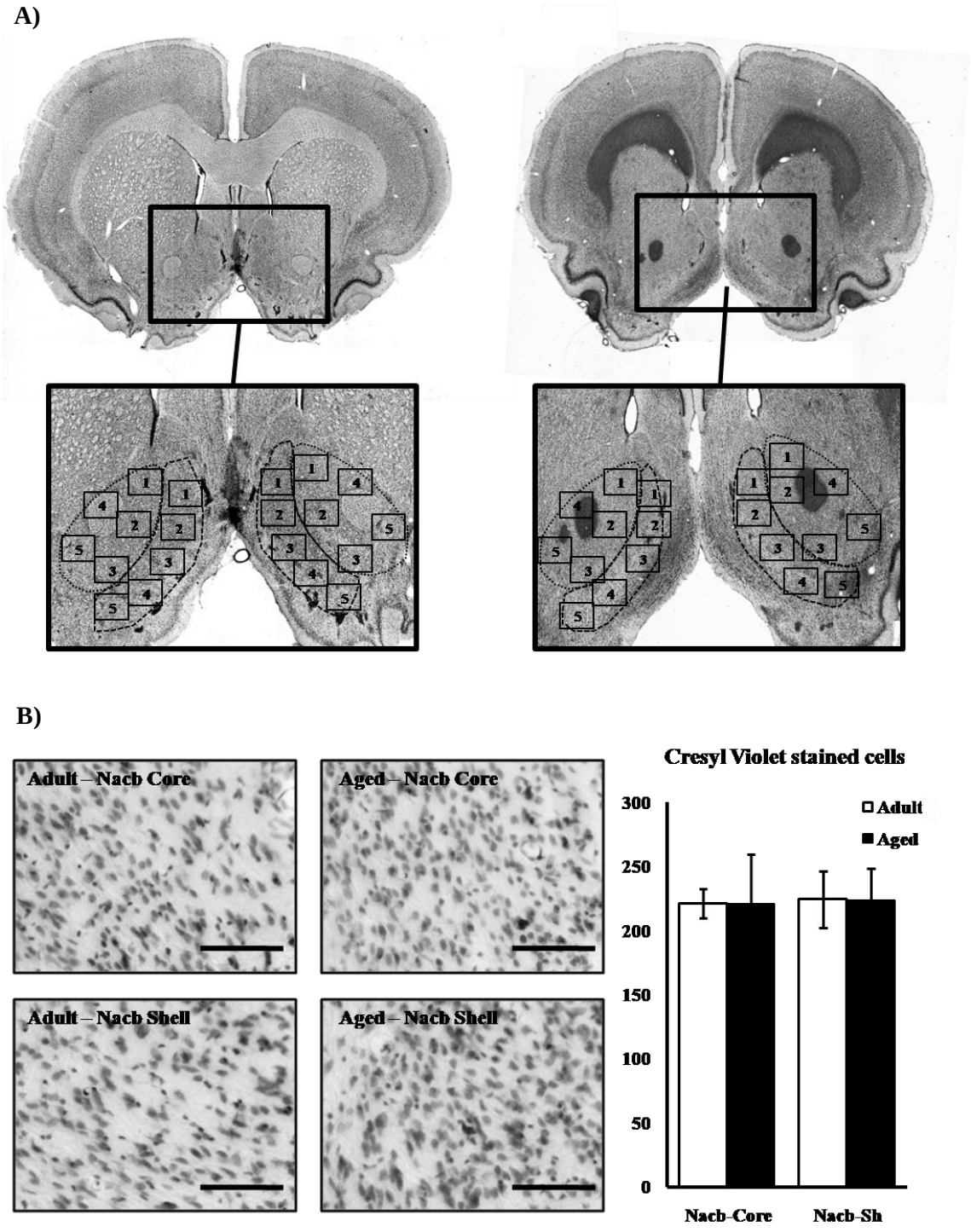

Figure 3: (A) Schematics of the microphotographs (40X) of the nucleus accumbens core and Shell that were stained with Cresyl Violet. All the microphotographs were captured following a dorso-ventral and

medio-lateral axis and were labeled with numbers from 1 to 5 in order to cover the entire extension of both nuclei for the adult (left panel) and aged (right panel) groups of age. **B)**Representative microphotographs obtained at 40X magnification of the nucleus accumbens core for adult and aged rats (top panel) and the nucleus accumbens shell for adult and aged rats (bottom panel). On the right, no differences in the counted cells stained with Cresyl Violet. Scale bars = 100 µm.

4. Discussion.

In this experiment we compared the number of c-Fos positive cells in the nucleus accumbens core and shell of adult and aged rats that were exposed to a novel flavor solution for one, two or six consecutive days. Consistent with the fact that the nucleus accumbens has been proposed to participate in flavor memory and the processing of flavor palatability, in adult rats we found an increased number of c-Fos positive cells in the NAcb-Shell after drinking the novel solution twice compared to the first and the sixth time. This increase was not evident in NAcbC, thus confirming our first hypothesis in adult rats. This is consistent with previous findings showing that reversible inactivation by anisomycin (Pedroza-Llinás et al., 2009) and neurotransmitter antagonists (Ramírez-Lugo et al., 2007) of NAcb-Shell prevented the attenuation of taste neophobia.

As it has been previously reported (Gómez-Chacón et al., 2015; Grau-Perales et al., 2019a), the behavioral results indicated that there was attenuation of the neophobic response to the novel flavor in both groups of

age. It can be conceived that since the novel flavor was not followed by negative consequences after the first exposure it was classified by the animals as safe so that the neophobic response was attenuated, increasing the biological significance of the flavor (Federico Bermúdez-Rattoni, 2004). The increased activity found in the NAcb-Shell during the second exposure supports its involvement in the formation of the safe flavor memory. Nevertheless, the attenuation of flavor neophobia was delayed in the case of aged rats. Aged rats did not increase consumption in the second exposure to the flavor, suggesting impairment of memory processes involved in familiarization. In fact, the attenuation of neophobia was evident at the third exposure and the neophobic response attenuated completely on day four while adult rats did it on day two.

The nucleus accumbens shell, but not the nucleus accumbens core, showed increased activity of the protein c-Fos when adult animals drank the novel flavored solution twice. This is relevant as in that specific day the animals showed an increase in consumption, indicating the existence of attenuation of the neophobic response. Moreover, once the neophobic response was completely attenuated on day six, the c-Fos activity was similar to that of the Novel group. This indicates that the activity found on the nucleus accumbens would not be related with the processing of flavor features such as taste, and also that the activity is not related to the detection of novelty or familiarity. Indeed, this activity seems to be related

to the formation of flavor memory and the consolidation of the safe taste memory. Similar patterns of dopamine activity have been described in the nucleus accumbens, as Bassareo and Di Chiara (1997) using microdialisys found a release of dopamine in the NAcb after the exposure to palatable and high-preferred food, but this response attenuated after two consecutive exposures to the same food. An alternative interpretation in terms of increased c-Fos expression induced by the motor activity during drinking is not feasible. As a matter of fact, the increase in the number of c-Fos positive cells does not parallel the consumption pattern of the flavored solution. The flavor solution intake increases throughout successive drinking sessions being significant such increase at the second exposure. However, the increase in c-Fos positive cells was only found at the second exposure (Familiar 1 group) while no differences were observed between Novel and Familiar 2 groups.

A different pattern of c-Fos activity was found in aged rats. While there were no differences between Novel and Familiar 1, c-Fos activity in NAcb-Shell but not NAcbC increased in the Familiar 2 group. An interpretation in terms of a delayed formation of the safe taste memory is consistent with the behavioral pattern as, they did not show a significant increase in vinegar consumption between days one and two. It can be conceived that, as vinegar is a non palatable flavor, the shifting in the hedonic value of the flavor would take more time to occur within the aged

rat brain. This would directly affect the attenuation of flavor neophobia, which would not happen at the second exposure to the flavor. Thus, recognition of the flavor as safe might require additional exposures to the flavor, resulting in an increased palatability of the flavor and thus, increasing the activity of the nucleus accumbens. However, it is important to note that aged rats as well as adult rats completely attenuated the neophobic response on day 6, but only aged rats exhibited that day increased number of c-Fos positive cells compared to the first day of exposure. It seems as if the nucleus accumbens of aged rats would not be processing only changes in flavor palatability but would be responding to the detection of flavor familiarity. In that case, it might be plausible that the nucleus accumbens would be participating in compensatory mechanisms and its activity would be necessary in order to achieve a behavioral response similar to that observed in adult rats.

The differential pattern of NAcb-Shell c-Fos activity in adult and aged rats allows us to exclude non-specific decline of brain activity induced by aging. In addition, cell quantification of Nissl stained cells using Cresyl Violet indicated no cell loss associated to aging. Therefore, the change of the brain activity patterns associated to aging could be interpreted as a reorganization of the brain circuit involved in processing flavor familiarity at advanced age. Such a reorganization could be expected taking into account the effect of the experience on a plastic brain along the life which

is evident also in taste related behaviors (Gámiz & Gallo, 2011; Morón & Gallo, 2007b). We have previously reported a similar change of the c-Fos activity pattern induced by flavor familiarity in the perirhinal cortex of aged rats (Gómez-Chacón et al., 2015). However, not all the areas involved in the attenuation of flavor neophobia are affected by aging in the same way since the pattern of changes is not uniform. In fact, we have also reported an overall increase of c-Fos activity of the posterior but not the anterior piriform cortex of aged rats exposed to a vinegar solution (Grau-Perales et al., 2019a).

In summary, it is conceivable that NAcb-Shell might be included together with amygdale (Gómez-Chacón, Gámiz, Foster, & Gallo, 2016; Gómez-Chacón et al., 2012), insular cortex (Bahar, Dudai, & Ahissar, 2004; Bermudez-Rattoni, 2014; Lin, Roman, Arthurs, & Reilly, 2012; Miranda, Ferreira, Ramírez-Lugo, & Bermúdez-Rattoni, 2003; Moraga-Amaro, Cortés-Rojas, Simon, & Stehberg, 2014), perirhinal cortex (Balderas et al., 2012; Gómez-Chacón et al., 2015; Gómez-Chacón et al., 2016; Morillas et al., 2017), piriform cortex (Grau-Perales et al., 2019a) and hippocampus (Grau-Perales et al., 2019b) as part of a flavor recognition memory brain circuit which shows complex reorganization throughout the lifespan. Further research on the specific role of the NAcb in recognition memory could help to understand dysfunctional restriction of dietary intake described in eating disorders often affecting to the older

population (Gallo, 2018) and it will contribute to advancements in diagnosis, assessment, behavioral intervention and nutritional management.

5. Acknowledgments.

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Capítulo 5

Serie Experimental 3. *Emotional component of taste recognition memory engaging a dopaminergic accumbens – amygdala network: role of D1 dopamine receptors*

1. Abstract

The attenuation of taste neophobia (AN) is a good model for studying the neural mechanisms of the emotional component of memory in rodents because taste recognition memory exhibits the rather unique feature of being necessarily linked to the hedonic properties of tastes. A single exposure to a novel taste not followed by aversive consequences is enough to attenuate the cautious neophobic response as the taste becomes safe and palatable. Given the involvement of the nucleus accumbens (NAcb) in reward and the amygdala (AMY) in emotional memories, we applied c-Fos immunohistochemistry as an index of neural activity in Wistar rats that were exposed to a vinegar solution for one, two or six days. An inverse pattern of increased NAcb versus decreased AMY activity was found on the second exposure day in which AN took place. There was a positive correlation between AN and the number of c-Fos positive cells in the NAcb shell but a negative correlation was found in the basolateral AMY. Furthermore the NAcb-AMY interplay relevant for AN seems to be mediated by dopamine D1 receptors (D1DR). The injection of SCH23390 (D1DR antagonist) in both NAcb and AMY during the second taste exposure resulted in impaired AN but injection of SFK-81297 (D1DR agonist) had opposite long term effects in each brain area. Furthermore, a combination of projection-specific DREADD expression and pharmacological intervention by D1DR agonists and antagonists revealed a dopaminergic NAcb-AMY network critical for adding the emotional component during the formation of a taste memory.

KEY WORDS: Accumbens, Attenuation of neophobia, Amygdala, Dopamine, Taste, DREADD, D1DR.

2. Introduction

There is a growth of interest in understanding the complex relationship among the emotional, motivational and cognitive components of memory. For this purpose taste recognition memory in rodents represents a choice model given that taste affective properties are intertwined with its discriminative properties along the sensory taste system (Yamamoto, 2006). Ingestive behavior relays heavily on the post-ingestional consequences of consumption that can be either aversive or appetitive but never neutral. In fact, rodents exhibit taste neophobia, i.e., a reluctance to ingest non-familiar tastes with unknown consequences. If the taste is followed by visceral distress a conditioned taste aversion (CTA) will take place and consumption will decrease. However, if the taste is not followed by aversive consequences the attenuation of taste neophobia (AN) will take place leading to the formation of an appetitive safe taste memory (Bermúdez-Rattoni, 2004). Thus, the taste memory contains hedonic, motivational and rewarding components determining consumption and offering a unique opportunity to study interactions with the cognitive component. Permanent (Morillas et al., 2017) and reversible (De la Cruz et al., 2008; Gutiérrez et al., 2004) lesions as well as immunohistochemical (Gómez-Chacón et al., 2015) studies have demonstrated the critical role of the perirhinal cortex in the cognitive component of safe taste recognition

memory, similar to that in object recognition memory (Warburton & Brown, 2015). Other brain areas such as the posterior piriform cortex (Grau-Perales et al., 2019a) and the dorsal hippocampus (Grau-Perales et al., 2019b) seem to be also involved in the cognitive component of taste recognition and its context-dependency. However, the brain mechanisms contributing to the emotional and rewarding component of the safe taste memory remain elusive. Candidate brain areas are the nucleus accumbens (NAcb) and the amygdala (AMY). It was proposed that NAcb contributes to the hedonic shifting of taste palatability by connections with the basolateral amygdala (BLA) (Yamamoto, 2006).

AMY is involved in emotional states associated to aggressive, maternal, sexual, and ingestive behaviors, being its role in fear conditioning one of the most thoroughly studied (LeDoux, 2007). Both taste and visceral sensory projections to BLA are associated with taste memory (Miranda, 2012; Reilly & Bornovalova, 2005; Yamamoto, 2006). The evidence pointing to its potential involvement in the formation of safe taste memories is scarce but BLA lesions interfere with the AN perirhinal activity pattern (Gómez-Chacón et al., 2012).

The two mayor NAcb subregions, core (NAcb-Core) and shell (NAcb-Sh), have been extensively investigated as a component of the reward system involved in different behaviors (Bassareo & Di Chiara, 1997, 1999; Bassareo et al., 2002; Yamamoto, 2006). Regarding taste

memory, the NAcb dopaminergic innervations plays also a role in CTA (S. Fenu et al., 2001; Ramírez-Lugo et al., 2006), preference learning (Shimura et al., 2002) and AN (Bassareo & Di Chiara, 1997). While the role of D1 dopamine receptors (D1DR) has been extensively studied in appetitive and aversive taste memories (Bodnar, 2018a; Fenu et al., 2001; Fenu & Di Chiara, 2003; Pedroza-Llinás et al., 2009; Ramírez-Lugo et al., 2006; Sclafani et al., 2011; Wichmann et al., 2012), its potential role in AN remains scarcely explored (Grau-Perales et al., 2019c).

In order to evaluate the role of the NAcb-AMY projection in the emotional/motivational component of the safe taste memory as well as the potential involvement of the dopaminergic innervations we propose: 1) to assess the activity of both areas during the taste familiarization process using c-Fos immunohistochemistry as an index of neural activity (**Exp. 1**); 2) to apply SCH23390 (D1DR antagonist) and SFK81297 (D1DR agonist) into the NAcb and the AMY in order to assess their effects on AN (**Exp. 2**); 3) to selectively modify the functional NAcb-BLA connectivity using the DREADD chemogenetic approach either by increasing AMY activity or decreasing NAcb activity during AN formation (**Exp. 3**); 4) to combine the chemogenetic inhibition of the NAcb-BLA pathway with pharmacological activation or blockade of D1DRs in the amygdala (**Exp. 4**).

3. Statistics

All data are presented as mean ± SEM. All statistical analyses are described in ***Supporting Information*** and were performed using SPSS software (IBM Corp. Released 2015. Version 23.0. Armonk, NY. IBM Corp.). The number of animals is indicated by "n". Comparisons between data were performed using repeated measures analyses of variance (ANOVA) followed by appropriate Bonferroni-corrected post-hoc comparisons when significant effects or interactions were observed. The null hypothesis was rejected at the $p<.05$ level.

4. Results.

4.1. Experiment 3.1.: c-Fos activity pattern in the nucleus accumbens and amygdala during taste recognition memory.

We began by testing whether the nucleus accumbens and the amygdala play a role in safe taste recognition memory. For this purpose, we used immunohistochemistry of the protein c-Fos as a marker of neural activity, a method which has been shown to be useful for exploring the brain circuits involved in taste learning (Bermúdez-Rattoni, 2004; Gómez-Chacón et al., 2012, 2015; Grau-Perales et al., 2019a, 2019c). Thirty-one male Wistar rats were exposed to 15-minute daily drinking sessions and the consumption was recorded. After five days of baseline in which all animals

were exposed to water, the animals were randomly assigned to the following groups: *Water* (Water; n=10) which was exposed only to water for six consecutive days; *Novel* (Novel; n=7), which had only one exposure to a cider vinegar solution (3%); *Familiar-1* (F-I; n=7) which was exposed to the vinegar solution for two consecutive days and *Familiar-2* (F-II; n=7) which was exposed to the vinegar solution for six consecutive days. After the last day of consumption of each group, the animals were sacrificed in order to perform immunohistochemistry of the protein c-Fos (see **Experimental procedures**).

The Novel group showed a lower intake of the non-familiar vinegar solution on Day 1 compared to water (BL) and also on the first day compared to Day 2. On day 3, the consumption of the vinegar solution reached an asymptote, thus indicating that the attenuation of taste neophobia already occurred on day 2 (see **Figure 1A**).

The mean number of c-Fos positive cells (±SEM) for the four familiarity groups (Water, Novel, F-I and F-II) during the drinking sessions is shown in the **Figure 1D (**NAcb-Core, NAcb-Sh) and **1E** (CeA and BLA). Both CeA and BLA showed a higher number of c-Fos positive cells in the Novel group than the F-I group (vinegar solution on Day 2). However, on Day 2 (the day in which AN took place) we found an inverse pattern of c-Fos expression in the NAcb-Shell consisting in an increase in the number of c-Fos positive cells.

We then wanted to measure to what extent those differential patterns of c-Fos expression observed at the different days of exposure to vinegar were related to behavior. For this purpose, we calculated Pearson´s correlations between the number of c-Fos positive cells in the four nuclei (NAcb-Shell, NAcb-Core, CeA and BLA) and three behavioral parameters (*Direct consumption (ml))* for both Novel and F-I groups separately), *Neophobia Index* which calculated the neophobic response to vinegar based on consumption on the previous water day, and *Attenuation of Neophobia (AN) Index*which calculated the increase in vinegar consumption on Day 2 compared to the previous day 1. Additional correlations between the activity of the four nuclei were performed in order to study potential relationships between c-Fos activity in the NAcb and AMY and the drinking behavior observed in an one-by-one animal basis (see **Statistical Analyses**).

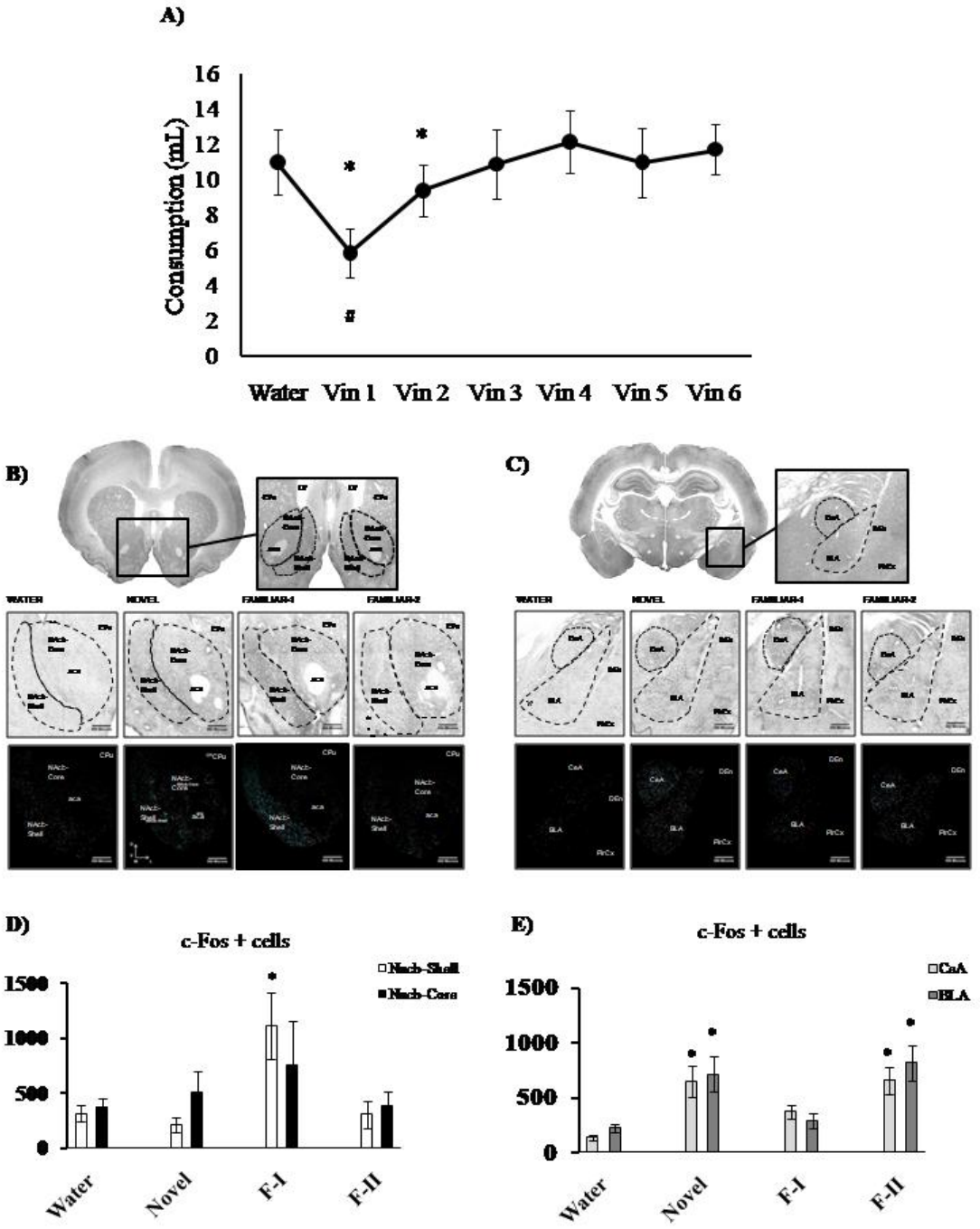

Figure 1: A) Mean (±SEM) of consumption in milliliters. * symbol represents statistically significant differences (p<.05) compared to vinegar 6. # symbol represents statistically significant differences (p<.05) compared to Water (baseline). **B)** Representative microphotographs of the nucleus accumbens (Core and Shell) and **C)** the amygdala (BLA and CeA) and the automatic counting performed by *imageJ* software. **D)** Mean (±SEM) of c-Fos positive cells in the four familiarity groups of the nucleus accumbens core and shell **(E)** and the BLA and CeA. * symbol represents statistically significant differences (p<.05) compared to Water group.

The results indicated statistically significant positive correlations between the parameter *Direct consumption* of vinegar in the Novel group and c-Fos positive cells in BLA (r=0.83, *p*=.021), showing more c-Fos positive cells those animals that drank more vinegar solution. No other correlation was found significant (see **Figure 2A**). Consistently, it was found a negative correlation between the *Neophobia Index* and c-Fos positive cells in BLA (r=-0.79, *p*=.035). Thus, the lower neophobia to vinegar, the higher vinegar consumption, resulting in more c-Fos cells observed in the BLA. This is also consistent with previous research indicating that vinegar is a taste that induces c-Fos expression in the AMY (Lin et al., 2012) (see **Figure 2A**).

However, regarding the *AN Index* and the number of c-Fos positive cells a positive correlation was found in the NAcb-Shell (r=0.80, *p*=.029), indicating that the higher AN, the higher number of c-Fos positive cells found in NAcb Shell, while a negative correlation was found in BLA (r=-0.77, *p*=.041), indicating an inverse activity pattern since the higher AN, the lower number of c-Fos positive cells found in BLA (see **Figure 2B**). No other correlations reached significance.

Additional correlations were performed between the activity of the four nuclei in each familiarity group in order to explore possible interactive relationships between them. The correlations performed for the Novel

group did not yield any significant result, meanwhile a significant negative correlation between c-Fos positive cells in NAcb-Shell and BLA was found in the F-1 group (r=-0.81, *p*=.026), indicating that in this group, the more cells found in NAcb-Shell, the less cells counted in BLA. This observation confirms the previous finding and points to a potential antagonistic relationship between the activity in NAcb-Sh and BLA that is specifically related to the attenuation of taste neophobia (see **Figure 2C left panel**).

In order to study the relationship between the changes observed in the NAcb-Shell and the BLA, we calculated an additional index called *c-Fos Estimated Change* (*c-Fos EC*). As it is impossible to obtain within subjects measures across days using immunohistochemistry data, we calculated the deviation of the expression of c-Fos observed on the F-I group (day two of vinegar) from the average number of c-Fos positive cells of the Novel group (day one of vinegar) (see **Statistical Analyses**). This estimation index allows us to analyze the magnitude of the variation of the F-I group over the mean observed on the Novel group as well as its directionality (either positive or negative) as values close to zero indicate that the number of c-Fos that does not vary between groups. Then we performed Pearson correlations between the *c-Fos EC* observed in the four areas. The results showed a negative correlation between the NAcb-Shell and the BLA (r=-0.86; *p*=.027), indicating that the more positive deviation from the average number of c-Fos positive cells in the NAcb-Shell

observed on vinegar day 1, the more negative deviation from the average number of c-Fos cells in the BLA (**See 2C and 2D**).

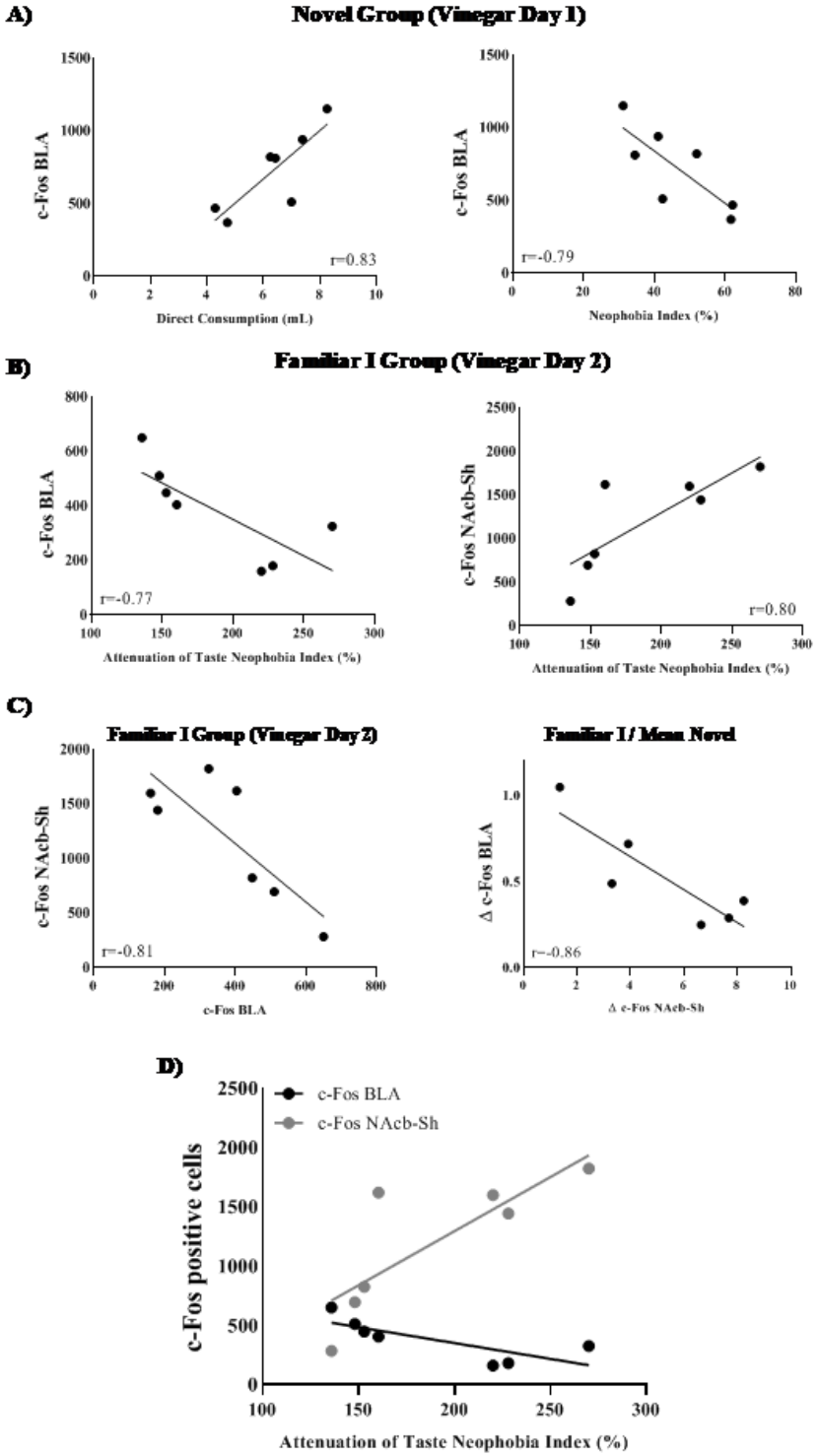

Figure 2: Representation of statistically significant Pearson´s correlations (p<.05) in **A)** the Novel group: the total number of c-Fos positive cells in the BLA was positively correlated with the direct consumption of vinegar (left panel) and negatively correlated with the neophobia observed on vinegar day 1; **B)** the F-I group: the attenuation of taste neophobia was positively correlated with the total number of c-Fos positive

cells in the NAcb-Sh and negatively correlated with c-Fos positive cells in the BLA; **C)** the total number of c-Fos positive cells in the BLA and the NAcb – Shell, showing a negative relation between these nuclei on the vinegar day 2 (left panel) andthe c-Fos Estimation Change (EC index) observed in the F-I group in the BLA and the NAcb – Shell, showing that the more deviation from the mean c-Fos positive cells of the Novel group observed in the NAcb – Shell, the less deviation observed in the BLA (right panel); **D)** c-Fos positive cells in the BLA and the NAcb – Shell as a function of the attenuation of taste neophobia, indicating that the more attenuation of taste neophobia observed, the more c-Fos positive cells observed in the NAcb – Shell and the less c-Fos positive cells observed in the BLA.

To sum up, it was found that when the taste was novel the number of c-Fos positive cells correlated with the total amount of vinegar intake within the BLA. However, in the second exposure day, the number of c-Fos positive cells was negatively correlated with AN in the BLA but positively correlated in the NAcb-Shell, suggesting an opposite role of these brain areas in the taste familiarization process.

4.2. Experiment 3.2.: D1 dopamine receptors and taste recognition memory.

In this series of experiments we aimed to explore the role of D1DR during the process of attenuation of taste neophobia in both the NAcb and the AMY by the intracerebral administration of the D1 dopamine antagonist (SCH-23390) or D1dopamine agonist (SFK-81297). In order to do so, male Wistar rats were exposed to 15-minute daily drinking sessions similar to those described in Experiment 3.1. One week before the experiment, all the animals were implanted bilaterally with guide cannulae

either in the NAcb-Shell or the AMY (**Figure 4**) (see **Experimental Procedures**).

Experiment 3.2.A: D1DR in the NAcb-Shell and the second exposure to the taste solution.

On **Experiment 3.2.A** the animals were randomly assigned to the following six groups: *vehicle + vinegar* (n=10), *SCH-23390 + vinegar* (n=10), *SFK-81297 + vinegar* (n=10), *vehicle + water* (n=6), *SCH-23390 + water* (n=14) and *SFK-81297 + water* (n=9). On vinegar day 2 all the animals received i.c. injections in the nucleus accumbens shell of either vehicle (phosphate-buffered saline, PBS; pH=7.4), dopamine D1/D5 receptors antagonist SCH-23390 or dopamine D1/D5 receptors agonist SFK-81297. The injections were performed 15 minutes before the drinking session.

The i.c. injection of PBS, SCH-23390 or SFK-81297 into the NAcb-Shell did not affect drinking behavior of those groups that were exposed to water (see **Figure 3C**). The i.c. injection of PBS did not caused any effect on AN as the animals increased consumption of vinegar on day 2 ($p<.05$). However, the i.c. injection of D1DR antagonist and agonist resulted in opposite effects on AN. On the one hand, SCH-23390 resulted in an impaired AN as those animals did not show increased consumption on vinegar day 2 (maintaining the same level of consumption observed on day

1). On the other hand, SFK-81297 resulted in enhanced AN as the animals increased consumption of vinegar on day 2. It is important to note that this increase in consumption was greater than that observed in the PBS group (See **Figure 3A**).

Experiment 3.2.B: D1DR in the AMY and the second exposure to the taste solution.

On **Experiment 3.2.B** the animals were randomly assigned to the following six groups: *vehicle + vinegar* (n=8); *SCH-23390 + vinegar* (n=10); *SFK-81297 + vinegar* (n=7); *vehicle + water* (n=8); *SCH-23390 + water* (n=9) and *SFK-81297 + water* (n=2). On test day 2 all animals received i.c. injections in the amygdala of either vehicle (phosphate-buffered saline, PBS; pH=7.4), dopamine D1/D5 receptors antagonist SCH-23390 or D1/D5 receptors agonist SFK-81297. The injections were performed 15 minutes before the drinking session on test day 2.

The i.c. injection of PBS, SCH-23390 or SFK-81297 into the AMY did not cause any effect over the drinking behavior of those groups that were exposed to water (all *p´s*>.31) (see **Figure 3D**). The i.c. injection of PBS did not affect the attenuation of taste neophobia as the animals increased consumption of vinegar on day 2 ($p<.001$). The i.c. injection of SCH-23390 resulted in impaired AN on day 2 that was also maintained the following day, as rats drank significantly less vinegar on test days 1, 2 and

3 compared to water baseline (p<.001; p<.01 and p<.01 respectively) and there were no differences between these three test days (all p´s>.05). The i.c. injection of SFK-81297 disrupted the typical AN pattern, as although consumption of vinegar increased on test day 2 compared to test day 1 (p<.01), an unexpected decrease in consumption was observed the day following the injection, as no differences were found between test days 1 and 3 (p=1) (See **Figure 3B**).

Experiment 3.2.C: D1DR in the AMY during the first exposure to the taste solution.

As the AMY has been reported to participate in the expression of neophobic response and its excitotoxic lesion results in the absence of taste neophobia (Beatriz Gómez-Chacón et al., 2012), on **Experiment 3.2.C** we aimed to investigate whether the activity of D1DR in the AMY is not only related to the attenuation of taste neophobia, but also to the expression of the neophobic response to a new taste *per se*. To do that, animals were randomly assigned to the following three groups: *vehicle* + *saccharin* (n=9); *SCH-23390* + *saccharin* (n=9) and *SFK-81297* + *saccharin* (n=7). On test day 1 all the animals received i.c. injections in the amygdala of either vehicle (phosphate-buffered saline, PBS; pH=7.4), dopamine D1/D5 receptors antagonist SCH-23390 or D1/D5 receptors agonist SFK-81297. The use of sodium saccharin (0,5%) as a novel taste instead of vinegar

allows us to obtain a wider behavioral range in order to detect a potentiated attenuation of taste neophobia avoiding floor effects. The injections were performed 15 minutes before the drinking session on test day 1.

The i.c. injection of PBS did not cause any effect on neither the neophobic response nor its attenuation as rats drank lesser amounts of saccharin on test day 1 compared to the water baseline ($p<.01$) and the rest of test days (all $p<.01$). The i.c. injection of SCH-23390 did not cause any effect on the expression of the neophobic response, but resulted in a mild impairment on the attenuation of taste neophobia, as rats drank lesser amounts of saccharin on test day 1 compared to water baseline ($p<.01$) and the rest of test days (all $p<.01$) and also drank lesser amounts of saccharin on test day 2 compared to test day 3 ($p<.05$). The i.c. injection of SFK-81297did not affect the neophobic response to saccharin but impaired AN completely, as rats did no increase consumption of saccharin during any of the following test days ($p>.05$) (See **Figure 3E**). This indicates that pharmacological activation of D1DR in the AMY before the first encounter with a novel taste results in the formation of a taste aversion substituting AN.

Experiment 3.2.D: D1DR in the AMY after the second exposure to the taste solution.

Due to the long-lasting effects over the attenuation of taste neophobia observed in Experiment 3.2.B, on **Experiment 3.2.D** we wanted to explore the role of D1DR in the consolidation of a safe taste memory by post-drinking injections. In order to do that, the animals were randomly assigned to the following three groups: *vehicle + vinegar* (n=9); *SCH-23390 + vinegar* (n=9) and *SFK-81297 + vinegar* (n=7). After drinking on test day 2 all animals received i.c. injections in the amygdala of either vehicle (phosphate-buffered saline, PBS; pH=7.4), dopamine D1/D5 receptors antagonist SCH-23390 or D1/D5 receptors agonist SFK-81297. The injections were performed 15 minutes after the drinking session on test day 2.

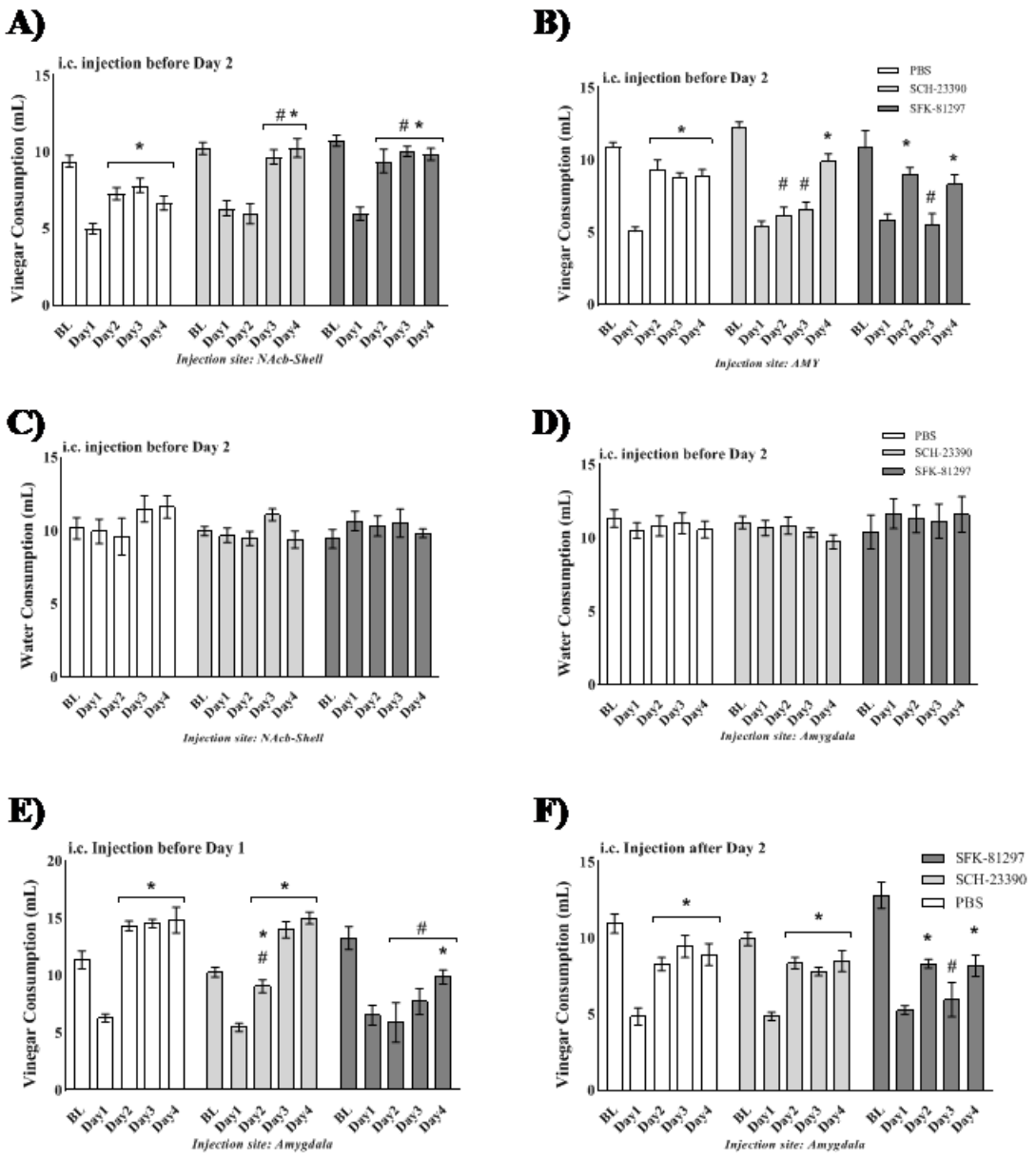

Figure 3: Mean (±SEM) of the consumption of the three groups that were exposed to vinegar and were injected with PBS, SCH-23390 and SFK-81297 inthe NAcb – Shell **(A)** and the Amygdala (CeA and BLA) **(B)** 15 minutes prior to drink vinegar on Day 2; the control groups that were exposed to water and were injected with PBS, SCH-23390 and SFK-81297 in the NAcb- Shell **(C)** and in the Amygdala (CeA and BLA) **(D);** the groups that were exposed to saccharin and were injected in the Amygdala prior to drink saccharinon Day 1 **(E)**;the groups that were exposed to vinegar and were injected in the Amygdala after drinking vinegar on day 2 **(F)**. The symbol * represents statistically significant differences (p<.05) with Day 1 within the same injection group. The symbol # represents statistically significant differences (p<.05) with the same Day of the PBS group.

The i.c. injection of either PBS or SCH-23390 did not affect AN on day 3 as the rats drank lesser amounts of vinegar on test day 1 compared to the water baseline (p<.01) and the rest of test days (all p<.01) in both cases. The i.c. injection of SFK-81297 resulted in a dramatic decrease of vinegar intake on day 3. The rats drank lesser amounts of vinegar on test day 1 compared to water baseline (p<.001) and test days 2 and 4 (all p´s <.01) and no differences in consumption were observed between test days 1 and 3 (p=1) (See **Figure 3E**).

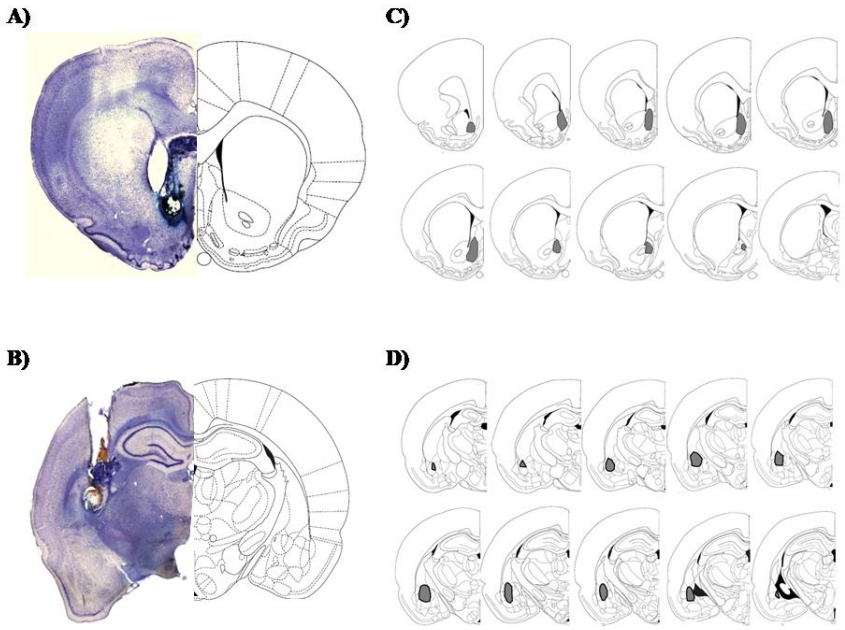

Figure 4. Example photomicrographs of the injection site in **A)** the nucleus accumbens shell (NAcb-Sh) and **B)** the amygdala (AMY). On the right, the panels show brain schematics with shading indicating the extent of the drug infusion for the NAcb-Sh **C)** and the AMY **D)**.

4.3. Experiment 3.3.: NAcb-Shell – AMY pathway: chemogenetic manipulation.

In the previous series of experiments we observed an inverse relationship between the nucleus accumbens shell and the amygdala during AN. We observed using c-Fos immunohistochemistry a higher BLA activity during the neophobic response, and an inverse pattern of activity in NAcb-Shell and BLA during AN formation, being AN highly positively correlated with the c-Fos expression in the NAcb-Shell and negatively correlated with the c-Fos expression in BLA. Additionally, this activity seems to be mediated by D1 dopamine receptors, whose pharmacological blockade or activation result in opposite behavioral effects depending on the brain area. Taking all these data in account, it could be hypothesized that this antagonistic relationship is required for safe taste learning. However, using the previous approach does not allow us to explore the directionality of this relationship. For this purpose, we used the DREADD chemogenetic approach to identify the dopaminergic circuit involved (Boender et al., 2014; Roelofs et al., 2017). A combination of two viral vectors was infused into the BLA and the NAcb-Shell in order to be able to manipulate the activity of this specific pathway.

In one condition, the retrograde CAV-Cre recombinase expressing virus was targeted to the BLA and the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the NAcb-Shell. In a second

condition, the retrograde CAV-Cre recombinase expressing virus was targeted to the NAcb-Shell and the excitatory DREADD pAAV2-hSyn-DIO-hM3D(Gq)-mCherry was targeted to the BLA (see **Figure 5**). Four weeks after the surgery, the animals were subjected to the same behavioral procedure consisting on four days of water (baseline phase) and four days of exposure to a 3% cider vinegar solution (test phase). All the animals received i.p. of Clozapine N-Oxide dihydrochloride (CNO- DHCL) 1.5 hours before the drinking session on test day 2. Thus, this manipulation allowed us to selectively silence the firing of neurons in the NAcb-Shell that are projecting directly to BLA (condition 1) and to selectively induce the firing of neurons in the BLA that are projecting directly to the NAcb-Shell (condition 2), since those are the neurons that are expressing the DREADD through the enhanced cre-recombinase expression produced by the CAV-cre vector. The expression of the DREADDs was assessed using immunofluorescence of anti mCherry four weeks after the viral infusions. As a control, some animals were injected with the DREADD (either excitatory in the BLA or inhibitory in the NAcb-Shell), but were not injected with CAV-cre, thus impeding the expression of the DREADDs.

The predictions are that if the increased activity of NAcb-Shell neurons receiving BLA projections is required on day 2 for a normal AN, then its inhibition on that day should result in impaired AN. Additionally, as a reduced activity in the BLA has been shown to occur on day 2 for a

normal attenuation of taste neophobia, the selective activation BLA neurons receiving NAcb-Shell projections would also impair AN on day 2. Additionally, this approach will also lead us to a better comprehension of the specific role of both nuclei, by the examination of the behavioral response on the specific day in which CNO-DHCL was injected and its long-term consequences.

In order to exclude non-specific effects of the CNO-DHCL injection over AN, a third group of animals was used as a control. In this group, one half of the animals were DREADD+/CAV-cre- and were i.p, injected with CNO-DHCL and the other half were DREADD+/CAV-Cre+ and were i.p. injected with sterile 0.9% NaCl solution.

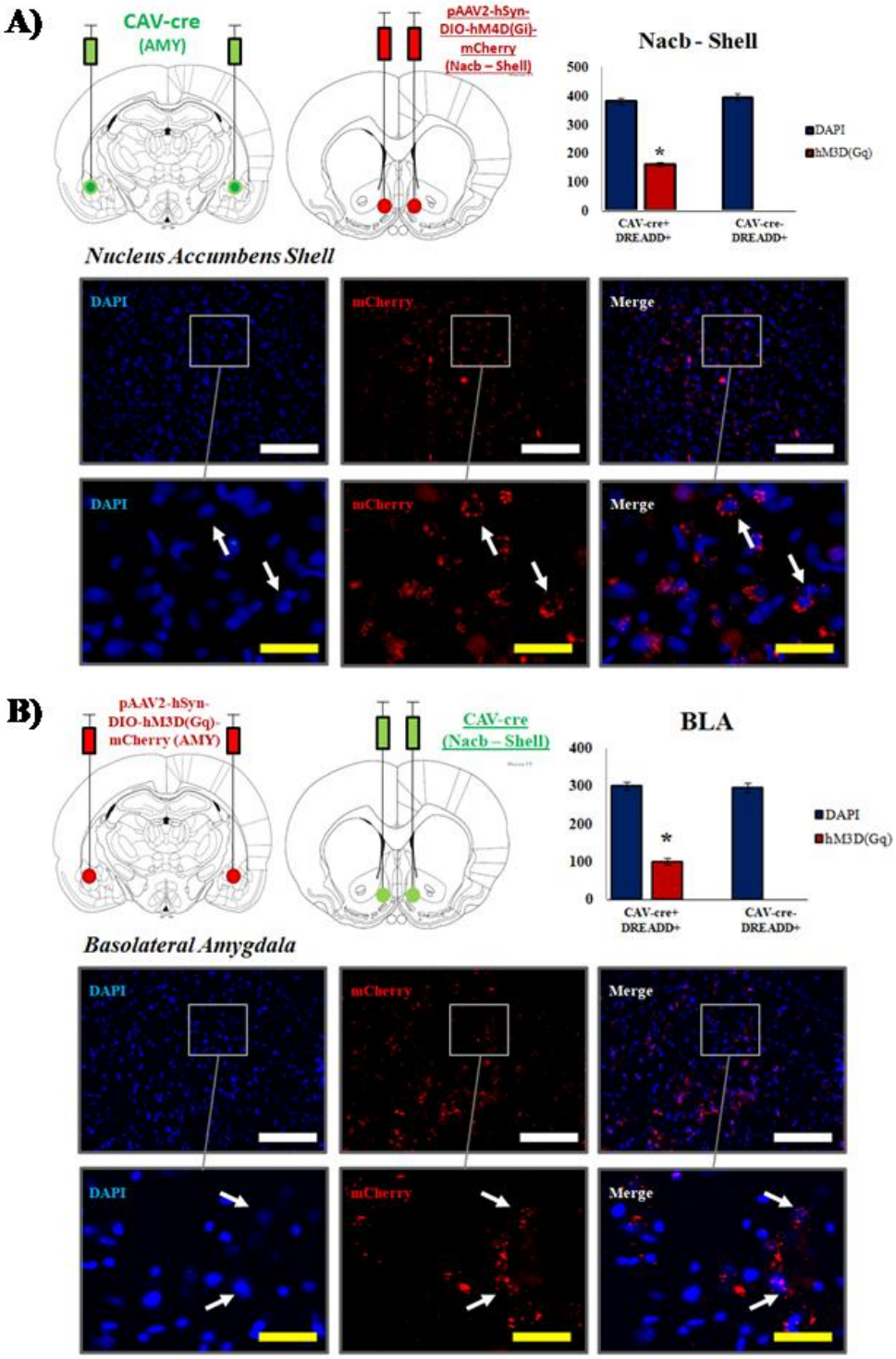

Figure 5. Representation of the two conditions used in Experiment 3.3. Panel **A)** represents the condition in which the CAV-Cre recombinase expressing virus was targeted to the BLA and the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the NAcb-Shell. Panel **B)** represents the condition in which the retrograde CAV-Cre recombinase expressing virus was targeted to the NAcb-Shell and the excitatory DREADD pAAV2-hSyn-DIO-hM3D(Gq)-mCherry was targeted to the BLA. The bottom panels from A) and B) represent the assessment of both DREADDs using

immunofluorescence of anti-mCherry. White scale bars = 20X magnification. Yellow scale bars = 40X magnification.

The results showed that the Control group showed normal attenuation of taste neophobia, as they were able to increase consumption of vinegar on day 2 compared to day 1 and reached the asymptote in consumption (p<.001). However, the chemogenetic manipulation of the NAcb-Shell–AMY pathway resulted in different effects over the attenuation of taste neophobia. On the one hand, the animals receiving NAcb-Shell inhibition on day 2 showed a delayed pattern of attenuation of taste neophobia, which occurred on day 3, indicating that the activity of NAcb-Shell is necessary for normal safe taste learning. On the other hand, the animals receiving AMY activation on day 2 did not show attenuation of taste neophobia neither that day nor the following days of exposure. Moreover, they decreased consumption of vinegar on day 3 compared to day 1 (p=.03), indicating the development of a strong aversive response to the taste **(Figure 6A).**

The effect of the DREADD infection and CNO-DHCL injection over consumption were also assessed with additional control groups that were exposed only to water. The animals were previously infused with both DREADDs in the same way that the animals exposed to vinegar (half of them injected with Gi-DREADD in NAcb-Shell and the other half with Gq-DREADD in the AMY) and they i.p. injected 1.5 hours before drinking

water with either CNO-DHCL or 0,9% NaCl. No differences were found in water consumption neither between the injection groups nor between drinking days (all *p´*s>.3). This allowed us to exclude any alternative interpretations in terms of both the DREADDs and the i.p. administration of drugs having general or unspecific effects over consumption **(Figure 6B)**.

These results point to a differential involvement of these two brain areas in AN, being the NAcb-Shell selectively related to the formation of an appetitive taste memory and BLA to the formation of an aversive taste memory. Thus, it can be conceived that the activation of NAcb-Shell during the second exposure to the taste would induce a global inhibition of BLA, preventing the formation of an aversive memory and resulting in increased consumption of the taste.

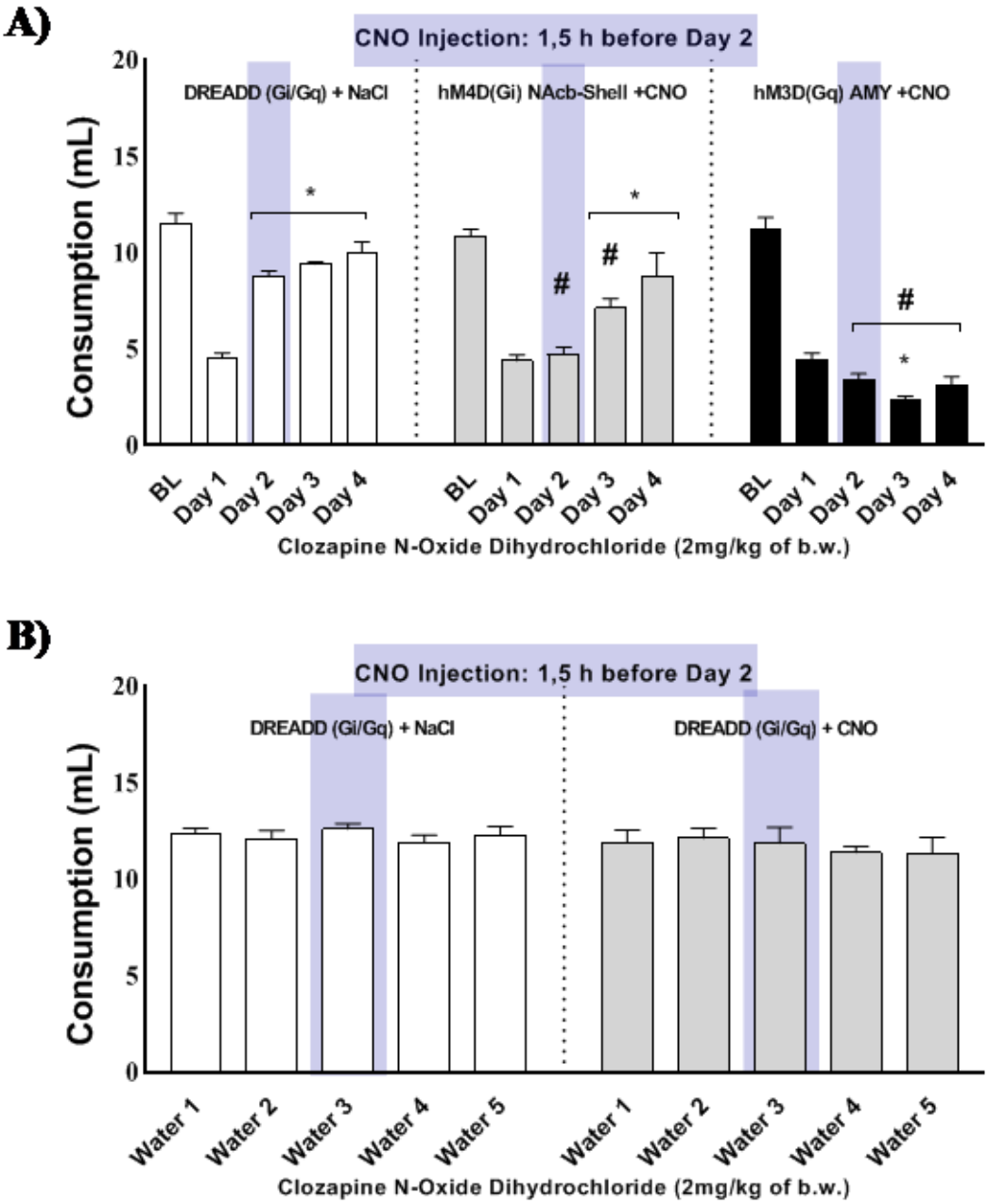

Figure 6. Mean (±SEM) of the consumption of **(A)** the three groups that were exposed to vinegar and were i.p. injected with either NaCl or CNO DHCL in order to activate the DREADD (either excitatory or inhibitory)and**(B)** the two groups that were exposed to water and were i.p. injected with either NaCl or CNO DHCL in order to activate the DREADD (either excitatory or inhibitory). The i.p. injection of CNO was performed 1.5 hours before the corresponding drinking session (yellow box). The symbol * represents statistically significant differences (p<.05) compared to **(A)** Day 1 and **(B)** Water 2 within the same injection group. The symbol # represents statistically significant differences (p<.05) with the same Day of the Saline control group.

4.4 Experiment 3.4.: Chemogenetic inhibition of the NAcb-Shell and D1DR activity in AMY.

In the previous experiment we observed that BLA chemogenetic activation resulted in the formation of an aversion to the novel taste. However, the chemogenetic inactivation of NAcb-Shell resulted in a delayed AN. Taking all this data into account it might be conceivable to hypothesize that NAcb-Shell dopaminergic projections are required to inhibit BLA in order to allow the formation of a safe taste memory, leading to increased consumption of the familiar safe taste, i.e., leading to the attenuation of taste neophobia.

In order to test this hypothesis, we combined the DREADD chemogenetic approach to selectively inhibit the NAcb-Shell-AMY connectivity similarly to Experiment 3.3. with i.c. injections of D1DRs antagonist (SCH-23390), agonist (SFK-81297) and vehicle (PBS) into AMY. For this purpose, the retrograde CAV-Cre recombinase expressing virus was targeted to the BLA, the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the NAcb-Shell and the animals were also implanted guide cannulae for *in vivo* i.c. administration of drugs into AMY (see **Experimental procedures**). Four weeks after the viral injection and cannulae implantation, the animals were subjected to the same behavioral procedures used in Experiment 3.3. The i.p. CNO-DHCL incjection was performed 1.5 hours before the drinking session on taste day

2. Additionally, the animals were randomly assigned to the following conditions according to the i.c. infusion they received in the amygdala: *SFK-Pre Day 2*, *Vehicle-Pre Day 3*, or *SCH-23390-Pre Day 3*.

Our predictions are that the chemogenetic inhibition of the NAcb-Shell would result in an impaired AN on day 2 as previously observed (*Vehicle-Pre Day 3* group). The i.c. administration of SFK-81297 15 minutes before the drinking session should mimic the effect of the inhibited NAcb-Shell-AMY projection, thus reverting the effect of the chemogenetic inhibition, and promoting normal AN on day 2. And finally, the administration of SCH-23390 previous to taste day 3 should block the delayed connectivity between NAcb-Shell and AMY, thus preventing the increased consumption of vinegar on Day 2 (due to chemogenetic inhibition of the NAcb-Shell) and Day 3 (due to pharmacological blockade of D1DRs in AMY).

The results showed that the chemogenetic inhibition of the nucleus accumbens on day 2 resulted in delayed AN, which occurred on day 3 as previously observed in Experiment 3.3. However, the additional AMY pharmacological intervention had different effects on AN. On the one hand, the animals that received i.c. injection of SCH-23390 (D1DR antagonist) on day 3 did not increase consumption of vinegar until day 4. This is consistent with the effect observed of SCH-23390 on AN observed in Experiment 3.2.B (**Figure 7A**). On the other hand, the animals that

received the D1DR agonist SFK-81297 on day 2 showed normal AN (p<.001) (**Figure 7B**). This suggests that the D1DR activity that is necessary for normal attenuation on day two is dependent on the activity of the NAcb-Shell. Thus, the administration of D1DR agonist would be inducing an artificial effect on the AMY similar to that induced by the projections coming from the NAcb-Shell and thus, reverting the effect of the NAcb-Shell chemogenetic inhibition.

Due to the unexpected decreased consumption of vinegar on day 3 observed in Experiment 3.2.D, we also aimed to investigate the effect of the chemogenetic inhibition of the NAcb-Shell and i.c. administration of SFK-81297 after drinking on day 2. The previous result could be showing that the AMY might also be related to memory updating of the taste memory. If this were the case, reduced consumption of vinegar on Day 3 should be found. The results showed that these animals did not increase consumption of vinegar neither on day 2, due to the chemogenetic inhibition of the NAcb-Shell (as expected), nor on Days 3 and 4 (**Figure 7B**). In fact, the animals showed the development of a taste aversion to the vinegar. These results might point to the existence of a differential timing in the activity of the amygdala. Thus, if D1DRs in the AMY are activated *during* the taste exposure at the drinking session, this might result in a global inhibition of the AMY and a safe taste memory is formed. However, if D1DRs in the AMY are activated *after* the taste exposure, the

development of a taste aversion occurs. This is highly consistent with the notion that BLA receives vagal projections containing information about post-ingestional consequences of what has been consumed. This information would take time to reach BLA and would activate it minutes – even hours – after the drinking session. The results obtained on Experiment 3.2.D might take in account for this explanation, as the pharmacological activation of D1DRs might last for almost 40 minutes due to the high dosage of SFK-81297 used. This would have resulted in D1DRs activation during the taste drinking session (thus showing increased consumption on day 2), but also D1DRs activation minutes after the drinking session, showing in aversion on the next encounter with the taste, However, on Experiment 3.2.D the decreased consumption of vinegar only lasted for 24 hours and was not as strong as the results obtained on Experiment 3.4. This could be explained by the appetitive response observed on day 2 (due to the activation of D1DRs *during* the taste session), causing a phenomenon of latent inhibition to the taste aversion, resulting in a mild aversion rather than a global avoidance of the taste. These results are consistent with recent findings showing the co-existence of two different populations of neurons within the BLA and CeA, being one related to the formation of appetitive memories and the other to aversive memories (J. Kim, Pignatelli, Xu, Itohara, & Tonegawa, 2016; J. Kim, Zhang, Muralidhar, LeBlanc, & Tonegawa, 2017).

In all, these data suggest that the NAcb-Shell–AMY pathway relevant for AN highly relies on the activity of D1DRs. Thus, the dopaminergic projections from the accumbens that target the amygdala and produce the activation of D1DR during the second encounter with the now familiar taste would be inducing the previously observed inhibition of the amygdala, which is necessary for safe taste memory formation. If this activation is prevented (either by chemogenetic inhibition of the NAcb-Shell or by the administration of D1DR antagonists) the AMY activity increases and this maintains the neophobic response to the taste, thus impairing AN on that specific day. In this case, AN would occur the following day, once the dopaminergic neurotransmission would recover its normal activity on day 3. This would explain the delayed AN observed in those animals that were injected with SCH-23390 on day 3, as they would not have recovered yet the normal activity of dopamine.

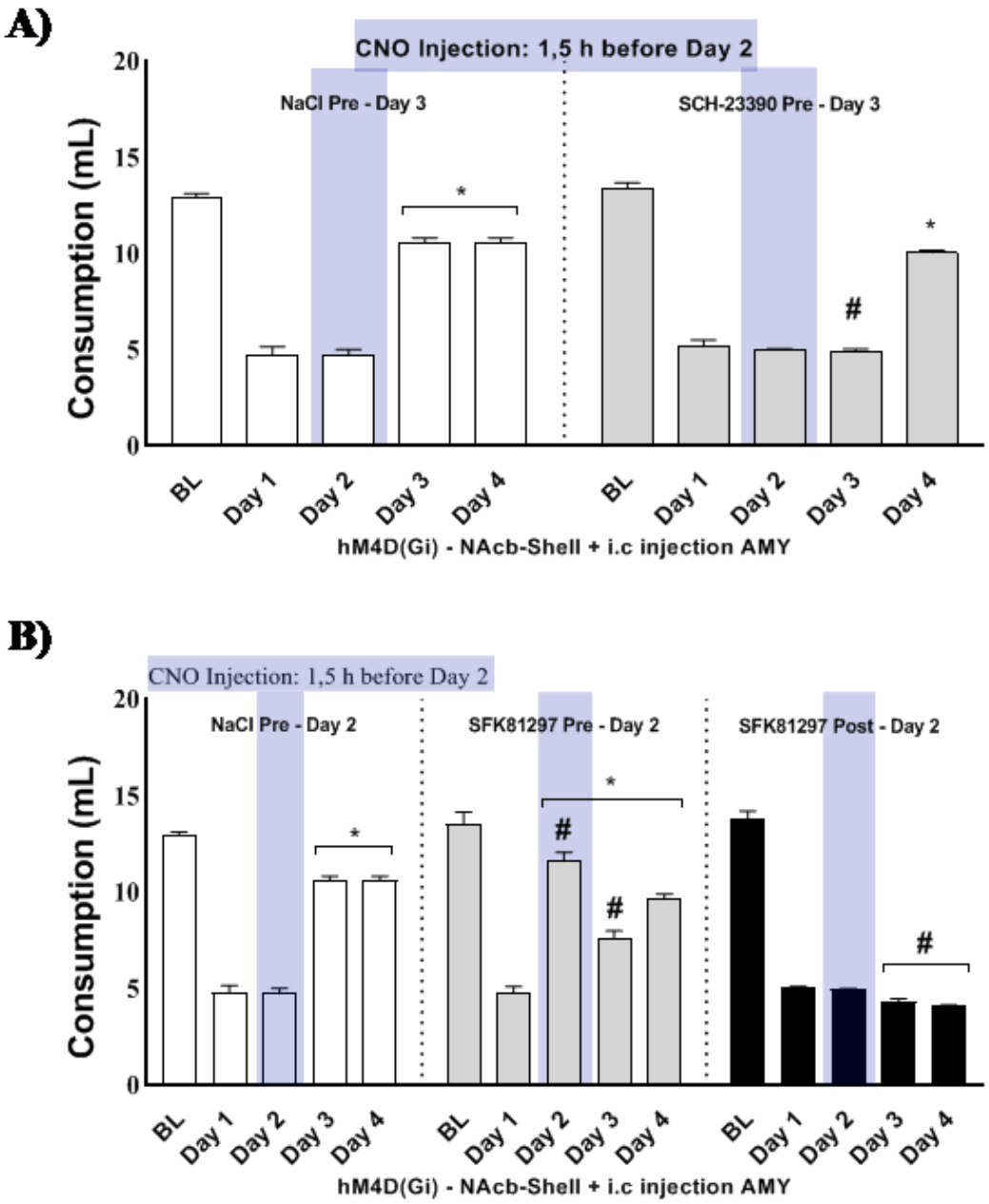

Figure 7. Mean (±SEM) of the consumption of **(A)** the groups that were exposed to vinegar and were i.p. injected with CNO DHCL in order to activate the inhibotory DREADD in the NAcb-Shelland were i.c. injected with either NaCl or SCH-23390 in the AMY **(B)** the groups that were exposed to vinegar and were i.p. injected with CNO DHCL in order to activate the inhibitory DREADD located in the NAcb-Shell and were also i.c. injected with SFK-81297 in the AMY. The i.p. injection of CNO was performed 1.5 hours before the corresponding drinking session (yellow box) and the i.c. administration of the drugs were performed **(A)** 15 minutes before the corresponding drinking session and **(B)** either 15 minutes before or after the corresponding drinking session. The symbol * represents statistically significant differences (p<.05) compared to Day 1 within the same injection group. The symbol # represents statistically significant differences (p<.05) with the same Day of the Saline control group.

5. Discussion

The present series of experiments identify a NAcb-Shell-BLA dopaminergic network mediated by D1-like receptors relevant for the affective component of taste recognition memory, which depends on the interplay of opposite activity patterns between NAcb-Shell and BLA during the formation of the safe taste memory.

In the first experiment we found an increased number of c-Fos positive cells in NAcb-Shell after the second exposure to a vinegar solution compared to the first and sixth exposures (see **Figure 1E**). This suggests the NAcb-Shell involvement at the beginning of the familiarization process. However, once the taste becomes fully familiar the NAcb-Shell activity returns to the previous level. This is consistent with the proposal of a NAcb role in the hedonic shifting once the taste is classified as safe after confirming the absence of negative visceral consequences (Yamamoto, 2006). As this takes time (minutes and even hours) the behavioral change would be evident at the second taste exposure. Thus, the increased NAcb-Shell activity during the second drinking session in which the formation of an appetitive safe taste memory takes place is consistent with the role of the area in the rewarding processes leading to behavioral reinforcement.

However, the inverse pattern of c-Fos expression was found in two nuclei of the amygdala, the BLA and the CeA. In both nuclei, there was an increased expression of c-Fos after consuming the novel taste for the first

time compared to the second exposure. This is in accordance with previous research relating the BLA and the CeA with novelty detection and the neophobic response to the novel tastes (Gómez-Chacón et al., 2012; Reilly & Bornovalova, 2005). Moreover, after the second exposure to the taste, we found a decreased expression of c-Fos compared to both the first and the sixth exposure, coinciding with the increase observed in the NAcb-Shell. Accordingly, it has been reported a decreased expression of c-Fos in the AMY after consuming a familiar taste compared to a novel taste (Lin et al., 2012). This seems to be consistent with the role of the AMY in the formation of taste aversive memories. It is conceivable that, in absence of aversive consequences after consumption of a novel taste, the AMY activity could be inhibited facilitating the formation of a safe taste memory.

The specific relationship between the expression of c-Fos and the attenuation of neophobia is further supported by within-subjects correlational analyses. There was a significant positive correlation between AN index and the number of c-Fos positive cells selectively in NAcb-Shell but not NAcb-Core. On the contrary, there was a negative correlation between the same index and the number of c-Fos positive cells selectively in BLA but not CeA. Thus, the higher AN, the higher c-Fos expression in the NAcb-Shell, meanwhile the opposite was found for the BLA as the higher AN, the lower number of c-Fos positive cells in the BLA was found (**Figure 2B**). Furthermore, the number of c-Fos positive cells in NAcb-

Shell was negatively correlated with the number of c-Fos positive cells in BLA (**Figure 2C and 2D**).

In order to understand the neurochemical mechanisms underlying this inverse relationship found, the second experiment focused on the dopaminergic system because of its role in emotion and specifically on D1DRs have previously been reported to participate in taste learning phenomena such as CTA (Valentina Bassareo et al., 2002) and conditioned flavor preferences (Touzani et al., 2008). Our results confirm the relevance of D1DRs during the second exposure to the taste in both NAcb-Shell and AMY for safe taste recognition memory in order to take place.

Regarding the NAcb-Shell, in the experiment 3.2.A pharmacological blockade of D1DRs using the dopamine antagonist SCH-23390 before the second exposure to the taste prevented AN, while pharmacological activation of D1DR using the dopamine agonist SFK-81297 facilitated AN as the consumption of the flavor increased (Experiment 3.2.A). In fact, the consumption was significantly higher than that observed in the control vehicle group. The effect of the dopaminergic drugs cannot be attributed to non-specific effects on drinking behavior because they did not alter water intake in control groups (**Figure 3C and D**).

Regarding the AMY, in the Experiment 3.2.B our results indicate that pharmacological blockade of D1DRs using SCH-23390 before the second taste exposure also prevented AN while it did not affect water

consumption. This is consistent with previous reports indicating that D1DR neurotransmission is necessary for the acquisition of conditioned flavor preferences(Touzani et al., 2013). We also evaluated a potential effect of modifying D1DR neurotransmission into AMY during different stages of the familiarization process. The experiment 3.2.C showed that injecting SCH-23390 prior to the first encounter with the novel taste did not prevent the neophobic response but mildly altered AN on day 2 since this group drank significantly less than the control group. This supports the notion of the D1DR as a modulator of other neurotransmission systems, although its activity would not be necessary for the neophobic response to take place. In the experiment 3.2.D the animals received post-drinking injections of D1DR agonist and antagonist on day 2 (**Figure 3D**). The administration of SCH-23390 did not have any effect on taste recognition memory as animals consumed similar amounts of the taste solution that the control group 24 hours later. However, the administration of the agonist SFK-81297 resulted in a reduced consumption of saccharin 24 hours later. This would support a role for the D1DRs in the AMY in the updating process of the safe taste memory. The modulation of taste consumption observed during the following session might be attributed to the relevance of D1DRs in the AMY for memory updating processes (Osorio-Gómez, Guzmán-Ramos, & Bermúdez-Rattoni, 2016, 2017).

Therefore, D1DR agonist had opposite effects on AN depending on the brain area. While in the NAcb-Shell the AN was favored, in the case of the AMY the AN formation was prevented. This further supports the differential involvement of these brain areas in opposite hedonic shifts, being the amygdala related to aversive taste learning and the nucleus accumbens to safe taste learning processes (Bassareo & Di Chiara, 1999; Bassareo et al., 2002; Gómez-Chacón et al., 2012; Touzani et al., 2008).

In the third experiment we applied projection-specific DREADD expression to identify the role of the NAcb-AMY pathway in AN. The results confirmed that both chemogenetic inhibition of the NAcb-Shell neurons projecting to BLA and chemogenetic activation of BLA neurons projecting to NAcb-Shell during the second taste exposure disrupted AN formation with a greater impact of the latter intervention, as BLA activation required a longer recovery period for AN to take place. Thus, it seems that NAcb-Shell activation is required for the formation of a safe taste memory while BLA activation is related with the formation of an aversive taste memory **(Figure 6)**.

Finally, the fourth experiment confirmed that the NAcb-AMY pathway relevant for AN formation was mediated by D1DR neurotransmission through the combination of the chemogenetic and pharmacological approaches. Thus, we were able to reverse the behavioral effect of the chemogenetic NAcb-Shell inhibition by the i.c. infusion of

D1DR agonist SFK-81297 into AMY. Additionally, we prevented AN formation on Day 3 by i.c. injection of the D1DR antagonist SCH-23390 into the AMY **(Figure 7).**

This is to our knowledge the first evidence relating the safe taste memory formation with the dopaminergic neurotransmission through D1-like receptors. Our results support the relevance of a NAcb-AMY dopaminergic network mediated by D1DRs responsible of adding the affective component to the taste memory during the taste memory formation. This does not discard the interaction among the dopaminergic and other neurotransmitter systems, such as the cholinergic (Gutiérrez et al., 2004; Miranda et al., 2003; Ramírez-Lugo, Zavala-Vega, Pedroza-Llinas, Núñez-Jaramillo, & Bermúdez-Rattoni, 2015) and glutamatergic (Ramírez-Lugo et al., 2006) systems involved in the cognitive component of taste recognition memory. Also, pharmacological studies have demonstrated the interaction between D1DR and glutamatergic neurotransmission through NMDA receptors in the NAcb during conditioned flavor preferences (Touzani et al., 2008). This interaction seems to occur also in BLA and CeA (Touzani et al., 2013) as well as in the lateral hypothalamus (Amador et al., 2014; Touzani et al., 2009) resulting in a direct control of the consumption of palatable tastes. This is consistent with the co-localization of post-synaptic D1DR and NMDA

receptors that has been reported in NAcb and AMY (Tarazi & Baldessarini, 1999).

In all, we might conclude that the emotional component of taste recognition memory crucially relies in the interplay between NAcb-shell and BLA throughout a D1DR-mediated dopaminergic network, which might interact with other neurotransmitter systems responsible of the cognitive component in the formation of the safe taste memory. The relevance of the dopaminergic system in recognition memory opens new opportunities to understand the structural and neurochemical mechanisms underlying the relationship between emotion and cognition. Also, a better knowledge of the brain mechanisms of taste recognition memory might help to diagnose and treat eating disorders and other psychopathologies (Gallo, 2018).

6. Experimental procedures.

6.1. Behavioural Procedures

On **Experiment 3.1.**thirty-one adult male Wistar rats were used. All the rats were subjected to the same behavioral procedure which consisted on 15-minute daily drinking sessions. Water intake during the morning drinking period was recorded for five days during the acclimation period in

order to get the animals used to the deprivation schedule. Once the water intake was stabilized as baseline consumption (BL) all rats had access to a novel 3% cider vinegar solution during the morning 15-minute drinking session. The amount ingested was recorded by weighing the tubes containing the solution with a microgram balance before and after each drinking session. In order to perform immunohistochemistry of the protein c-Fos as a marker of neural activity, all rats were randomly assigned to the following familiarity groups: 7 rats were euthanized after drinking the vinegar solution on day 1 (Novel group), 7 rats were euthanized after drinking the vinegar solution on day 2 (Familiar-1 group) and the remaining 15 rats were euthanized after drinking the vinegar solution on day 6 (Familiar-2 group). In order to compare changes in expression of c-Fos due to the taste solution, another group of animals (n=10) was used in this experiment which was subjected to the same behavioral procedure but received water instead of the vinegar solution for six consecutive days (Water group).

On **Experiment 3.2.,** one week after surgery, all the rats were subjected to the same behavioral procedure as in the Experiment 3.1., consisting on 15-minute daily drinking sessions. Water intake during the morning drinking period was recorded. Once the water intake was stabilized as baseline consumption (BL) all rats had access to either tap water or a 3% cider vinegar solution during the 15-minute drinking session

for four consecutive days (test phase). The amount ingested was recorded by weighing the tubes containing the solution with a microgram balance before and after each drinking session. Due to requirements of the Ethics for Animal Research Committee, four and a half hours after the drinking session all the animals received a rehydration session which consisted on free access to tap water for thirty minutes. Indeed the rehydration sessions allowed the animals to be completely hydrated and thus, avoided the animals being extremely thirsty during the behavioral procedure. For this reason, changes in solution intake during the morning 15-minute drinking sessions may be attributed to the processing of taste features and cannot be merely explained by the animals´ need of drinking. On **Experiment 3.2.C** animals were exposed to a 0.5% saccharin solution instead of a 3% cider vinegar solution as these animals were used in a previous experiment involving the vinegar taste.

Experiments 3.3.and3.4.were conducted according to the same behavioral protocol used in Experiment 3.2. All the animals were injected with the combination of viral vectors 4 weeks before the start of the experiment and were injected with CNO-DHCL (2mg/kg of body weight) 1.5 hours before the drinking session of the vinegar phase.

All procedures were approved by the University of Granada Ethics Committee for Animal Research and were in accordance with the European Communities Council Directive 86/609/EEC.

6.2. Immunohistochemistry.

All the animals were euthanized 90 minutes after the corresponding drinking session depending on the c-Fos immunohistochemical group they were assigned to (Water, Novel, F-I or F-II). They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde 90 minutes after drinking the vinegar solution the first (Novel group), second (Familiar-1) and sixth day (Familiar-2). The brains were removed and placed in a 4% paraformaldehyde solution for 4 h at 4ºC before being transferred to a 30% sucrose solution until they sank for cryoprotection. Coronal sections were cut at 20µm in a cryostat (Leica CM1900).

Tissue sections were then rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4), incubated for 15 minutes with 3% hydrogen peroxide, rinsed again and incubated in a solution of 3% normal goat serum and 0.4% Triton X-100 in PBS for 30 minutes. Slices were transferred to a c-Fos primary antibody (1:10,000; Calbiochem) for 24 h at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Biotinylated goat anti-rabbit IgG, 1:500; Calbiochem) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, 0.4% Triton X-100 and PBS. The sections were rinsed, then processed using the ABC-kit (Vector

Laboratories, Burlingame, CA), and the reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA). Finally they were rinsed, mounted on gelatine-bubbed slides, rehydrated with ethanol and xylenes and finally they were cover-slipped.

6.3. Immunofluorescence of DREADDs.

For Experiment 3.3..and3.4., the animals´ brains were removed following the same protocol described for Immunohistochemistry. After being cut in coronal sections in a cryostat, tissue sections were rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4) and incubated in a solution of 3% normal goat serum and 0.5% PBS tween20 for 30 minutes. Slices were transferred to a mouse anti-mCherry primary antibody (1:500; Abcam, UK) overnight at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Alexa-594 labelled goat anti-mouse IgG, 1:500; Molecular Probes, USA) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, 0.5% PBS tween20. The sections were rinsed, then mounted on gelatine-bubbed slides they were cover-slipped using Fluorosheild Mounting Medium with DAPI (Abcam, UK) for nuclear counterstaining.

6.4. Data Acquisition

Images of both hemispheres from brain slices containing NAcb-Core, NAcb-Sh, CeA and BLA were captured using a light microscope (Olympus BX41). Slices containing the NAcb-Core and NAcb-Sh and slices containing the CeA and BLA were identified using the *Stereo Investigator* Software (mbf Bioscience) from two coronal sections of the NAcb located at the level of the apparition of the lateral ventricles, approximately at +2,52 and +2,28mm relative to Bregma (Section 1 and Section 2 respectively) and two coronal sections of the AMY located at the level of the apparition of the ventral horn of the IV ventricle, approximately at -3,2 and -3,5mm relative to Bregma according to Paxinos and Watson (2009). Within each section microphotographs at 20X magnification were captured for the NAcb-Core, NAcb-Sh, CeA and BLA that covered the entire extension of all the nuclei (See **Figure 1B and 1C**).

The number of c-Fos positive cells was obtained using the *Image J* Software (National Institute of Mental Health). For each microphotograph threshold objects (black circular dots over the white background) having specific size (35-150 μm^2) and circularity (0.35-1.00) values matching those c-Fos positive nuclei were automatically identified by the software as c-Fos positive cells. In order to equalize all the microphotographs and cancel out possible background noise, they were previously converted into

8-bit type image and the background was lightened (150.0 pixels) and the Threshold was set up to 0-150 for all images.

Due to the immunohistochemical procedure used when the brain slices were mounted in the slides, it was impossible to determine to which hemisphere belonged. For this reason, in order to randomize a possible confounding effect, for each section the mean number of c-Fos positive cells was calculated in every microphotograph for both hemispheres.

The expression of the DREADDs was assessed using a Fluorescence Microscope (Olympus BX41). Slices containing the NAcb-Sh and slices containing the BLA were identified using the *Stereo Investigator* Software (mbf Bioscience) from coronal sections of the NAcb located at the level of the apparition of the lateral ventricles, approximately at +2,52 and +2,28mm relative to Bregma (Section 1 and Section 2 respectively) and two coronal sections of the AMY located at the level of the apparition of the ventral horn of the IV ventricle, approximately at -3,2 and -3,5mm relative to Bregma according to Paxinos and Watson (2009). Within each section microphotographs at 20X magnification were captured using the blue (DAPI) and red (mCherry) filters for the NAcb-Sh and BLA that covered the entire extension of all the nuclei.

The Blue and Red images were then processed using the *Image J* Software (National Institute of Mental Health). The Blue and Red images were processed according to the same protocol followed for the

immunohistochemistry of c-Fos. Both images were then merged in order to obtain the ratio of DAPI neurons labeled with anti-mCherry in order to measure the number of neurons expressing the DREADDs.

To assess the correct location of the microphotographs, the perimeter (µm) and area (µm^2) of every region selected as NAcb-Core, NAcb-Sh, CeA and BLA was measured. Global mixed ANOVAs were performed for the parameter area for each nucleus and any of them revealed a significant effect of familiarity (all F<1).No other effect or interaction was found. Global mixed ANOVAs were performed for the parameter perimeter of each nucleus and any of them revealed a significant effect of familiarity (all F<1) and no other effect of interaction was found. This allowed us to ensure that all the microphotographs were captured in the correct location as all the regions selected for every group had the same perimeter (NAcb-Core perimeter = 4439,64 ± 20.22 µm; NAcb-Sh perimeter = 5251,78 ± 21.35 µm; CeA perimeter = 1023,98 ± 30,24 µm; BLA perimeter = 2214,28 ± 28,23 µm) and area (NAcb-Core area = 1091601,13 ± 417 µm^2; NAcb-Sh area = 1023460,59 ± 368 µm^2; CeA area = 89380,60 ± 352 µm^2; BLA area = 1644653,89 ± 426 µm^2).

5.5. Surgery

Animals were anesthetized with i.p. injection with a mixture of ketamine and xilacine (0,1% of body weight) and were placed in the

stereotaxic apparatus (Stoelting. Co. Instrument, Word Dale, IL, USA). For the Experiment 3.2., the animals were bilaterally implanted with handmade stainless guide cannulas (10mm long, 20G wide) in the nucleus accumbens Shell [coordinates of the ventral tip of the guide cannulas: anterior (A) +2.00; lateral (L) ±1.1; ventral (V) -3mm from Bregma for the cannulas implanted in the nucleus accumbens, and (A) -2.5; (L) ±4.4; (V) -4mm from Bregma for the cannulas implanted in the amygdala according to Paxinos and Watson 2009]. Two additional screws were also implanted and the guide cannulas were fixed to the skull using dental cement. All the animals received s.c. injections of Baytril 5% and Bupag (0.3 ml) after the surgery and they received additional injections throughout the following five consecutive days in order to ensure a good recovery from surgery. Additionally, during recovery animals were daily handled for five minutes and handmade cannule-cleaners (10mm long, 30G wide) were inserted into both guide cannulas in order to keep the cannulas clean and prevent obturations. Once the animals were completely recovered from the surgery, the guide cannulas were cleaned every two days until the end of the experiment.

For **Experiment 3.3.**, a combination of two viral vectors was used in order to perform the DREADD chemogenetic experiment. Animals were placed in the stereotaxic apparatus, and half of them received i.c. injection of 1 µl/hemisphere containing $1x10^9$pp ofthe retrograde CAV-Cre

recombinase expressing virus (Plataforme de Vectorologie de Montpellier, France) which was targeted to the BLA and 1µl/hemisphere containing $5x10^{12}$ vg/mL of the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry (Addgene, USA; #44362-AAV2) was targeted to the NAcb-Shell. The other half was injected with the same retrograde CAV-Cre recombinase expressing virus was targeted to the NAcb-Shell and 1µl/hemisphere containing $6x10^{12}$vg/mL of the excitatory DREADD pAAV2-hSyn-DIO-hM3D(Gq)-mCherry (Addgene, USA; #44361-AAV2) was targeted to the BLA. The coordinates from Bregma were the same as used in Experiment 2 and the infusion of the vectors was controlled by a Harvard Infusion Pump at a rate of 0.5µl per minute. The injectors were kept in place for additional 5 minutes before slowly withdrawn.

For **Experiment 3.4.** the animals were injected with 1 µl/hemisphere containing $1x10^{9}$pp of the retrograde CAV-Cre recombinase expressing virus (Plataforme de Vectorologie de Montpellier, France) which was targeted to the BLA and 1µl/hemisphere containing $5x10^{12}$ vg/mL of the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry (Addgene, USA; #44362-AAV2) was targeted to the NAcb-Shell (see Experiment 3.3.) and they were also implanted with guide cannulas in the AMY following the same surgical procedure as Experiment 3.2.

6.6. Drugs and substances

Dopamine D1/D5 receptors antagonist SCH-23390 (R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride. Sigma Aldrich, Spain) was dissolved in PBS at a concentration of 5µg/µl (0.015M). Dopamine D1 receptors agonist SFK-81297 ((±)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide. Sigma Aldrich, Spain) was dissolved in PBS at a concentration of 10µg/µl for the NAcb and 3.2µg/µl for the AMY. We used these doses as they have been previously used elsewhere and have demonstrated to be efficient for occupying dopamine receptors (André & Manahan-Vaughan, 2015).

In Experiments 3.3. and 3.4., the drug used to activate both DREADDs was Clozapine N-Oxide Dihydrochloride (8-Chloro-11-(4-methyl-4-oxido-1-piperazinyl)-5*H*-dibenzo[b,e][1,4]diazepine dihydrochloride; Tocris: #6329) and was dissolved in distilled H_2O at a concentration of 2mg/ml. We used this dose as it has been previously used elsewhere (Boender et al., 2014; Garner et al., 2012; Zhu et al., 2014).

The novel taste used in experiments 3.1., 3.2.A, 3.2.B and 3.2.C consisted on a cider vinegar solution (5º of acidity) dissolved at 3% in tap water. The taste used in experiment 3.2.D consisted on a saccharin solution dissolved at 0.5% in tap water. All solutions were daily prepared by the

experimenters ten minutes before the drinking session in order to prevent the loss of taste and smell properties.

5.7. Intracerebral in vivo microinjections

For the i.c. injections, either 15 minutes before or after the corresponding drinking session, the rats were held by the experimenter and microinjectors (14.8mm long, 30G wide for the injections in the accumbens shell and 13.5mm long, 30G wide for the injections in the amygdala) connected to 10µl Hamilton syringe by polyethylene tubing were carefully inserted into the guide cannulas. A total volume of 1µl was infused into both hemispheres at the same time at the speed rate of 0.5µl/min [coordinates of the tip of the microinjector from Bregma: (A) +2.00; (L) ±1.1; (V) -7.8mm for the accumbens shell and (A) -2.50; (L) ±4.4; (V): -7.5mm for the amygdala according to Paxinos and Watson (2009)]. The speed rate of the drug infusion was automatically controlled by a Harvard infusion pump. Ninety seconds after the infusion the microinjectors were carefully pulled out from the guide cannulas a few millimeters and completely removed 30 seconds later. Then, the animals were placed in their home cages.

5.8. Histology

All the animals were euthanized after the last drinking session. They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.). In order to evaluate the diffusion of the drugs in the nucleus accumbens shell and AMY, microinjectors (14.8mm long for the NAcb-Shell and 14.5mm long for the AMY, 30G wide) were inserted in their guide cannulas and a total volume of 1µl of Toluidine Blue (1% diluted in MiliQ water) was bilaterally infused at a speed rate of 0.5µl/min through 10µl Hamilton syringes controlled by Harvard infusion pumps. After that, the animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were removed and placed in a 4% paraformaldehyde solution for 4 hours before being transferred to a 30% sucrose solution until they sank for cryoprotection. Brains were maintained at -80ºC until coronal sections were cut at 20µm in a cryostat (Leica CM1900). The brain sections were mounted on gelatin-bubbed slides and stained with cresyl violet, a Nissl stain, using a standard protocol before being cover slipped. The Neurolucida system (Micro Bright Field Inc., Williston, USA) was used to quantify the extension of the drug infusion in each rat using a light microscope (Olympus BX41) with a motorized stage interfaced to a computer (See **Figure 4**).

7. Supporting information

7.1 Statistical analyses:

Attenuation of neophobia: behavioral analyses.

A One-Way analyses of variance (ANOVA) (*Water* X *Day*) revealed no differences in water consumption on the last day of BL between the three familiarity groups [F(2, 18)=0,32; *p*=.72].

Regarding vinegar consumption, a One-Way ANOVA (*Familiarity* X *Vinegar day-1*) revealed no significant differences between the three groups on day 1 of vinegar [F(2, 18)=0,25; *p*>.7]. An independent-samples T-test (*Familiarity* X *Vinegar day-2*) revealed no significant differences between the Familiar-1 and Familiar-2 groups on day 2 of vinegar (*p*=.57). For this reason, data from the Familiar-2 were included in the behavioral statistical analyses, as this was the group that completed the behavioral procedure.

A Repeated-Measures ANOVA (*Solution* X *Day*) comparing the amount of water (BL) and vinegar drank on day 1 revealed a significant effect of *Solution*, showing the existence of a neophobic response to vinegar. It corresponded to a decrease in consumption of vinegar on day one compared to the last day of water (BL) [F(1,13)=11,43; *p*=.005]. The attenuation of the neophobic response to vinegar was analyzed separately.

A Repeated-Measures ANOVA (*Consumption* X *Day*) revealed a significant effect of *Day* [F(5, 30)=6,815; *p*<.001]. Further post hoc

analyses of the factor *Day* revealed that rats drank lesser amounts of vinegar on day 1 compared to day 2 (p=.014), day 3 (p=.014), day 4 (p=.007), day 5 (p=.039) and day 6 (p=.001) and also that rats drank lesser amounts of vinegar on day 2 compared to day 6 (p=.036) (See **Figure 1A**).

Attenuation of neophobia: c-Fos analyses.

One-Way Analyses of Variance (ANOVAs) (*Familiarity* X *c-Fos*) were used to compare differences in c-Fos positive cells between the three familiarity groups for each region (NAcb-Core, NAcb-Sh, CeA and BLA). The analysis performed for the NAcb-Core did not reveal any significant effect of familiarity for the nucleus accumbens core [$F(3, 23)=0.808$; p=.503]. The analysis performed for the NAcb-Sh revealed a significant effect of *Familiarity* [$F(3, 23)=8.74$; p<.001]. Post hoc analyses using Bonferroni Tests revealed an increased number of c-Fos positive cells in the Familiar-1 group compared to the water (p=.002), Novel (p=.001) and Familiar-2 group (p=.004). See **Figure 1E.**

The analysis performed for the CeA revealed a significant effect of *Familiarity* ($F(3, 25)=14.79$; p<.001]. Further post hoc analyses using Bonferroni Tests revealed an increased number of c-Fos positive cells in the Novel and the Familiar-2 groups compared to Water (p<.001 and p<.001 respectively). The analysis performed for the BLA revealed a significant effect of *Familiarity* [$F(3, 25)=10.65$; p<.001]. Further post hoc

analyses using Bonferroni Tests revealed an increased number of c-Fos positive cells in the Novel group compared to Water (p=.002) and also an increased number of c-Fos positive cells in the Familiar 2 group compared to water (p=.008). See **Figure 1F.**

Correlation Analyses.

The first behavioral parameter calculated was the *Neophobia Index* as a rate of the difference between the amount that was ingested in the first encounter with the novel taste (Vinegar day 1) and the amount of water ingested in the last day of the baseline, as **Equation 1.**

The second behavioral parameter calculated was the *Attenuation of taste neophobia Index* basing on the definition of attenuation of taste neophobia, which consists on the increase in consumption observed over repeated exposures compared to the first encounter with the substance, as **Equation 2.**

The third parameter calculated was de *c-Fos Estimated Change Index* by computing the deviation of expression in c-Fos observed on the F-I group (day two of vinegar) from the mean number of c-Fos positive cells of the Novel group (day one of vinegar) for each animal as **Equation 3.**

All the correlations performed between *Direct Consumption* and c-Fos positive cells for he Novel and F-I group can be found in **Table 1.**

Only those correlations with a $p<.05$ were considered statistically significant.

Experiment 3.2.A: D1DR in the NAcb-Sh and retrieval of safe taste.

A One-Way ANOVA revealed that all the rats did not differ in water consumption the last baseline day [$F(2, 57)=3,17$; $p>.05$]. A global Mixed 2 X 3 X 6 Repeated Measures ANOVA (*Solution* X *Injection* X *Day*) comparing the amount of fluid intake throughout the test phase between groups revealed a significant effect of *Solution* [$F(1, 57)=25.73$; $p<.001$]; *Day* [$F(6, 57)=9.56$; $p<.001$] and the interactions *Solution* X *Injection* [$F(2, 57)=4.37$; $p<.05$]; *Solution* X *Day* [$F(6,57)=7.78$; $p<.001$] and the third-order interaction *Solution* X *Injection* X *Day* [$F(12, 57=1.9$; $p<.05$].

In order to understand the triple interaction a Mixed 3 X 6 Repeated Measures ANOVA (*Injection* X *Day*) was performed for those rats that only received water and also for those that received the vinegar solution separately. The mixed ANOVA used for the rats that received water did not reveal any significant effect or interactions (all $p>.05$) indicating that the infusion of any of the drugs caused no effect on the drinking behavior. The ANOVA used for the rats that drank the vinegar solution revealed a significant effect of *Injection* [$F(2, 27)=7.76$; $p<.01$]; *Day* [$F(6, 27)=20.89$; $p<.001$] and the interaction *Injection* X *Day* [$F(2, 27)=3.67$; $p<.001$] (**Figure 3C**).

In order to study the attenuation of neophobia of those rats that drank the vinegar solution, additional Repeated Measures ANOVAs (*Daily consumption* X *Day)*were performed separately for the three injection groups (PBS, SCH-23390 and SFK-81297):

The i.c. injection of PBS did not caused any effect on the attenuation of taste neophobia as the ANOVA revealed a significant effect of *Day* [$F(6, 10)=10.31$; $p<.001$] and further post-hoc analyses using Bonferroni Tests revealed that rats drank less on vinegar day 1 compared to vinegar day 2 ($p<.05$).

The i.c. injection of SCH-23390 resulted in an impairment of the attenuation of taste neophobia as ANOVA revealed a significant effect of *Day* [$F(6, 10)=12.08$; $p<.001$] and further post-hoc analyses using Bonferroni Tests revealed that rats drank less on vinegar days 1 and 2 compared to water (BL) ($p<.001$ and $p<.001$ respectively) and no differences were found between vinegar day 1 and vinegar day 2 ($p=1$).

The i.c. injection of SFK-81297 resulted in a facilitation of the attenuation of taste neophobia as the ANOVA revealed a significant effect of *Day* [$F(6, 10)=7.394$; $p<.001$] and further post-hoc analyses using Bonferroni Tests revealed that rats drank less test day 1 compared to water (BL) and test day 2 ($p<.001$ and $p<.01$, respectively) and no differences were found between vinegar day 2 and water (BL) ($p=1$) (See **Figure 3A**).

Experiment 3.2.B: D1DR in the AMY and retrieval of safe taste memory.

A One-Way ANOVA revealed that all the rats did not differ in water consumption the last baseline day [$F(5, 39)=0,88$; $p>.05$]. A global Mixed 2 X 3 X 6 Repeated Measures ANOVA (*Solution* X *Injection* X *Day*) comparing the amount of fluid intake throughout the test phase between groups revealed a significant effect of *Day* [$F(3, 114)=9.39$; $p<.001$]; *Day* X *Injection* [$F(6, 114)=3.28$; $p<.01$]; *Solution* X *Day* [$F(3, 114)=6.44$; $p<.001$] and the third-order interaction *Solution* X *Injection* X *Day* [$F(6, 114)=4.3$; $p<.01$].

In order to understand the triple interaction a Mixed 3 X 6 Repeated Measures ANOVA (*Injection* X *Day*) was performed for those rats that only received water and also for those that received the vinegar solution separately. The mixed ANOVA used for the rats that received water did not yield any significant effect or interactions (all *p´s* >.31) indicating that the infusion of any of the drugs caused no effect on the drinking behavior (See **Figure 3D**). The ANOVA used for the rats that drank the vinegar solution revealed a significant effect of *Day* [$F(3, 69)=31.57$; $p<.001$] and the interaction *Injection* X *Day* [$F(6, 69)=8.66$; $p<.001$].

In order to study the attenuation of neophobia of those rats that drank the vinegar solution, additional Repeated Measures ANOVAs (*Daily*

consumption X *Day)* were performed separately for the three injection groups (PBS, SCH-23390 and SFK-81297):

The i.c. injection of PBS did not caused any effect on the attenuation of taste neophobia as the ANOVA revealed a significant effect of *Day* [$F(3, 24)=23.29$; $p<.001$] and further post-hoc analyses using Bonferroni Tests revealed that rats drank less on vinegar day 1 compared to vinegar day 2, and 3 (all $p´s <.01$).

The i.c. injection of SCH-23390 resulted in an impairment of the attenuation of taste neophobia as ANOVA revealed a significant effect of *Day* [$F(3, 27)=17.78$; $p<.001$] and further post-hoc analyses using Bonferroni Tests revealed that rats drank less on vinegar days 1 and 2 compared to water (BL) (both$p´s <.001$) and no differences were found between vinegar day 1 and vinegar day 2 ($p=1$).

The i.c. injection of SFK-81297 resulted in a facilitation of the attenuation of taste neophobia as the ANOVA revealed a significant effect of *Day* [$F(6, 10)=7.394$; $p<.001$] and further post-hoc analyses using Bonferroni Tests revealed that rats drank less test day 1 compared to water (BL) and test day 2 ($p<.001$ and $p<.01$, respectively) and no differences were found between vinegar day 2 and water (BL) ($p=1$).

Experiment 3.2.C: D1DR in the AMY and acquisition of safe taste memory.

A One-Way ANOVA revealed that all rats did not differ in water consumption the last baseline day [$F(1,22)=1.476$; $p>.05$]. A Repeated Measures ANOVA (*Injection* X *Day*) comparing the amount of fluid intake between the last day of baseline and the first day of saccharin revealed a significant effect of *Day* [$F(1,22)=159.98$; $p<.001$], indicating a lesser consumption of saccharin than water regardless the injection received.

In order to study the attenuation of taste neophobia, a 3 X 6 Mixed ANOVA (*Injection* X *Day*) comparing the amount of saccharin consumed during the test days revealed a main effect of *Day* [$F(3, 51)=60.21$; $p<.001$] as well as the interaction *Day* X *Injection* [$F(6, 51)=8.84$; $p<.001$]. In order to understand this interaction additional Repeated Measures ANOVAs (*Daily consumption* X *Day*)were performed separately for the three injection groups (PBS, SCH-23390 and SKF-81297).

The i.c. injection of PBS did not cause any effect on the attenuation of neophobia as the ANOVA showed a significant effect of *Day* [$F(3,21)=43.83$; $p<.001$] and further analyses using Bonferroni Tests revealed that rats drank lesser amounts of saccharin on test day 1 compared to the rest of test days (all $p´s<.01$).

The i.c. injection of SCH-23390 did not cause a mild impairment on the attenuation of taste neophobia, as the ANOVA showed a significant

effect of *Day* [F(3,21)=43.83; $p<.001$] and further analyses using Bonferroni Tests revealed that rats drank lesser amounts of saccharin on test day 1 compared to water baseline ($p<.01$) and the rest of test days (all $p<.01$) and also that rats drank lesser amounts of vinegar on test day 2 compared to test day 3 ($p<.05$).

The i.c. injection of SFK-81297 resulted in a complete impairment of the attenuation of neophobia, as the ANOVA did not reveal a significant effect of *Day* [F(3, 9)=3.32; $p<.05$], indicating a complete absence of increase in consumption of saccharin over the test days (**Figure 3B)**.

Experiment 3.2.D: D1DR in the AMY and consolidation of safe taste memory.

A One-Way Analyses of Variance (ANOVA) revealed that all the rats did not differ in water consumption the last baseline day [F(2, 22)=0.53; $p=.59$]. A Repeated Measures ANOVA (*Injection* X *Day*) comparing the amount of fluid intake throughout the test phase revealed a significant effect of *Day* [F(3,60)=45.39; $p<.001$] and the interaction *Day* X *Injection* [F(6,60)=3.50; $p<.01$]. In order to understand this interaction additional Repeated Measures ANOVAs (*Daily consumption* X *Day*)were performed separately for the three injection groups (PBS, SCH-23390 and SKF-81297).

The i.c. injection of PBS did not cause any effect over the attenuation of taste neophobia on day 3 as the ANOVA showed a significant effect of *Day* [$F(3,24)=25.57$; $p<.001$] and further analyses using Bonferroni Tests revealed that rats drank lesser amounts of vinegar on test day 1 compared to the water baseline ($p<.01$) and the rest of test days (all $p<.01$).

The i.c. injection of SCH-23390 not cause any effect over the attenuation of taste neophobia on day 3 as the ANOVA showed a significant effect of *Day* [$F(3,24)=18.22$; $p<.001$] and further analyses using Bonferroni Tests revealed that rats drank lesser amounts of vinegar on test day 1 compared to water baseline ($p<.001$) and test days 2 and 3(all $p´s<.001$).

The i.c. injection of SFK-81297 caused an effect over consumption of vinegar on day 3, as the ANOVA revealed a significant effect of *Day* [$F(3, 12)=16.32$; $p<.001$] and further analyses using Bonferroni Tests revealed that rats drank lesser amounts of vinegar on test day 1 compared to water baseline ($p<.01$) and also that rats drank lesser amounts of vinegar on test day 1 compared to test day 2 ($p=.001$) and no differences were found between test day 1 and 3 ($p=1$) (**Figure 3D**).

Experiment 3.3. Chemogenetic manipulation of the NAcb-Shell-AMY pathway.

A global repeated measures ANOVA comparing the amounts of vinegar intake between the three chemogenetic groups (*Gi-NAcb-Shell; Gq-AMY and Control*) revealed a significant effect of *DREADD Location* [$F(2, 15)=52.16$; $p<.001$], *Day* [$F(3, 45)=20.33$; $p<.001$] and the interaction *DREADD Location* X *Day* [$F(6, 46)=13.24$; $p<.001$]. In order to understand the interaction, separate repeated measures ANOVAs were performed for each of the chemogenetic groups.

The ANOVA performed for the *Control* group revealed a significant effect of *Day* [$F(3, 12)=41.01$; $p<.001$], and further posthoc analyses using Bonferroni-corrected tests revealed a lesser amount of vinegar intake on day 1 compared to the rest of days 2, 3 and 4 (all $p´s<.001$), thus showing normal attenuation of taste neophobia on day 2.

The ANOVA performed for the *Gi-NAcb-Shell* group revealed a significant effect of *Day* [$F(3, 18)=13.10$; $p<.001$] and further posthoc analyses using Bonferroni corrected tests revealed no differences in consumption between vinegar days 1 and 2 ($p=1$), and lesser amounts between vinegar days 1 and 2 compared to day 3 ($p=.02$ and $p=.03$ respectively) and day 4 (both $p´s<.001$), indicating a delayed attenuation of taste neophobia, which occurred on day 3.

The ANOVA performed for the *Gq-AMY* group revealed a significant effect of *Day* [F(3, 15)=3.54; *p*=.040]. Further posthoc analyses using Bonferroni corrected tests revealed a decreased consumption of vinegar on day 3 compared to day 1 (*p*=.03) and no differences between any other day was found. This indicates that the chemogenetic activation of the AMY prevents the attenuation of taste neophobia and even results in avoidance of the taste 24 hours after its activation.

The global analysis of the rats that were exposed only to water as a control for the DREADD and drug administration revealed no significant effect or interactions between the DREADD type (either inhibitory or excitatory) and the i.p. injection (either CNO-DHCL or 0,9% NaCl) (all *p´*s>.35).

Experiment 3.4.: Chemogenetic inhibition of the NAcb-Shell and pharmacological activation of D1DRs in the AMY.

The global repeated measures ANOVA comparing the amounts of vinegar between the three injection groups (*SCH-23390, SFK-81297* and *NaCl*) revealed a significant effect of the factors *Injection Group* [F(2, 7)=20.21; *p*=.001], *Day* [F(3 21)=207.51; *p*<.001] and the interaction *Injection Group* X *Day* [F(6, 21)=98.48; *p*<.001]. In order to understand the interaction, separate repeated measures ANOVAs were performed for each of the injection groups.

The ANOVA performed for the *NaCl* control group revealed a significant effect of the factor *Day* [F(3, 6)=412.05; *p*<.001] and further analyses using Bonferroni corrected tests revealed no differences between the vinegar days 1 and 2 (p=1) and lesser amounts of vinegar consumption between days 1 and 2 with respect to days 3 and 4 (all *p´*s<.001). This replicates the effect of the chemogenetic inhibition of the NAcb-Shell over AN as previously observed in Experiment 3.

The ANOVA performed for the group injected with SFK-81297 previous to drinking vinegar on day 2 revealed a significant effect of the factor *Day* [F(3, 9)=94.83; *p*<.001] and further analyses using Bonferroni corrected tests revealed a lesser conumsption of vinegar on day 1 compared to the rest of days (all *p´*s<.001), indicating an increased consumption of vinegar, and thus attenuation of taste neophobia on day 2.

The ANOVA performed for the group injected with SCH-23390 previous to drinking vinegar on day 3 revealed a significant effect of *Day* [F(3, 6)=139.53; *p*<.001] and further analyses using Bonferroni corrected tests revealed no differences in vinegar consumption between days 1 to 3 (all *p´*s=1) and increased consumption of vinegar on day 4 compared to the previous days (all *p´*s<.001). This indicated that these animals did not show attenuation of taste neophobia until day 4.

7.2 Equations:

Equation 1: Neophobia index calculated for each subject of the F-I group as an inverse function of consumption observed on the last day of water baseline. A low score on the Neo Index indicates the existence of low neophobia to vinegar, while a high score indicates a strong neophobia to vinegar.

$$\boldsymbol{Neo\ Index = 100 - \frac{Vinegar\ Day\ 1\ (ml)}{Last\ day\ of\ water\ (BL)\ (ml)}\ x\ 100}$$

Equation 2: Attenuation of Neophobia index calculated for each subject of the F-I group as a function of the consumption observed on the first day of vinegar. Low scores on the AN Index indicate absence of attenuation of neophobia, while high scores indicate strong attenuation of neophobia to vinegar.

$$\boldsymbol{AN\ Index = \frac{Vinegar\ Day\ 2\ (ml)}{Vinegar\ Day\ 1\ (ml)} x100}$$

Equation 3: c-Fos Estimated Change index calculated by computing the deviation of expression in c-Fos observed on the F-I group (day two of vinegar) from the mean number of c-Fos positive cells of the Novel group (day one of vinegar) for each animal.

$$\boldsymbol{c - Fos\ EC}$$

$$= \frac{(\boldsymbol{c - Fos\ Vinegar\ Day\ 2}) - (\boldsymbol{mean\ c - Fos\ Novel\ Group})}{(\boldsymbol{c - FosVinegarDay2}) + (\boldsymbol{mean\ c - Fos\ Novel\ Group})}$$

8. Acknowledgments:

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Capítulo 6

Experimento 4. *Dorsal hippocampal damage disrupts the auditory context-dependent attenuation of taste neophobia in mice.*

1. Abstract

Rodents exhibit neophobia for novel tastes, demonstrated by an initial reluctance to drink novel-tasting, potentially-aversive solutions. Taste neophobia attenuates across days if the solution is not aversive, demonstrated by increased consumption as the solution becomes familiar. This attenuation of taste neophobia is context dependent, which has been demonstrated by maintained reluctance to drink the novel tasting solution if the subject has to drink it after being brought to a novel environment. This spatial context-dependent attenuation of taste neophobia has been described and likely depends on the integrity of the dorsal hippocampus because this brain area is crucial for representing space and spatial context associations, but is unnecessary for processing taste memories *per se*. Whether changing the non-spatial auditory context causes a similar effect on attenuation of taste neophobia and the potential role of the dorsal hippocampus in processing this decidedly non-spatial information has not been determined. Here we demonstrate that changing the non-spatial auditory context affects the attenuation of taste neophobia in mice, and investigate the consequence of hippocampal lesion. The results demonstrate that the non-spatial auditory context-dependent attenuation of taste neophobia in mice is lost following NMDA excitotoxic lesions of the CA1 region of the dorsal hippocampus. These findings demonstrate that the dorsal hippocampus is crucial for the modulation non-associative taste learning by auditory context, neither of which provide information about space.

Key Words: context, hippocampus, lesion, memory, neophobia, taste.

2. Introduction

Taste recognition memory is a robust ethologically-grounded paradigm that has been exploited for studying neural mechanisms of learning and memory in rodents (Federico Bermúdez-Rattoni, 2004). Taste neophobia is an unconditioned response that can be measured as an attenuation of fluid intake that is induced by a novel taste. Learning about the consequences of food and fluid ingestion leads to recognition of either aversive or safe tastes that manifests as changes in consumption. Specifically, safe taste recognition memory manifests as an attenuation of taste neophobia (ATN), measured as an increase in intake upon repeated exposures as a harmless taste becomes familiar.

Taste neophobia, along with taste aversion have been investigated for decades as neuroethologically-founded, non-associative types of learning that depend on non-declarative memory according to the declarative versus non-declarative memory dichotomy proposed by Squire (2004). However, recent evidence indicates that rats with excitotoxic lesions of the perirhinal cortex exhibit impairments of ATN that are comparable to the lesion-induced deficits that are observed in the novel object recognition memory task (Morillas et al., 2017), providing evidence that ATN also shares neural circuits that have traditionally been associated with declarative memory. Moreover, aging, which has been associated with selective alteration and decay of declarative memory (Dardou et al., 2008, 2010), leads to impaired

ATN (Gómez-Chacón et al., 2015) in addition to other changes of taste learning (Gámiz & Gallo, 2011; Manrique, Gámiz, et al., 2009; Manrique, Morón, et al., 2009; Moron et al., 2002).

The declarative versus non-declarative memory dichotomy is founded on the hypothesis that the hippocampal system is crucial for declarative memory and not required for non-declarative memory. Furthermore, a somewhat alternative conception of the hippocampal function, the "cognitive mapping" theory, asserts that a central role of the hippocampus system is in computing and evaluating spatial information that is central to making spatially-informed and adaptive behavior. Attenuation of neophobia provides an opportunity to evaluate both cognitive mapping theory and the declarative-memory hypothesis for hippocampal function because evaluating taste memory is naturally accomplished without any overt physical changes to the testing environment, and without any conditioning or explicit reward. Thus, both the dominant declarative memory and cognitive mapping theories of hippocampal function predict no role of hippocampus in attenuation taste of neophobia.

In the present experiment we first investigated whether the attenuation of taste neophobia in mice is modulated by non-spatial changes to the auditory background. After observing that changing the auditory background reduces ATN, we investigated whether the non-spatial auditory

modulation of this non-spatial taste recognition memory is sensitive to dorsal hippocampal lesion, thereby testing the two dominant theories of the hippocampal function.

3. Materials and methods

3.1 Subjects

Forty-eight adult male BALB/c mice (weighing 20-24g, Charles River, France) were used in this experiment. They were housed individually and maintained on a 12-hour dark-light cycle (lights from 8:00 am to 8:00pm). All the experimental procedures were performed during the light cycle at the same time each morning in the home cage. Mice were given *ad libitum* access to food and water until the experiment started, at which time access to water was restricted to a daily 10-minute morning drinking session. Four hours afterwards, all mice got additional access to water for one hour.

All procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (CEEA17-02-15-195) and were in accordance with the European Communities Council Directive 86/609/EEC.

3.2 Surgery

Surgery was performed under general anesthesia with a mixture of ketamine and medetomidine (0.1% b.w.). The animals were randomly assigned to one of two groups: *Lesion* and *Sham*. They were placed in a stereotaxic apparatus (Stoeling Co. Instrument, Word Dale, IL, USA)with bregma and lambda at the same height. Small trephine openings were drilled in the exposed skull in order to perform bilateral injections of either NMDA (NMDA, Sigma–Aldrich, 0.077 M) or sterile 0.9% saline solution through 30-gauge injection needles that were connected to 10-µl Hamilton syringes, so that 0.4-µl of the NMDA (M3262 – 25mg, Sigma Aldrich, Spain) solution was infused in each hemisphere at a rate of 0.2µl/min using an injection pump (Harvard Apparatus, Holliston, MA, USA). The needles were left in place for an additional 90 seconds before being slowly withdrawn. The stereotaxic coordinates targeted dorsal CA1 according to Paxinos and Watson´s mouse brain atlas (2001). The coordinates relative to bregma were: AP: -1.70mm; ML: ±1.00mm; DV:-1.50mm. The skin was sutured and covered with povidone. After the surgery, all animals received an i.p. injection of 4% atipamezole (0.5% b.w.) in order to reverse the effects of anesthesia. They also received additional s.c. injections of 5% Baytril and Bupac (0.1ml) for four consecutive days in order to reduce post-surgical pain and prevent infection.

3.3 Behavioral Procedure

One week after surgery, all the animals were subjected to the same behavioral procedure consisting of baseline (4 days), Phase I (one day) and Phase II (3 days) protocol. Liquid was available from a drinking tube during daily 10-min drinking sessions and the amount ingested was recorded.

An experimentally-controlled auditory background was continuously present during the 10-min drinking sessions in all protocol phases. In a separate room adjacent to the colony room, two speakers were used to deliver the auditory background. They were positioned one meter from the mouse homecages. The speakers were separated by 50 cm, and slightly angled apart from each other, so that each speaker faced half of the rack that held the homecages. Two different tones created using MATLAB were used and counterbalanced amongst the subjects. One tone was a pure 600 Hz tone (PT) consisting of 3-secondpulses with an inter stimulus interval (ISI) of 3 seconds. The second tone was Gaussian white noise (WN) consisting of 2-second pulses with an ISI of 4 seconds. Each tone was delivered by the two speakers simultaneously.

Dorsal hippocampus and sham lesion were randomly assigned to experimental groups specified by the taste solution (Water or Vinegar) and whether the auditory background was the same or different in Phases I and II. Two sham groups received sham surgery to assess the impact of

changing the auditory background on drinking behavior: *Water-Same Tone* (*n*=8) and *Water-Different Tone* (*n*=8). Four other groups were used to assess the impact of hippocampus lesion: *Sham-Vinegar-Same Tone* (*n*=8), *Sham-Vinegar-Different Tone* (*n*=8), *Lesion-Vinegar-Same Tone* (*n*=8) *and Lesion-Vinegar-Different Tone* (*n*=8). (**see Table 1**).

Table 1

Table depicting the study groups as defined by the drinking solution and the auditory background.

Groups	Surgery	Baseline (-4 to 0 days)	Day 1 (Phase 1)	Day 2 (Phase 2)	Day 3 (Phase 2)	Day 4 (Phase 2)
Same Tone	Lesion or Sham	Water Tone A	Vinegar Tone A	Vinegar Tone A	Vinegar Tone A	Vinegar Tone A
Different Tone	Lesion or Sham	Water Tone A	Vinegar Tone A	Vinegar Tone B	Vinegar Tone B	Vinegar Tone B
Same Tone	Sham	Water Tone A	Water Tone A	Water Tone A	Water Tone A	Water Tone A
Different Tone	Sham	Water Tone A	Water Tone A	Water Tone B	Water Tone B	Water Tone B

Tones A and B were counterbalanced: half the animals experienced the PT (600 Hz) and the other half the WN. If Tone A was PT, Tone B was WN, and vice versa.

During Phases I and II all mice assigned to the *Vinegar* groups had access to the3% cider vinegar solution (5º acidity) instead of water during the 10-min drinking sessions. The groups assigned to *Water* continued to be exposed to water. The mice assigned to the *Same Tone* groups were only exposed to one of the two auditory cues (either the PT or the WN). The mice assigned to the *Different Tone* groups experienced a change in the

auditory background in Phase II. Due to counterbalancing half of the animals changed from PT to WN and the other half changed from WN to PT (**see Table 1**).

3.4 Histology

All the animals were euthanized after the last drinking session. They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.). The animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were removed and placed in a 4% paraformaldehyde solution for 48 hours before being transferred to a 30% sucrose solution until they sank for cryoprotection. Brains were maintained at -80ºC until 20µm coronal sections were cut on a cryostat (Leica CM1900). The brain sections were mounted on gelatin-coated slides, stained with cresyl violet, and cover slipped, using a standard protocol. The Neurolucida system (Micro Bright Field Inc., Williston, USA) was used to quantify the extent of the hippocampal lesions in each mouse using a light microscope (Olympus BX41) with a motorized stage interfaced to a computer (See **Figure 1**).

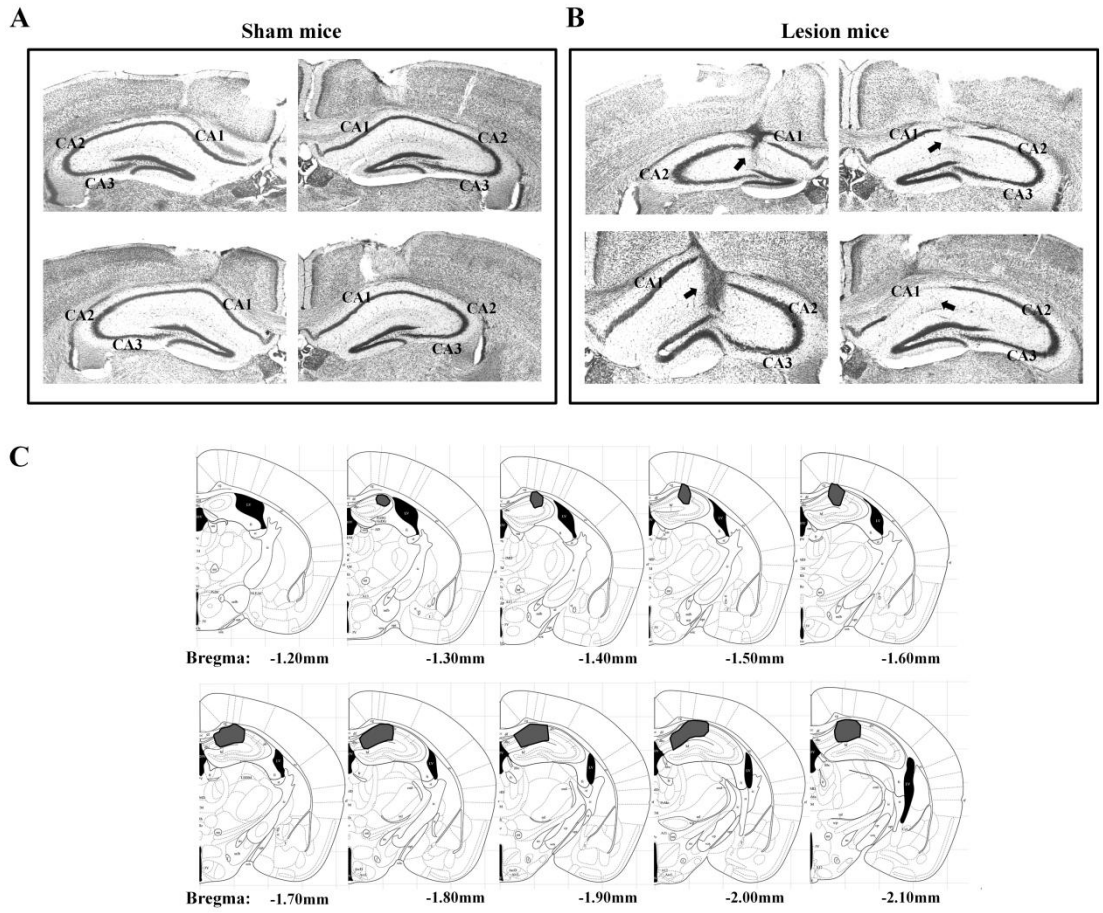

Figure1.Example photomicrographs of the hippocampus in A) Sham and B) CA1 lesion mice. C) Mouse brain schematics with shading indicating the extent of the lesion.

4. Results.

4.1 Water consumption: Phases I and II

We began by testing whether water consumption is affected by changing the background auditory noise (Fig. 2A). A global Mixed 4 x 2 x 2 (*Day* X *Tone Change* X *Counterbalance Order*) Repeated-Measures ANOVA comparing the water intake of the *Water-Same Tone* and *Water-Different Tone* groups on the four days after the baseline period did not

reveal any significant effects or interaction (all *p's* >.2). This indicates that changing the auditory background did not itself alter drinking (See **Figure 2A,B**) and allowed us to test the effect of changing the auditory background on taste neophobia.

4.2 Vinegar consumption: Phases I and II

We tested the effects of changing the auditory background and dorsal hippocampus lesion on taste neophobia, using vinegar as a novel taste **(Figures 2C,D).** By inspection, taste neophobia is clearly observed in response to introducing vinegar, and reduced drinking appears to persist longer if the auditory background is changed in control animals (**see Figure2C**), but not in mice with dorsal hippocampus lesions **(Figure 2D).** We confirmed these impressions starting with a global Mixed 4 X 2 X 2 X 2 (*Day* X *Lesion* X *Tone Change* X *Counterbalance Order*) Repeated Measures ANOVA that compared the intake of vinegar amongst the groups on the four days after the baseline period. There was a significant effect of the main factors *Days* [F(3,60)=104.51; *p*<.001], *Tone Change* [F(1,20)=11.5; *p*=.003], the interactions *Day* X *Tone Change* [F(3,60)=10.12; *p*<.001], *Tone Change* X *Lesion* [F(1,20)=7.32; *p*=.014] and *Day* X *Tone Change* X *Lesion* [F(3,60)=8.60; *p*=.004].

To analyze the interactions, additional 4 X 2 (*Day* X *Tone Change*) Repeated Measures ANOVAs were performed for the *Sham* and *Lesion*

groups separately. The analysis performed for the *Sham* groups confirmed a significant effect of the main factors *Day* [F(3,36)=72.07; $p<.001$] and *Tone Change* [F(1,12)=26.7; $p<.001$] as well as the *Day* X *Tone Change* interaction [F(3,36)=24,64; $p<.001$]. Analysis of the interaction by Repeated Measures ANOVAs of the vinegar consumption was performed on the factor *Day* for each of the *Tone Change* groups separately. The analyses confirmed a significant effect of *Day* in the *Sham-Vinegar-Same Tone* group [F(3,18)=48.92; $p<.001$] as well as the *Sham-Vinegar-Different Tone* [F(3,18)=47.81; p<.001], indicating attenuation of neophobia. Further comparisons using Bonferroni-corrected tests identified significantly less vinegar was consumed on Day 1 compared to Days 2, 3 and 4 (all *p's*<.001) in the *Sham-Vinegar-Same Tone* group, and this confirms that the neophobic response to the vinegar taste was completely attenuated on Day 2 and its consumption remained stable across the rest of days. In contrast, the same analysis performed in the *Sham-Vinegar-Different Tone* group identified that the amount of vinegar consumed on Days 1 and 2 were indistinguishable ($p=1$) and less than on Days 3 and 4 ($p's \leq .009$). Thus unlike the mice that did not experience a change of auditory background, the animals that experienced the change maintained the neophobic response for one more day; the attenuation of taste neophobia occurred on Day 3, when the novel auditory background became familiar.

Because hippocampus is specialized for spatial computations, and declarative-type learning and memory, and neither taste novelty nor changes in auditory background carry information about space, we investigated whether dorsal hippocampus lesion affects the attenuation of taste neophobia and its delay by changing the auditory background, a test of non-declarative memory. We repeated the above analysis for the *Lesion* groups. There was a significant effect of *Day* [$F(3,24)=39.195$; $p<.001$] and no other effect or interaction (all $p's>.6$). Post-hoc analysis of the effect of *Day* using Bonferroni-corrected t tests confirmed less vinegar intake on Day 1 compared to Days 2, 3 and 4 (all $p's<.001$) but no other comparisons were significant. This indicates that unlike the sham mice, the lesion animals attenuated the neophobic response to the vinegar taste on Day 2, regardless of whether the background tone was or was not changed (see **figure 3**). These results demonstrate that lesions of dorsal CA1 impairs the auditory background-dependent attenuation of taste neophobia (see **Figure 2D**).

4.3 Baseline: water consumption.

Finally, we examined whether the differences between the *Sham* and *Lesion* groups or any other groups for that matter, could be due to group differences in baseline water consumption. A global Mixed 4 x 2 x 2 x 2 x 2 (*Day* X *Lesion* X *Tone Change* X *Counterbalance Order*) Repeated

Measures ANOVA comparing the amount of water intake between all the groups during the four days of baseline (BL) revealed only a significant effect of *Day* [F(3,93)=85.86; p<.001]. No other effect or interaction was significant (all p's>.2). Further analyses of the main effect *Day* using Bonferroni-corrected t tests revealed that all groups consumed less amounts of water on BL Day 1 compared to BL Days 2, 3 and 4 (all p's<.001). This indicates adaptation to the water deprivation procedure was indistinguishable across the groups, and so cannot easily account for the observed differences.

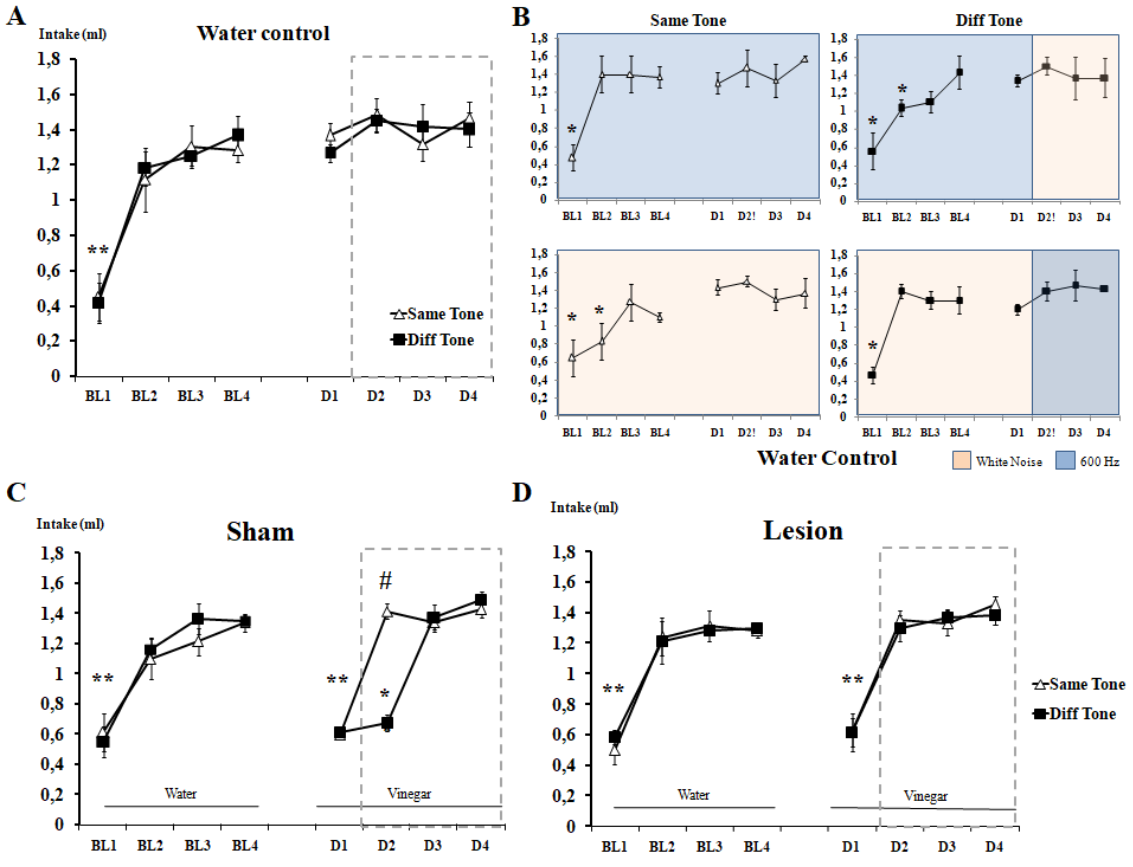

Figure 2. Water intake (±SEM) across the experimental days A) in the water only control mice demonstrating no effect of changing the auditory background, and B) that there is no effect of the specific auditory backgrounds; and demonstrating attenuation of taste neophobia in C) sham and D) hippocampus

lesion mice. *Symbol represents statistically significant differences compared to Day 4.****** indicates that both the *Same Tone* and *Diff Tone* groups were statistically significant compared to Day 4. # Symbol represents statistically significant differences between groups. Dashed-line boxes represent the days in which the tone was changed for the *Different* groups only (in a counterbalanced way).

5. Discussion

The present findings demonstrate for the first time that the auditory background influences attenuation of neophobia, a non-associative form of recognition memory and that dorsal hippocampus integrity is required for this influence of the auditory background. Because the auditory background can provide contextual information, we interpret these findings as evidence that the auditory context can influence the attenuation of taste neophobia and that the hippocampus is crucial for this effect, despite the absence of spatial information in the taste or auditory background.

The modulation of ATN by auditory context was assessed using two different auditory backgrounds. Changing the auditory background reduced ATN in the Sham control groups while the group of mice that experienced a constant auditory background exhibited rapid ATN on day 2.These findings are consistent with a prior demonstration of the spatial context dependency of ATN (De la Casa & Díaz, 2013) and they extend the phenomenon to non-spatial auditory background as a contextual cue. To our knowledge, there is only one previous report using an auditory background as part of the context in taste learning (Bonardi, Honey, & Hall, 1990). Most previous research used visual cues (De la Casa & Díaz,

2013; Quintero et al., 2011), as well as temporal information to define context (De la Casa, Diaz, & Lubow, 2003; Manrique et al., 2004; Moron et al., 2002).

The auditory backgrounds were distinct, differing in frequency (600 Hz versus Gaussian white noise), duration (three versus two seconds) and ISI (three versus four seconds). As we were interested in the effect of changing the auditory background on taste neophobia attenuation, the context change occurred after the mice had consumed the novel taste for the first time with the same auditory background as the baseline period. The fact that the ATN was delayed by changing the auditory background on the second exposure day confirms that the animals were able to distinguish the auditory backgrounds. Also, all groups exhibited similar consumption of water and eventually vinegar, regardless of the auditory background. This indicates that the modulation of the taste memory by changing the auditory background was not specific for a single auditory frequency; the presentation order was counterbalanced and this had no effect (see **Figure 3**) allowing one to conclude that the influence of auditory background is not unique to a particular tone and that this influence is specific to attenuation of neophobia, rather than a general disruption of drinking behavior (see **Figure 2A,B**).

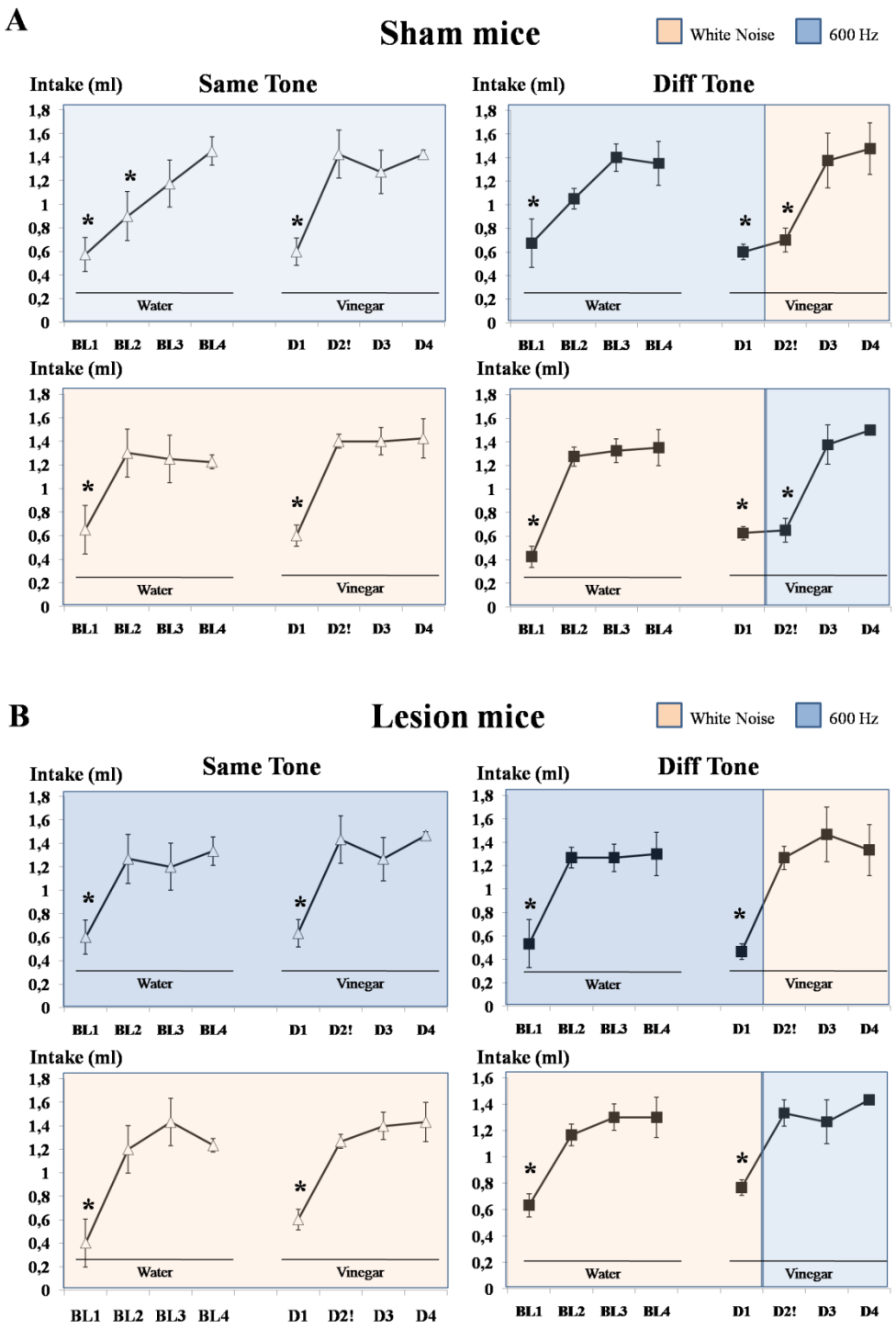

Figure 3. Water and vinegar intake (±SEM) across the experimental days A) in *Sham* animals demonstrating that both *Different* groups continued to show taste neophobia to vinegar on Day 2 regardless the presentation order of the auditory background and B) that there is no effect of the specific auditory backgrounds in the *Lesion* animals, regardless the presentation order of the auditory background, demonstrating attenuation of taste neophobia on Day 2. *Symbol, represents statistically significant differences compared to Day 4.

Prior work has indicated a role for hippocampal function in complex taste learning phenomena, such as blocking (Gallo and Cándido, 1995; Moron et al., 2002) and in taste learning tasks that critically depend on contextual information (Gallo et al., 1999). Electrolytic lesions of the dorsal hippocampus impaired both learned taste aversions to the physical context and the blocking of the context in taste aversion learning (Aguado et al., 1998). The context-dependence of taste aversion's extinction was also disrupted by electrolytic lesions of the dorsal hippocampus (Fujiwara et al., 2012) as well as the context-dependent extinction itself (Garcia-Delatorre et al., 2010). Finally, excitotoxic dorsal hippocampal lesions disrupted the context dependency of both taste aversions and latent inhibition of taste aversion (Manrique, Gámiz, et al., 2009; Manrique, Morón, et al., 2009; Molero et al., 2005).

What defines a context? Context is commonly defined as the set of background stimuli that comprises the environment during a behavior. These same stimuli can of course also become foreground conditioned stimuli, depending on the task (Luis Gonzalo De la Casa et al., 2018; Nadel & Willner, 1980).The study of context in different taste recognition memory tasks has primarily investigated spatial contexts, often defined only by visual cues (De la Casa & Díaz, 2013; Quintero et al., 2011), as well as temporal contexts, defined either as time elapsed (De la Casa et al., 2003) or the time of day (Manrique et al., 2004; Moron et al., 2002). In this

context, it is important that memory, spatial and temporal task information are signaled in the discharge of hippocampus CA1 cells, as well as other hippocampus subfields (Howard Eichenbaum, 2017; Jezek, Henriksen, Treves, Moser, & Moser, 2011; Lenck-Santini, Fenton, & Muller, 2008; Pastalkova, Itskov, Amarasingham, & Buzsáki, 2008; van Dijk & Fenton, 2018). This, as well as other robust behavioral evidence that hippocampus is crucial for context-based memory (J. J. Kim & Fanselow, 1992), is consistent with the present finding that dorsal hippocampus lesions interfere with the auditory context modulation of ATN.

What other evidence is there for a role of hippocampus in the auditory context modulation of ATN? A role for dopamine has been reported in the consolidation of contextual memories in hippocampus (Kempadoo, Mosharov, Choi, Sulzer, & Kandel, 2016; Takeuchi et al., 2016; Yamasaki & Takeuchi, 2017a). Like we observed for the auditory background, the attenuation of the neophobic response to a novel saccharin solution was weaker when the novel taste was encountered in a novel cage compared to the familiar homecage. In that work the contexts differed in spatial (size of the cages), visual (red vs white light) and somatosensory (different bedding) dimensions but a crucial role for hippocampus was not established. At present there is no evidence that specific hippocampal subfields have a particular role in contextual taste learning, and frankly this would not be expected given that hippocampal subfields have distinctive

computational roles such as pattern separation, pattern completion, and model-data comparisons that transcend specific classes of information and learning (Aronov et al., 2017; Colgin et al., 2009; Dvorak et al., 2018; Guzowski et al., 2004; Lenck-Santini et al., 2008). The effect of changing contexts on ATN is disrupted by systemic administration of the D1/D5 dopamine receptor antagonist SCH-23390 (De la Casa & Díaz, 2013), but rather little is known about the contextual modulation of ATN and the brain areas involved.

We observed, to our knowledge for the first time, that dorsal CA1 subfield lesions disrupt the non-spatial contextual dependence of ATN, which on the surface appears to contradict cognitive mapping theory (O'Keefe and Nadel, 1978), but is consistent with the view that hippocampus is critical for processing complex associative representations of stimuli involving context (Eichenbaum et al., 1999; Eichenbaum, 2017; Jezek et al., 2011; Lenck-Santini et al., 2008; Pastalkova et al., 2008; van Dijk and Fenton, 2018). There are of course, also non-associative explanations for the differential role of auditory context, which when changed, could increase levels of arousal, and lead to the recovery of taste neophobia. This is supported by the finding thatif the context is familiar, neophobia can persist despite the change of context (Honey, Pye, Lightbown, Rey, & Hall, 1992).

We find that the relationship between taste and auditory cues whatever its nature, requires dorsal hippocampus. Indeed, the present findings suggest that changes in the auditory background has similar effects on taste learning as what was previously observed by manipulating the physical properties of the environment. To our knowledge, this is the first evidence that mice use the auditory information that is present in the environment to define context sufficient to modulate attenuation of taste neophobia. Although more research is needed to identify the particular procedural features that might be critical for auditory modulation of taste memory, the present results introduce a new paradigm for exploring the hippocampus-dependent mechanisms that underlie how non-spatial memories are stored and modulated by non-spatial environmental cues.

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this manuscript state that there are no actual or potential conflicts of interest.

Capítulo 7

Serie Experimental 5. ***Chemogenetic and pharmacological identification of a dopaminergic accumbens-hippocampal pathway underlying taste-context interactions in recognition memory.***

1. Abstract

Taste recognition memory is evident in rodents because the initial neophobia to novel tastes attenuates across days as the taste becomes familiar and safe. The attenuation of taste neophobia (AN) is context-dependent. An auditory background change induces a recovery of the neophobic response. The AN auditory context-dependency requires the hippocampal integrity but the pathways underlying the interaction with the taste memory circuit remain unexplored. We have applied pharmacological interventions by systemic agonists and antagonists of the D1 dopamine receptor (D1DR) and 6-hidrosydopamine (6-OHDA) hippocampal lesion as well as chemogenetic silencing for assessing the role of the accumbens-hippocampal pathway. We found that systemic administration of the D1DR antagonist SCH-23390 mimicked a context change. It disrupted AN in mice whether or not the auditory context changed, even if the drug was given a day after the context change. Moreover, a disruption of the context-dependent AN was induced by intracerebral administration of 6-OHDA targeted to ventral CA1 hippocampus (vCA1) in rats. Finally, the chemogenetic DREADD approach by combining two viral vectors allowed us to identify the relevant dopaminergic accumbens-hippocampal pathway involved. The retrograde Cre-recombinase expressing CAV-Cre was targeted to ventral CA1, and the DREADD pAAV-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the nucleus accumbens shell (NAcb-Shell). Chemogenetic silencing of this pathway 2 hours before the taste drinking session disrupted AN mimicking a context change similarly to the effect of D1DR antagonism. We conclude that the ability of the auditory context to modulate taste recognition memory requires the activity of a dopaminergic NAcb-Shell-vCA1 circuit involving D1DRs.

KEY WORDS: Accumbens, Attenuation of neophobia, Dopamine, DREADD, D1DR, Hippocampus, Taste,

2. Introduction

Taste recognition memory is a robust paradigm for studying the neural mechanisms of learning and memory processes regulating intake in rodents (Bermúdez-Rattoni, 2004). Familiar tastes are recognized as either aversive or safe depending on the consequences of previous encounters. Safe taste recognition memory is evident as the initial neophobia to novel tastes is attenuated upon repeated exposures. The attenuation of taste neophobia (AN) has proven to be context-dependent since it is disrupted by a spatial context change in rats (De la Casa & Díaz, 2013). Moreover, we have previously reported the non-spatial auditory context-dependency of AN in mice which is hippocampal-dependent (Grau-Perales et al., 2019b).

The role of the hippocampus in context processing has been related to its relevance for the formation of multiple complex representations of stimuli (Dvorak & Fenton, 2014; Fenton, 2015; Kelemen & Fenton, 2016). Although the hippocampal role in the contextual information of taste learning has been thoroughly demonstrated in taste aversion learning (Aguado et al., 1998; Fujiwara et al., 2012; Garcia-Delatorre et al., 2010; Manrique et al., 2009), the relevant circuits mediating its interaction with the brain areas involved in safe taste learning remain unexplored. Taste neophobia and AN depend on a brain circuit that includes insular cortex (Bahar et al., 2004; Bermudez-Rattoni, 2014; Lin et al., 2012), basolateral amygdala (Gómez-Chacón et al., 2012), piriform cortex (Grau-Perales et

al., 2019a), perirhinal cortex (Gómez-Chacón et al., 2015; Gutiérrez et al., 2004) and nucleus accumbens (Ramírez-Lugo et al., 2007; Pedroza-Llinás et al., 2009; Grau-Perales et al., 2019c).

The relevant interaction might be mediated by the dopaminergic activity, since D1 dopamine receptors (D1DRs) play a critical role in the contextual memory formation (Kempadoo et al., 2016; Takeuchi et al., 2016). Also, dopamine has been linked to AN (Ramírez-Lugo et al., 2007) and to other types of taste learning (Sclafani et al., 2011). Specifically, D1DRs have been reported to be crucial for both taste aversion learning (S. Fenu et al., 2001; Fenu & Di Chiara, 2003) as well as conditioned flavor preferences (Bodnar, 2018b; Touzani et al., 2013, 2008). However, little is known about the implication of D1DRs in the context dependency of AN. De la Casa and Díaz (2013) studied the role of D1DRs in the effect of changing the physical context on AN. The administration of the D1DR antagonist SCH-23390 disrupted AN when tested in a novel cage. Accordingly, the hippocampal-ventral tegmental area projections mediated by D1DRs have been proposed as a novelty-triggered memory consolidation mechanism (Lisman & Grace, 2005). However, a potential role of the reciprocal dopaminergic accumbens-hippocampal interaction in AN remains unexplored.

In the present series of experiments we first investigated whether the auditory modulation of taste recognition memory is sensitive to i.p.

administrations of the D1DR antagonist SCH-23390 (Experiments 5.1., 5.2. and 5.3.). Adult male C57BL/6 mice were exposed to an auditory background during daily drinking sessions in which a cider vinegar solution was available. An auditory background change disrupted AN. We found that SCH-23390 administration mimicked the effect of changing the auditory background.

In Experiment 5.4., the catecholaminergic terminals of the ventral CA1 subfield of the hippocampus were depleted by i.c. administration of 6-hydroxydopamine (6-OHDA) in order to explore the role of the hippocampal dopaminergic activity in the auditory context-dependency of AN in rats. Hence, if hippocampal dopaminergic innervations are required for the context modulation of AN, then it should be disrupted.

Finally, in Experiment 5.5., we explore the dopaminergic accumbens-hippocampal circuit using DREADD (designer receptor exclusively activated by designer drug)chemogenetics by combining intracerebral injection of two viral vectors. The retrograde Cre-recombinase expressing CAV-Cre was targeted to ventral CA1, and the DREADD pAAV-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the nucleus accumbens shell (NAcb-Shell). This approach allowed us to specifically manipulate the activity of the nucleus accumbens neurons projecting to the hippocampus at specific time points which led us to a better comprehension of the temporal dynamics of the taste-context association.

3. Results.

3.1 Experiment 5.1.: Effect of changing auditory background and D1 dopamine receptors activity on the attenuation of taste neophobia.

We began by testing the effects of changing the auditory background and the D1 dopamine antagonism and agonism on taste neophobia, using vinegar as a novel taste (**Figure 1**).By inspection, taste neophobia is clearly observed on Day 1 in response to introducing vinegar and it attenuates on Day 2 (**Figure 1A left**). The animals that were injected with saline drank significantly less vinegar on Day 1 compared to Days 2, 3 and 4 and 5 (all *p's*<.001) in the *Saline-Same Context* group. This indicates that the neophobic response to the vinegar taste was completely attenuated on Day 2 and its consumption remained stable across the rest of days. Taste neophobia persists longer if the auditory background is changed in NaCl control animals (**Figure 1A right**). In the *Saline-Different Context* group the amount of vinegar consumed on Days 1 and 2 did not differ (p=1) and it was lower than that ingested on Days 3, 4 and 5 (*p's*<.001). Thus, unlike the mice that did not experience a change of auditory background, the animals that experienced the context change maintained the neophobic response for one more day and the AN occurred on Day 3, when the novel auditory background became familiar.

As D1DRs have been pointed as relevant for taste memory formation and for the AN spatial context dependency of the attenuation, we investigated whether the i.p. injection of D1DR antagonist SCH-23390 or agonist SFK-81297 affects AN and its delay caused by changing the auditory background. As we were not interested in the effect of D1DRs in taste neophobia but in its attenuation combined with the effect of the changing context, the drugs were injected 15 minutes before the drinking session on Day 2. The results showed that all animals that received i.p. injection of SCH-23390 did not increase consumption of vinegar on Day 2 regardless the auditory background they were exposed to (**Figure 1B**). The amounts of vinegar consumed on Days 1 and 2 did not differ (p=1) and they were lower than the amount of vinegar consumed on Days 3, 4 and 5 (p's<.001) either if the context remained constant (**Figure 1B left**) or after a context change (**Figure 1B right**). In contrast, all the animals that received i.p. injection of SFK-81297 showed AN on Day 2 regardless the auditory background they were exposed to (**Figure 1C**). The amounts of vinegar consumed on Day 1 was significantly lower than the amount of vinegar consumed on Days 2, 3, 4 and 5 (p´s<.001). This was evident not only in absence of the context change (**Figure 1C left**) but also even though the background sound changed (**Figure 1C right**), thus indicating absence of the AN context-dependency.

In brief, unlike the *Saline* mice, the animals injected with SCH-23390 without a context change did not attenuate the neophobic response to the vinegar taste solution until Day 3 while the animals injected with SFK-81297 exhibited AN on Day 2 in spite of being subjected to a context change. These results demonstrate that the blockade of D1DRs mimics the effect of an auditory background change over AN when the context remains constant and also that the pharmacological activation of D1Rss suppresses the modulation of the auditory background on AN.

In order to assess the potential impact of the D1 dopamine antagonist on the drinking behavior, a *WaterDrug-Same* group was added. This group was only exposed to water but it did not experience any change of the auditory context (they only were exposed either to the auditory context). Half of them were injected with SCH-23390 and the other half were injected with SFK-81297. The analysis of water consumption did not reveal any significant main effect or interaction (all p´s >.4). This indicates that the injection received did not alter water consumption and this allows us to discard unspecific effects of SCH-23390 or SFK-81297 in drinking behavior, motor activity or general motivation to drink, as these animals were subjected to the same water restrictions (**Figure 1D left**).

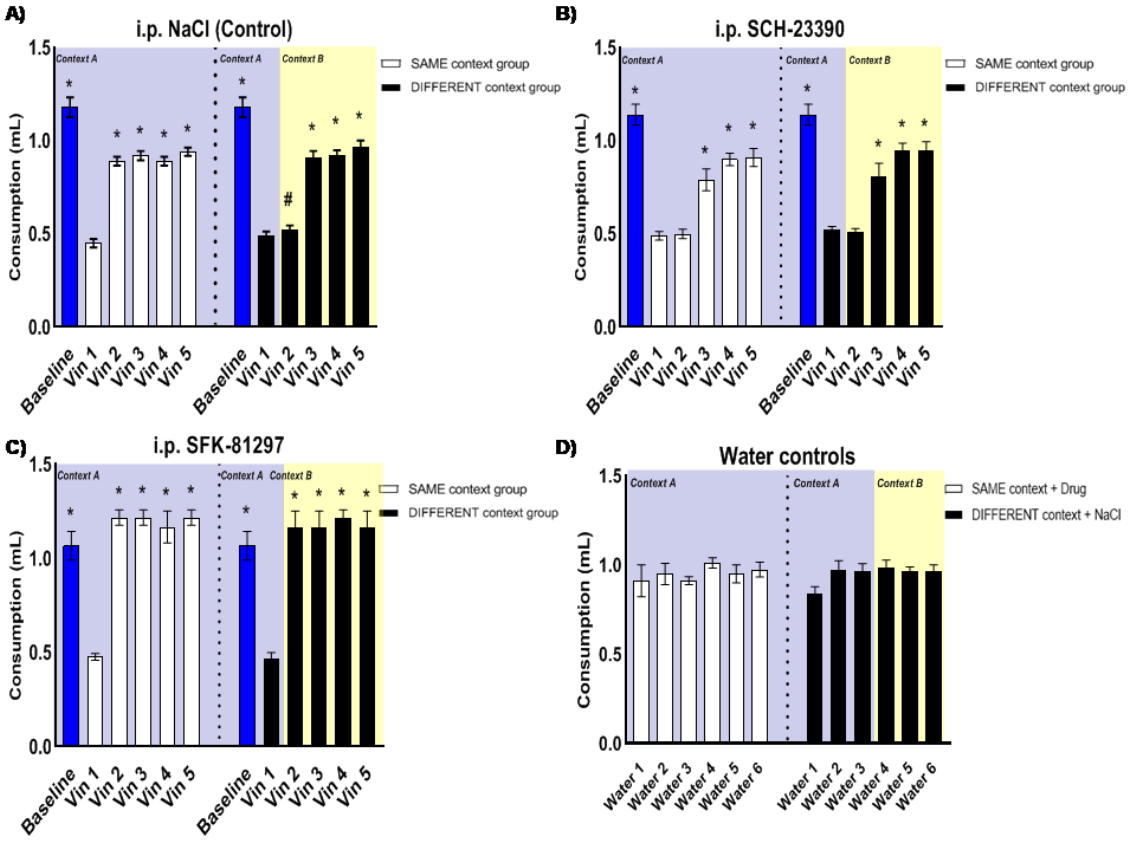

Figure 1. Mean (±SEM) of the consumption of **(A)** the groups of mice that were exposed to vinegar and were i.p. injected with NaCl **(B)** SCH-23390 and **C)** SFK-81297. Panel **D)** represents the groups the groups that were exposed to water and were i.p. injected with either SCH-23390 or SFK81297 (on the left) and NaCl (on the right) 15 minutes before Water 4. All the groups on the left were exposed to the same auditory background throughout the entire behavioral protocol (Context A, represented in blue) while the groups on the right received a change in the auditory background (Context B, represented in yellow) from vinegar day 2 onwards. The symbol * represents statistically significant differences (p<.05) compared to Vin 1 within the same injection group. The symbol # represents statistically significant differences (p<.05) compared to the same day of the NaCl control group.

3.2 Experiment 5.2.: D1 dopamine receptors activity mimics the effect of an auditory context change disrupting the attenuation of taste neophobia.

We then aimed to investigate whether the effect observed in the animals injected with SCH-23390 was comparable to the change in the auditory background. In Experiment 5.1. we observed that pharmacological

blockade of D1DRs caused an effect similar to an auditory background change on AN. The opposite was also observed, as pharmacological activation of D1DRs disrupted the AN auditory context –dependent modulation. Thus, it could be proposed that changing the auditory background would result in a decreased dopaminergic activity which is critical for disrupting AN. This would explain the effects of SCH-23390 and SFK-81297 injections on Day 2. Accordingly, once the novel auditory background becomes familiar on Day 3, dopamine neurotransmission would recover its normal activity and animals would be able to exhibit AN. Taking all this into account, we then hypothesized that if the contextual information and the dopaminergic activity are closely related, then changing the auditory background on Day 2 and blocking D1DRs on day 3 should still have a similar impact on AN, as dopamine activity would be affected by both contextual information on day 2 and the acute drug infusion on day 3.

We confirmed this hypothesis with the groups that experienced an auditory context change on Day 2 and were injected with either saline, SCH-23390 or SFK-81297 on Day 3 (**Figure 2**). The results showed that the context change disrupted AN in the three groups as vinegar consumption did not increase on Day 2 as predicted by the results observed on Experiment 1. However, on Day 3 the animals injected with Saline and SFK-81297 showed increased consumption of vinegar compared to Days 1

and 2 (both *p´s*<.001), meanwhile the animals injected with SCH-23390 did not increase consumption of vinegar until Day 4 and no differences in consumption were observed between Days 1, 2 and 3 (all *p´s*=1).

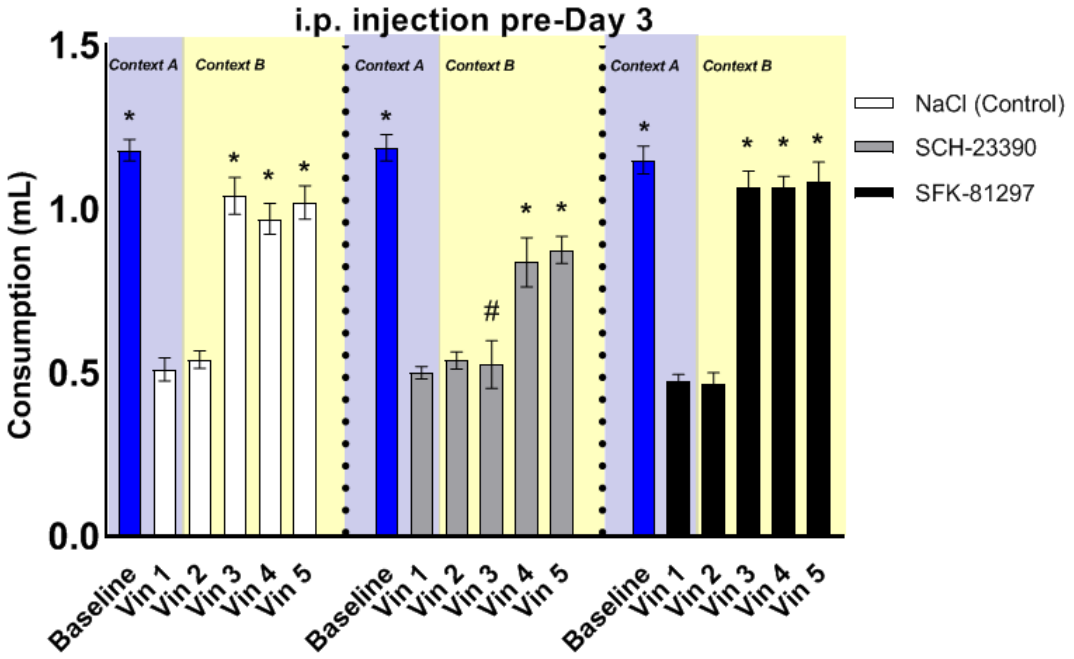

Figure 2. Mean (±SEM) of the consumption of the groups of mice that drank vinegar, received a change in the auditory background on vinegar day 2 and were i.p. injected with NaCl,SCH-23390 or SFK-81297. The symbol * represents statistically significant differences (p<.05) compared to Vin 1 within the same injection group. The symbol # represents statistically significant differences (p<.05) compared to the same day of the NaCl control group.

3.3 Experiment 5.3.: Effect of the auditory context change and the internal physiological state on the attenuation of taste neophobia.

The results of Experiment 5.1. showed that both the manipulation of the auditory background as well as the administration of D1DR antagonist SCH-23390 disrupted AN. Moreover, the results of Experiment 5.2. showed that the effect of these manipulations are independent from each

other, that is to say, the change in auditory background results in delayed AN, and the administration of SCH-23390 on a different day maintains the low consumption of the taste and prevents AN. As a potential explanation we have proposed that the context change might induce a decrease of the D1DR activity which is responsible of the AN context-dependency. However, an alternative explanation could be that the SCH-23390 injection might constitute itself an internal context change that could impair AN. Thus, the pharmacological changes induced by the injection could produce a state-dependent effect causing a disruption of AN. Hence, the dopaminergic system would not be critically involved in the AN context-dependency but it would be just causing a novel internal state acting as a context change. If this were the case, repeated exposures to the taste under the influence of SCH-23390 would avoid the effect as the internal physiological context caused by the SCH-23390 injection would become familiar and AN would take place.

In order to evaluate this possibility, two groups of mice were used. Both groups of animals were subjected to the same behavioral protocol than Experiments 5.1. and 5.2. They experienced a change in the auditory background on Day 2 but they were repeatedly i.p. injected with either SCH-23390 or sterile 0.9% NaCl on Days 2, 3 and 4. As expected, the animals injected with NaCl showed reduced consumption of vinegar on Day 2 ($p<.001$) – showing the disruptive effect of the auditory background

change – and increased consumption of vinegar on Days 3 4 and 5 (all *p´s*<.001). However, the animals injected with SCH-23390 showed reduced consumption of vinegar on Days 1, 2, 3 and 4 (all *p´s*<.001) and consumption of vinegar did not increase until Day 5, thus discarding a state-dependent effect (**Figure 3**) and supporting evidence for the implication of D1DRs in AN.

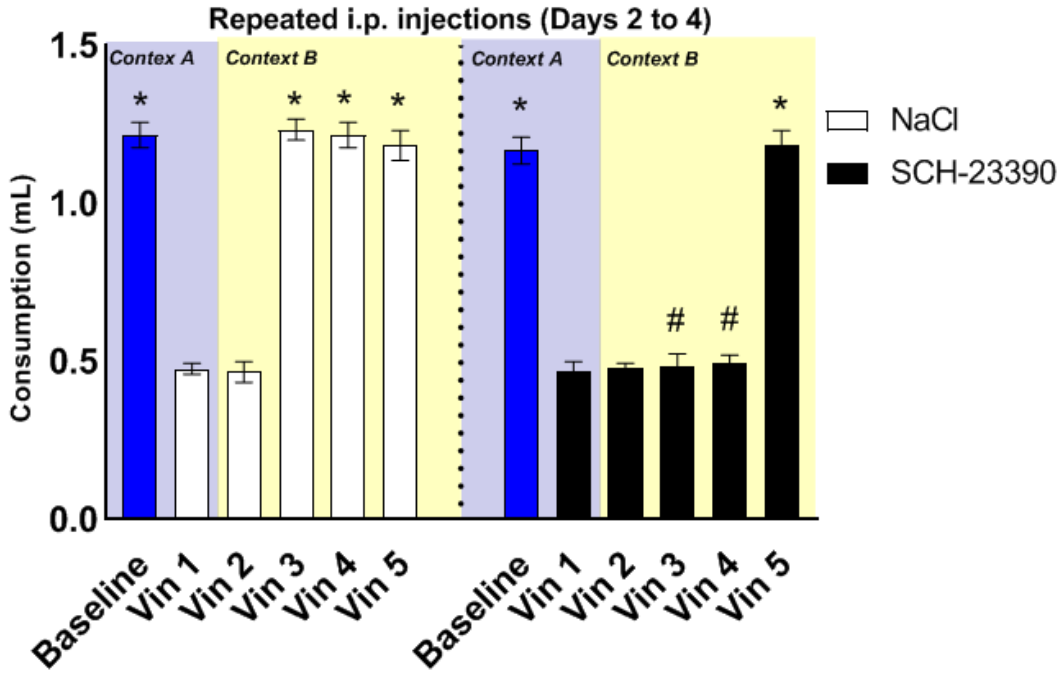

Figure 3. Mean (±SEM) of the consumption of the groups of mice that drank vinegar, received a change in the auditory background on vinegar day 2 and were repeatedly i.p. injected with NaCl orSCH-23390 15 minutes before the drinking sessions of days Vin 2, Vin 3 and Vin 4. The symbol * represents statistically significant differences (*p*<.05) compared to Vin 1 within the same injection group. The symbol # represents statistically significant differences (*p*<.05) compared to the same day of the NaCl control group.

3.4 Experiment 5.4.. Effect of hippocampal 6 hydroxidopamine lesions on the attenuation of taste neophobia.

As the AN contextual modulation by the auditory non-spatial context has been shown to depend on the integrity of the CA1 region of the hippocampus in mice (Grau-Perales et al., 2019b) and the above results suggest that the AN contextual modulation might also depend on the activity of the dopaminergic system throughout D1DRs, we aimed to investigate to what extent this dopaminergic activity is required at the hippocampus. For this purpose, we assessed the effect of the hippocampal catecholaminergic depletion on the auditory context dependency of AN, using the behavioral protocol described in Experiments 5.1., 5.2. and 5.3**.**

In this experiment we used rats instead of mice, given the bigger brain size which results more adequate for intracerebral interventions. The animals were randomly assigned to the following groups: *6-OHDA* group, which received bilateral i.c. injection into the ventral CA1 region of the hippocampus (vCA1), and *Sham* group, which received bilateral i.c. injections of sterile 0.9% NaCl solution into the vCA1. One week after the surgery, the animals were subjected to the same behavioral protocol described in Experiments 5.1., 5.2. and 5.3. involving the exposure to a vinegar taste solution and an auditory background that could either change on Day 2 or remain constant throughout the procedure.

There were no differences between Sham and 6-OHDA groups during the baseline (BL) of water consumption, thus discarding non specific effects of the lesion on drinking behavior. All groups consumed lower amounts of water on Day 1 compared to Days 2, 3 and 4 ($p<.001$) during the baseline. This indicates similar adaptation to the water deprivation procedure in both groups.

By inspection, neophobia to vinegar is clearly observed and reduced drinking persists longer if the auditory background is changed in control sham animals (**Figure 4A**). Thus, AN occurred on Day 3, when the novel auditory background became familiar, showing the same AN dependency of the auditory context previously reported in mice. However, the ventral hippocampal catecholaminergic depletion interfered with the context-dependency of AN (**Figure 4B**) since vinegar intake increased on Day 2 in spite of the context change. This result is similarly to that previously reported after hippocampal NMDA lesions (Grau-Perales et al., 2019b)**.**

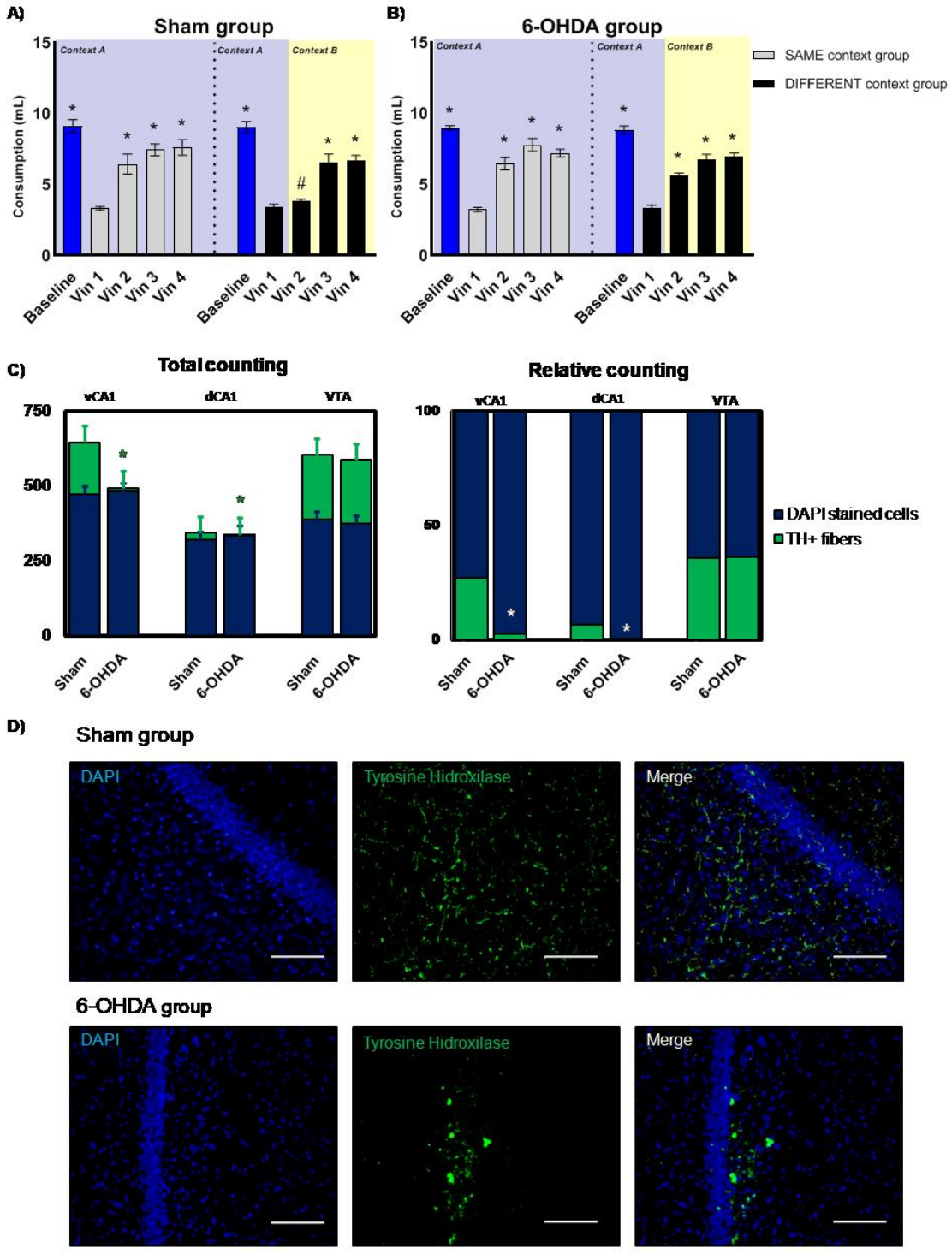

Figure 4. A) Represents the mean (±SEM) of the consumption of the Sham group of rats that were i.c. injected with NaCl and **B)** the 6-OHDA groups of rats that were i.c. injected with 6-Hydroxidopamine. On the left, the groups that kept the auditory background constant and on the right the groups that experienced an auditory background change on vinegar day 2. The symbol * represents statistically significant differences (p<.05) compared to Vin 1 within the same injection group. The symbol # represents statistically significant differences (p<.05) compared to the same day of the SAME context group. Figure **C)** represents the Total counting (left) and Relative counting (right) of the Tyrosine

Hydroxilase (TH+) fibers of different brain regions: dorsal and ventral CA1 region of the hippocampus and ventral tegmental area (VTA) between the Sham and 6-OHDA groups The symbol * represents statistically significant differences ($p<.05$) compared to the Sham group. Figure **D)** shows representative images of immunofluorescence of Tyrosine Hydroxilase within the ventral CA1 region of the hippocampus of both Sham and 6-OHDA groups.

Further analyses using immunofluorescence of tyrosin hydroxilase (TH+) terminals confirmed that the administration of 6-OHDA resulted in a selective decreased number of TH+ fibers within the hippocampus (both in the dorsal and ventral CA1 regions), but not in control areas such as the ventral tegmental area (VTA) in animals injected with 6-OHDA compared with sham-injected animals. Moreover, the analyses revealed no differences in the total number of DAPI stained cell nuclei present in the brain samples, indicating that the injection of 6-OHDA selectively affected catecholaminergic terminals rather than causing a global and unspecific lesion of the area (**Figure 4C and 4D**).

3.5 Experiment 5.5.: Chemogenetic silencing of the nucleus accumbens shell–ventral CA1 hippocampal region pathway.

The results of the previous experiment indicated that the hippocampal catecholaminergic activity is required for the formation of the safe taste memory to be modulated by the auditory context. Also the results of the first series of experiments (Experiments 5.1, 5.2. and 5.3.) point to the relevance of D1DRs in the effect of the auditory context over the

formation of a safe taste memory. However, little is known about the neural network supporting the interaction between the hippocampal system and the reward/emotional systems related with the change in the taste hedonic value. The hippocampus is highly innervated by dopaminergic terminals originated at the nucleus accumbens and the VTA area (Hansen & Manahan-Vaughan, 2014). It has been reported increased activity of NAcb-Shell at the second exposure to a novel taste (Grau-Perales et al., 2019c) which occurs the same day in which AN takes place.

Taking all these data into account, it could be hypothesized the relevance of the nucleus accumbens efferent projections to the hippocampus for the contextual modulation of AN. In order to selectively silence the NAcb-Shell-vCA1 pathway, we used the DREADD (designer receptor exclusively activated by designer drug) chemogenetic approach (Boender et al., 2014; Roelofs et al., 2017) in order to evaluate the ability to disrupt the effect of a context change at different stages of the safe taste memory acquisition. Thus, a combination of two viral vectors was infused into the vCA1 region of the hippocampus and the NAcb-Shell in order to be able to inhibit the activity of this specific pathway at very discrete time points. The retrograde CAV-Cre recombinase expressing virus was targeted to the vCA1 region of the hippocampus and the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the NAcb-Shell (**Figure 5A**). Four weeks after the surgery, the animals were subjected to

the same behavioral protocol described in the previous experiments. All the animals received i.p. of Clozapine No-Oxide dihydrochloride (CNO-DHCL) 1.5 hours before the drinking session on test Day 2. This allowed us to specifically silence the firing of neurons in the NAcb-Shell that are projecting directly to vCA1 since those are the neurons that are expressing the DREADD through the enhanced cre-recombinase expression produced by the CAV-cre vector. The expression of the hM4D receptor was assessed using immunofluorescence of anti mCherry four weeks after the viral infusions (**Figure 5B**).

The predictions were that since increased activity in the nucleus accumbens is required on day 2 for AN, the selective inhibition of the NAcb-Shell–vCA1 projecting neurons activity on that day should result in a AN impairment. However, the inactivation of NAcb-Shell–vCA1 pathway in a group of animals injected with CNO-DHCL before drinking on Day 3 should not impair AN. Furthermore, in order to exclude any unspecific effect of the CNO-DHCL injection on AN, one additional control groups was used, being half of the animals DREADD^{+}/CAV-cre^{-} and i.p, injected with CNO-DHCL and the other half DREADD^{+}/CAV-Cre^{+} and i.p. injected with sterile 0.9% NaCl solution.

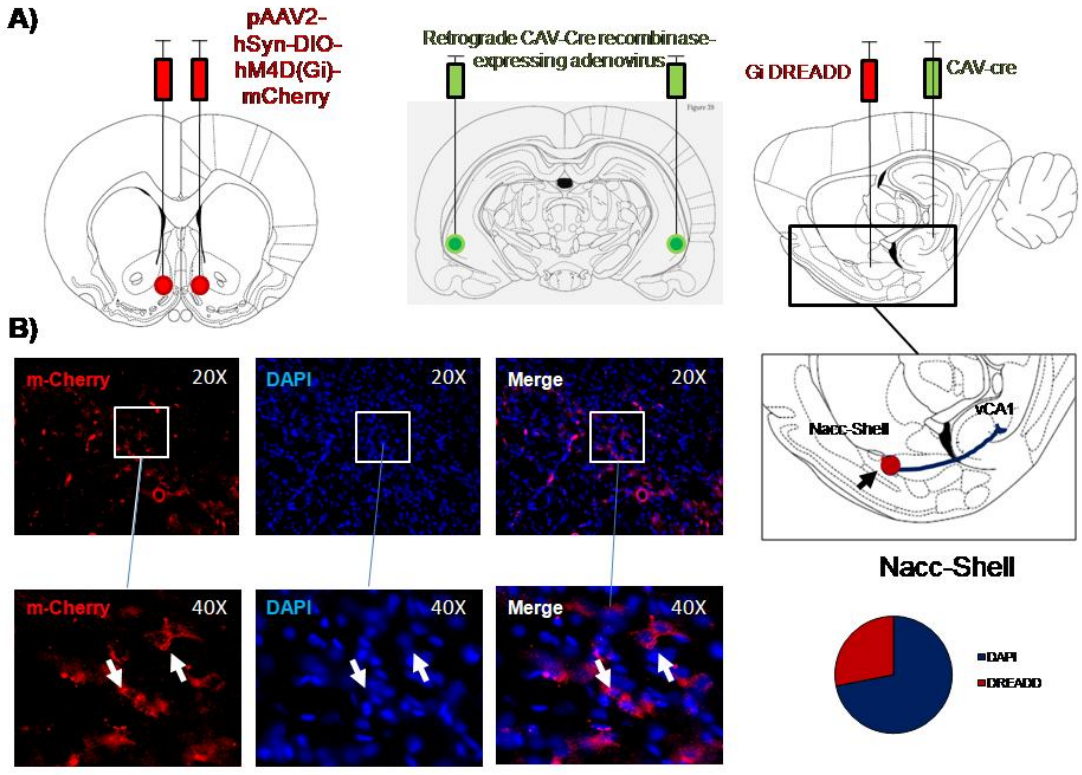

Figure 5. Panel **A)** represents the condition in which the CAV-Cre recombinase expressing virus was targeted to the vCA1 and the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry was targeted to the NAcb-Shell. Panel **B)**represents the assessment of the expression of the hM4D receptor using immunofluorescence of anti-mCherry.

Te results are shown in **Figure 6**. The Control No-DREADD groups showed normal AN when the auditory background remained constant, as they were able to increase consumption of vinegar on day 2 compared to day 1 and reached the asymptote in consumption (p<.001) (**Figure 6A and 6C left)**. The AN context-dependency was also evident because they exhibited delayed AN when the auditory background changed on Day 2. In fact no difference in consumption between Days 1 and 2 (p=1) and increased consumption on Days 3, 4 and 5 (all $p´s$<.001) was evident

(**Figure 6B and 6D left**). However, the chemogenetic silencing of the NAcb-Shell–vCA1 pathway ay on Day 2 delayed AN until Day 3 (p=1 between Days 1 and 2) in spite of the auditory background being kept constant, thus mimicking the context change (**Figure 6A right**). This indicates that the activity of NAcb-Shell–vCA1 pathway on Day 2 is required for the formation of a context-dependent safe taste memory. However, no effect was found when the the chemogenetic silencing of the NAcb-Shell-vCA1 pathway occurred on Day 3 (p=1) remaining the auditory background constant (**Figure 6C right**). In this case, both groups of animals maintained AN which had been evident already on Day 2 (p<.001 between Days 1 and 2 for both groups). This suggests that the activity of NAcb-Shell–vCA1 pathway is crucial during the formation of the safe taste memory but not in later stages.

On the other hand, no differences were found between the control group and the DREADD group on Day 2 when the auditory background was changed on Day 2 (p=1 for both groups). The chemogenetic inhibition of the NAcb-Shell-vCA1 projecting neurons had an effect on Day 3, when the control animals showed an increased consumption of vinegar (p<.001) and the DREADD animals did not increase consumption on Day 3 (p=1).

Additionally, the effect of the DREADD infection and CNO-DHCL injection over consumption was also assessed with additional control groups that were exposed only to water. The animals were previously

infused with the DREADD 1.5 hours before drinking water and they received i.p. injections of either CNO-DHCL or 0,9% NaCl ,. No differences were found in water consumption neither between the injection groups nor between drinking days (all *p´*s>.3). This allowed us to exclude any alternative interpretations in terms of both the DREADDs and the i.p. administration of drugs having general or unspecific effects on drinking behavior.

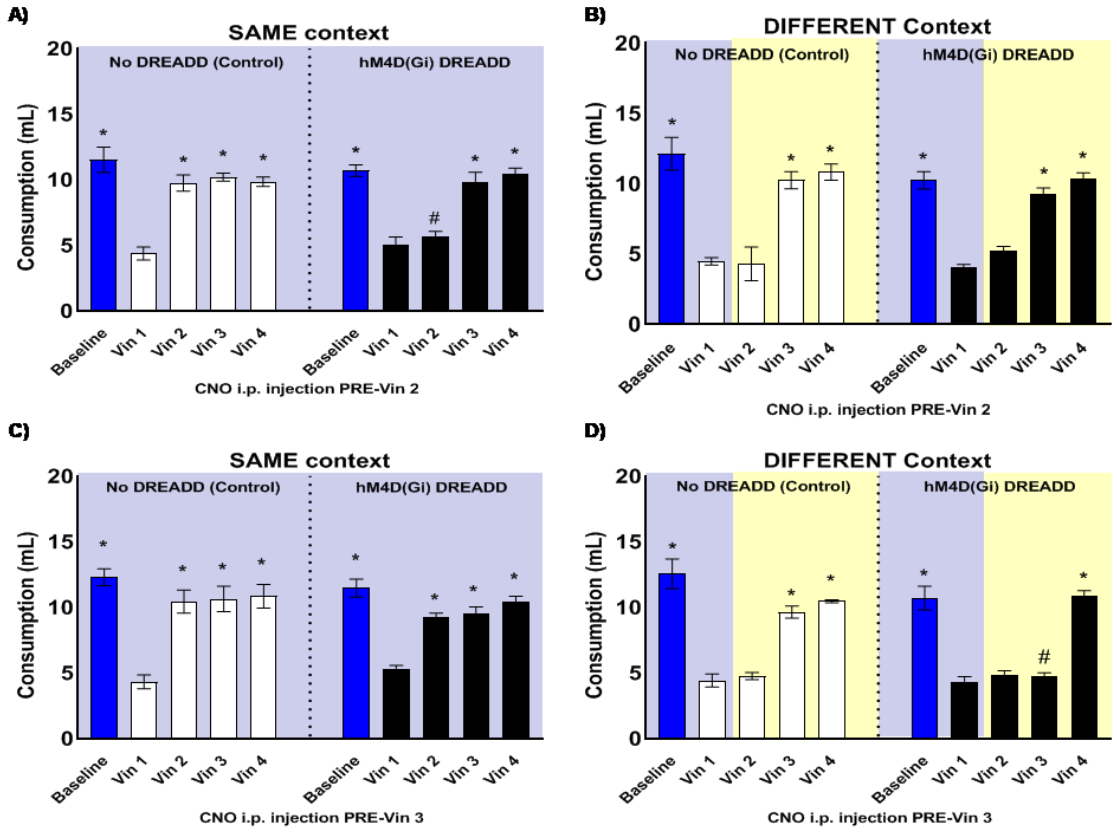

Figure 6. Mean (±SEM) of the consumption of **(A)** the groups of rats that were exposed to the same auditory background and were i.p. injected with CNO before Vin 2; **B)** the groups of rats that experienced a change in the auditory background on Day 2 and were i.p. injected with CNO before Vin 2; **C)** the groups of rats that were exposed to the same auditory background and were i.p. injected with CNO before Vin 3 and **D)** the groups of rats that experienced a change in the auditory background on Vin 2 and were i.p. injected with CNO before Vin 3. White bars represent control animals that were not expressing the inhibitory DREADD (No-DREADD group). Black bars represent the animals that expressed the hM4D receptor by the DREADD. The symbol * represents statistically significant differences (p<.05) compared

to Vin 1 within the same injection group. The symbol # represents statistically significant differences (p<.05) compared to the same day of the No-DREADD control group.

Hence, these results point to the involvement of the NAcb-Shell–vCA1 pathway in AN modulation by non-spatial contextual cues. In all, it can be proposed that the ability of the auditory context to modulate taste recognition memory requires the activity of a dopaminergic nucleus accumbens-hippocampal circuit that might be crucial for the taste-context interaction during the safe taste memory formation.

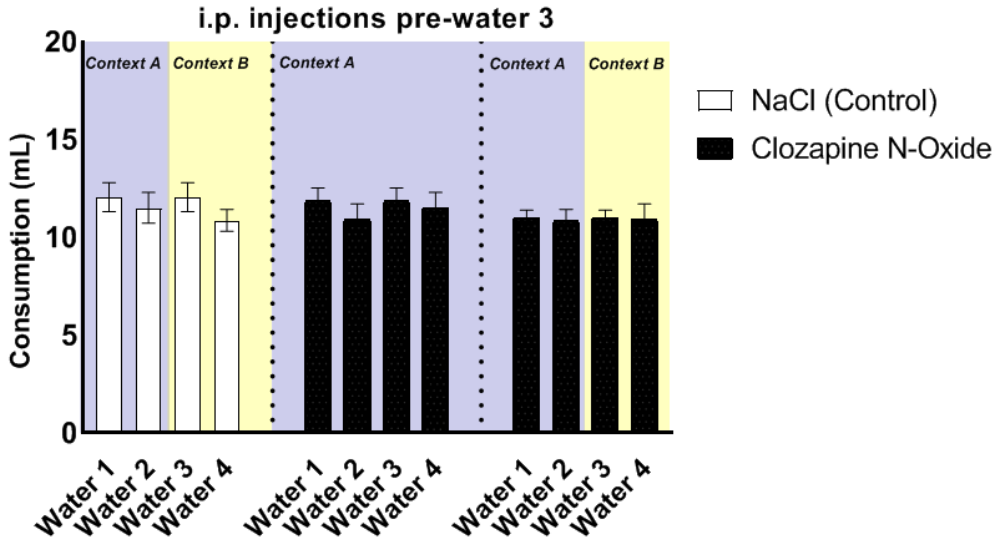

Figure 7. Mean (±SEM) of the consumption of the control groups of rats that were exposed to water. White bars represent the animals that experienced a change in auditory background and were i.p. injected with NaCl. Black bars represent the animals that were i.p. injected with CNO before Water 3, being half of them exposed to the same auditory background and the other half experienced a change in auditory background.

4. Discussion

Our results identify for the first time a NAcb-Shell–vCA1 pathway critical for the formation of the auditory context-dependent safe taste memory which is responsible of AN. Also our data suggest that this phenomenon requires dopaminergic signaling mediated by D1DRs in the NAcb-Shell–vCA1 pathway. The present data indicate that changes of the auditory contextual background modulate the AN behavioral expression and that this modulation depends on the D1DRs activity being the hippocampus the critical target of the accumbens projections relevant for it.

The fact that the auditory background provides contextual information influencing AN confirms our previous report (Grau-Perales et al., 2019b). As previously reported, the modulation of AN by the auditory context was assessed using two different auditory backgrounds. Changing the auditory background in the second taste solution exposure disrupted AN while the groups under a constant auditory background exhibited complete AN on day 2. Given that the groups subjected to the context change exhibited AN on day 3 in which the second context was already familiar, it can be conceived that the memories of both context and taste have been associated to form a context-dependent safe taste memory, whatever the learning mechanisms involved. This is in accordance with previous results demonstrating the spatial context dependency of AN (De la Casa & Díaz, 2013) and it extends the phenomenon to non-spatial auditory contextual

cues. Hence, this is consistent with a wide definition of context that includes the auditory modality in addition to the cues previously used such as visual (De la Casa & Díaz, 2013; Quintero et al., 2011) and temporal information (Moron et al., 2002; De la Casa et al., 2003; Manrique et al., 2004).

Accordingly to the view of dopamine as a critical neurotransmitter for taste learning processes as well as its relation with the context-dependency of AN (De la Casa & Díaz, 2013; Ramírez-Lugo et al., 2007), systemic administration of the D1 dopamine antagonist SCH-23390 prevented AN when the context was kept the same. Interestingly, both D1DR blockade and changing the auditory background resulted in a similar impairment of AN. Additionally, the administration of the D1 dopamine agonist SFK-81297 resulted in a facilitation of AN regardless that the auditory background changed or not.

These data suggest a relationship between D1DRs activity and the association of the taste with the context. When the animal drank vinegar on day 2 in the same context, D1DRs might have been critical for recognizing the auditory background. Thus, if D1DRs were blocked, the animal would not have been able to establish the context-dependent safe taste memory, resulting in maintained taste neophobia. Additionally, a change of the auditory background would have had similar effects on D1DR activity, resulting in maintained neophobia as well. However, since on day 3 the

second auditory background becomes familiar, then D1DRs activity should increase, thus resulting in increased taste consumption that indicates AN.

This hypothesis relating D1DRs activity and the context change was assessed with additional groups of mice that experienced a change of the auditory background on day 2 as well as i.p. administration of SCH-23390 or SFK-81297 on day 3. The behavioral results confirmed a similar additive effect of the context change and the D1DR antagonist SCH-23390 systemic injection as the animals did not show any increase in consumption of vinegar until day 4. Hence, it seems reasonable to propose that the change of the auditory background might have disrupted the dopaminergic neurotransmission which maintains the context-dependent safe taste memory trace.

It is important to mention that neither changing the auditory background nor the administration of SCH-23390 did have any effect on drinking behavior separately, as they did not affect water consumption. This allows us to rule out any explanation involving non-specific effects on drinking behavior. An alternative explanation in terms of a state-dependent effect attributed to a change of the internal physical state induced by the drug injection do not seem feasible, because in the Experiment 5.3. repeated SCH-23390 injections disrupted AN along several days in spite of the drug-induced internal state becoming familiar.

Therefore, it could be hypothesized that the AN disruption induced by changing the auditory background might be due to the proposed effect of the context change on the dopaminergic activity (De la Casa & Díaz, 2013; De la Casa et al., 2003) and that this activity takes place in the hippocampus. In fact, dopamine has been reported to be crucial for consolidating contextual memories in the hippocampus (Kempadoo et al., 2016; Takeuchi et al., 2016; Yamasaki & Takeuchi, 2017b). Also, we have previously reported that excitotoxic NMDA lesions of the hippocampal CA1 region impair the AN auditory context-dependency using an identical behavioral procedure to that applied in the present study (Grau-Perales et al., 2019b).

In the Experiment 5.4. we tested this hypothesis inducing the catecholaminergic depletion of the ventral hippocampus CA1 region (vCA1) using 6-hidroxydopamine (6-OHDA). The results indicated absence of the AN context-dependency since the familiar taste consumption increased on day 2 in spite of the auditory context change. Thus, it seems that in those 6-OHDA animals was impaired the association between taste and auditory cues whatever its nature. This is consistent with the widely accepted hippocampal critical role in learning and memory processes depending on contextual cues (O'Keefe & Dostrovsky, 1971). However, little is known about the anatomical pathways that mediate the

interaction between the hippocampus and the anatomical circuit responsible of the safe memory taste formation.

We have previously reported using an identical behavioral protocol that the attenuation of vinegar neophobia involves a circuit which includes the perirhinal cortex (Gómez-Chacón et al., 2015; Morillas et al., 2017), the piriform cortex (Grau-Perales et al., 2019a) and NAcb-Shell(Grau-Perales et al., 2019c). The NAcb is a main component of the dopaminergic reward system proposed as relevant for the hedonic shifting of taste palatability that takes place during AN (Yamamoto, 2006). Both increased NAcb-Sh activity (Grau-Perales et al., 2019c) and hippocampal dopaminergic innervations have been found to be related with the second exposure day to a vinegar solution. Thus, it could be hypothesized a NAcb-Shell dopaminergic projection targeting vCA1 as being crucial for the taste-context interaction during the safe taste memory formation on day 2.

In the Experiment 5.5. we inactivated the NAcb-Shell neurons projecting to vCA1 in order to test this hypothesis. Using a chemogenetic approach we were able to perform the inhibition of the nucleus accumbens neurons projecting to vCA1 region that expressed the inhibitory DREADD hM4D(Gi) by the i.p. administration of Clozapine N-Oxide (CNO) at discrete time points. The inhibition of the NAcb-Shell-vCA1 pathway resulted in the inability to increase consumption of vinegar, that is, impaired AN. Thus, chemogenetic silencing of the NAcb-Shell-vCA1

pathway performed on day 2 of vinegar exposure disrupted AN even if the auditory background remained constant. This effect was the same as that induced by the administration of the D1DR antagonist SCH-23390 and that of a context change. Also, inhibition of the NAcb-Shell-vCA1 pathway on day 3 impaired AN when there was a change in the auditory background on day 2. This is in line with the proposed explanation in terms of the auditory background change affecting the activity of the dopaminergic system during the formation of the safe taste memory, that results in impaired AN.

Furthermore, our approach allows us to refine the temporal involvement of theNAcb-Shell - vCA1 pathway in the AN context-dependency. In fact, under a constant context its inactivation impaired the formation of the safe taste memory on day 2 but not on day 3 once AN had already taken place. This is consistent with a wider view of the so called "hippocampus-VTA downward loop" which has been proposed to be crucial for the acquisition of complex learning phenomena, such as contextual learning (Lisman & Grace, 2005) having the NAcb specific relevance in taste learning. Accordingly, the NAcb dopaminergic innervations plays a role not only in AN (Bassareo & Di Chiara, 1997) but also in other types of taste learning such as aversive taste recognition memory (S. Fenu et al., 2001; Ramírez-Lugo et al., 2007), preference learning (Shimura et al., 2002).

A dorsal hippocampus-medial prefrontal cortex pathway critical for visual recognition memory has been identified using chemogenetic

inactivation (Tusscher et al., 2018). The chemogenetic approach has been also proven to be useful for understanding the neural mechanisms of contextual memory consolidation. Moreover, DREADD excitatory synaptic activity of a dopaminergic hippocampal-accumbens pathway can rescue memory deficits in a mouse model of Alzheimer's disease (Cordella et al., 2018).

Although more research is needed to understand how the NAcb-Shell-vCA1 pathway interact with the neural circuits involved in regulating consumatory behavior, these results contribute to outline the brain network that underlie the formation of complex taste memories modulated by the environmental cues that go beyond spatial information processing.

5. MATERIALS AND METHODS

5.1 Subjects

In the present series of experiments both mice and rats were used. A total number of one hundred and sixteen adult male C57BL/6 mice (weighing 20-24g, Charles River, France) and eighty adult male Wistar rats were used in this series of experiments. The use of mice was specifically selected for the experiments requiring systemic administration of drugs and the use of rats was selected for the experiments requiring stereotaxic surgery for a more precise intracerebral administration of substances. All animals were housed individually and maintained on a 12-hour dark-light

cycle (lights from 8:00 am to 8:00pm) during the behavioral procedure. All the experimental procedures were performed during the light cycle at the same time each morning in the home cage. All animals were given *ad libitum* access to food and water until the experiment started, at which time access to water was restricted to a daily 10-minute (for the mice) and 15-minute (for the rats) morning drinking session. Four hours afterwards, all animals could rehydrate as they got additional access to water for one hour.

All procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (CEEA17-02-15-195) and were in accordance with the European Communities Council Directive 86/609/EEC.

5.2 Behavioral Procedure

All the animals received the same behavioral procedure, a three phase protocol: baseline (5 days), Phase I (one day) and Phase II (3-4 days). Liquid was available from a drinking tube during daily drinking sessions (Grau-Perales et al., 2019b). The tube was gently bent and carefully placed in the home cage to prevent the loss of solution. The amount of ingested liquid was recorded using a microgram balance to weigh the tubes containing the solution before and after each drinking session.

An experimentally-controlled auditory background was continuously present during the 10-minute drinking sessions (including baseline, Phase I and Phase II). Two different sounds were used and counterbalanced amongst the subjects. One sound was a pure 600 Hz tone (PT) consisting of 3 second pulses with an inter stimulus interval (ISI) of 3 seconds. The second sound was Gaussian white noise (WN) consisting of 2 second pulses with an ISI of 4 seconds. Both sounds were created using MATLAB. Each day two speakers were positioned approximately one meter from the rack with the mouse homecages. Immediately after the sound started, the tubes containing the solution were placed in the home cages. After 10 minutes, the tubes were removed and the sound stopped. These sounds were not present during the rehydration sessions.

In **Experiment 5.1.**, the mice were randomly assigned to the following experimental groups according to the i.p. injection and auditory background pairing: *SCH-23390 – Same Sound* (*n*=10), *SCH-23390 – Different Sound* (*n*=10), *SFK-81297 – Same Sound* (n=10), *SFK-81297 – Different Sound* (n=10), *Saline-Same Sound* (*n*=10)and *Saline-Different Sound* (*n*=10). Three additional groups *Water-Different Sound – SCH-23390* (n=8), *Water-Different Sound – SFK-81297* (n=8) and *Water-Different Sound - Saline* (n=8) were used to assess the impact of the drug infusion on drinking behavior.

During Phase I and Phase II all mice had access to the 3% cider vinegar solution (5º acidity) instead of water during the 10-minute drinking sessions. The groups assigned to *Water* continued to be exposed to water and did not receive any flavored solution. The mice assigned to the *Same Sound* groups were only exposed to one of the two auditory cues (either the PT or the WN). The mice assigned to the *Different Sound* groups experienced a change in the auditory background in Phase II. Thus, the auditory cue present during Phase II was different from that used on baseline and Phase I (vinegar day 1). Due to counterbalancing half of the animals changed from PT to WN and the other half changed from WN to PT.

The animals assigned to *SCH23390* groups received a i.p. injection of SCH-23390 (0.5µg/µl in a volume of 0.1ml/100mg of body weight) 15 minutes before the drinking session of Day 2 of vinegar. The animals assigned to *SFK81297* groups received a i.p. injection of SFK-81297 (5mg/kg of b.w.) 15 minutes before the drinking session of Day 2 of vinegar The animals assigned to *Saline* received i.p. injection of 0.9% sterile NaCl solution (0.1ml/100mg) 15 minutes before the drinking session of Day 2 of vinegar. Additionally, two control groups were used to assess the impact of the context change and the drug infusion over consumption of vinegar.

In **Experiment 5.2.**the mice were subjected to the same behavioral procedure than performed on Experiment 1. The only difference was that

all the animals received a change in the auditory background on day 2 and the i.p. injection of either SCH-23390 (n=8), SFK-81297 (n=8) or sterile NaCl saline (n=8) was performed 15 minutes before drinking vinegar on day 3.

In **Experiment 5.3.** the mice were subjected to the same behavioral procedure as Experiments 5.1. and 5.2., but all mice received i.p. injection of SCH-23390 (n=6) or sterile 0.9% NaCl saline solution (n=6) 15 minutes before drinking vinegar on days 2, 3 and 4.

In **Experiment 5.4.** all the rats were subjected to the same behavioral protocol performed on mice on the three previous experiments. Ventral hippocampus and sham catehcolaminergic depletion were randomly assigned to experimental groups specified by the auditory background was the same or different in Phases I and II. Thus, four groups were used to assess the impact of hippocampus catehcolaminergic depletion: *Sham-Vinegar-Same Sound* (*n*=8), *Sham-Vinegar-Different Sound* (*n*=8), *6OHDA-Vinegar-Same Sound* (*n*=8)and *6OHDA-Vinegar-Different Sound* (*n*=8). For this purpose, all animals were i.c. injected with either 6-OHDA or sterile 0.9% NaCl solution into the ventral CA1 region of the hippocampus (see **Surgery**) one week before the behavioral procedure started.

In **Experiment 5.5.**all the rats were subjected to the same behavioral protocol performed on the previous experiments. All the animals were

injected with the combination of viral vectors: the retrograde CAV2-cre recombinase expressing virus (Plataforme de Vectorologie de Montpellier, France) that was targeted to the ventral CA1 region of the hippocampus and the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry (Addgene, USA; #44362-AAV2) which was targeted to the NAcb-Shell 4 weeks before the start of the experiment. On Experiment 5A rats were injected with CNO-DHCL (2mg/kg of body weight, n=8) or sterile 0.9% NaCl (n=8) 1.5 hours before the drinking session on vinegar day 2. On Experiment 5B rats were injected with CNO-DHCL (2mg/kg of body weight, n=8) or sterile 0.9% NaCl (n=8) 1.5 hours before the drinking session on vinegar day 3. Two additional control groups were also used to assess the impact of the chemogenetic inactivation of the nucleus accumbens – hippocampus projecting neurons over water consumption. These animals were injected with the combination of the two viral vectors and were exposed to water for five consecutive days. 1.5 hours prior to the drinking session of day 4, the animals received the i.p. injection of CNO-DHCL and then were exposed to water.

5.3 Surgery

-Experiment 5.4.: Catecholaminergic depletion of vCA1 using 6-hydroxidopamine.

Surgery was performed under general anesthesia with a mixture of ketamine and medetomidine (0.1% b.w.). The animals were randomly assigned to one of two groups: 6-OHDA and sham. They were placed in a stereotaxic apparatus (Stoeling Co. Instrument, Word Dale, IL, USA) with bregma and lambda at the same height. Small trephine openings were drilled in the exposed skull in order to perform two bilateral injections of either 6-OHDA (12 µg/µl dissolved in phosphate buffered saline (PBS) plus 0.01% L-ascorbic acid) or sterile phosphate buffered solution (PBS; pH= 7.4) through 30 gauge injection needles that were connected to 10µl Hamilton syringes, so that 0.5µl of 6-OHDA solution or PBS was infused in each hemisphere at a rate of 0.5µl/min using an injection pump (Harvard Apparatus, Holliston, MA, USA). The needles were left in place for an additional 90 seconds before being slowly withdrawn. The stereotaxic coordinates targeted dorsal CA1 according to Paxinos and Watson´s rat brain atlas (2001). The coordinates relative to bregma were: AP: -4.8; ML: ±5; DV:-7.6mm for the first injection and AP: -5.3; ML: ±5.4 and DV: -8mm for the second injection. The skin was sutured and covered with povidone. After the surgery, all animals received additional s.c. injections of 5% Baytril and Bupac (0.1ml) for four consecutive days in order to reduce post-surgical pain and prevent infection.

-Experiment 5.5.: Chemogenetic inhibition of the nucleus accumbens shell neurons rojecting to the ventral CA1 hippocampal region.

Animals were placed in the stereotaxic apparatus, and half of them received i.c. injection of 1 µl/hemisphere containing $1x10^9$pp of the retrograde CAV-Cre recombinase expressing virus (Plataforme de Vectorologie de Montpellier, France) which was targeted to the ventral CA1 region (coordinates relative to bregma were: AP: -4.8; ML: ±5; DV:-7.6mm) and 1µl/hemisphere containing $5x10^{12}$ vg/mL of the inhibitory DREADD pAAV2-hSyn-DIO-hM4D(Gi)-mCherry (Addgene, USA; #44362-AAV2) was targeted to the NAcb-Shell(coordinates relative to bregma: AP: +2.00; ML: ±1.1; DV: -7.8mm). The other half (the control no-DREADD group) was injected with the same retrograde CAV-Cre recombinase expressing virus was targeted to the vCA1 and 1µl/hemisphere containing sterile 0.9% NaCl solution targeted to the NAcb-Shell. The infusion of the vectors was controlled by a Harvard Infusion Pump at a rate of 0.5µl per minute. The injectors were kept in place for additional 5 minutes before slowly withdrawn. The skin was sutured and covered with povidone. After the surgery, all animals received additional s.c. injections of 5% Baytril and Bupac (0.1ml) for four consecutive days in order to reduce post-surgical pain and prevent infection.

5.4.Immunofluorescence of tyrosine hydroxilase.

All the animals were euthanized ~24 hours after the last drinking session. They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were removed and placed in a 4% paraformaldehyde solution for 4 h at 4ºC before being transferred to a 30% sucrose solution until they sank for cryoprotection. Coronal sections were cut at 20µm in a cryostat (Leica CM1900).

Tissue sections were then rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4) and incubated in a solution of 3% normal goat serum and 0.5% PBS twen20 for 1 hour. Slices were transferred to a rabbit anti-tyrosine hydroxilase primary antibody (1:1000, #AB152, MERCK USA) overnight at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Goat Anti-Rabbit IgG (H+L) cross-adsorved Secondary Antibody, Alexa fluor 488, 1:500, #A-11008) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, and 0.5% PBS tween20. Finally they were rinsed, mounted on gelatine-bubbed slides and cover-slipped using DAPI mounting medium.

The analysis of 6-OHDA induced lesion was assessed using a Fluorescence module on a Microscope (Olympus BX41). Slices containing the vCA1 region were identified using the *NeuroLucida* Software (mbf

Bioscience) from coronal sections located at the level of approximately at -5 mm relative to Bregma according to Paxinos and Watson (2009). Within each section microphotographs at 20X magnification were captured using the blue (DAPI) and red (mCherry) filters for the NAcc-Shell that covered the entire extension of all the nuclei.

5.5.Immunofluorescence for DREADD expression assessment.

For Experiment 5.5., the animals´ brains were removed following the same protocol described for Immunofluorescnce of tyrosine hydroxilase. After being cut in coronal sections in a cryostat, tissue sections were rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4) and incubated in a solution of 3% normal goat serum and 0.5% PBS tween20 for 30 minutes. Slices were transferred to a mouse anti-mCherry primary antibody (1:500; Abcam, UK) overnight at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Alexa-594 labelled goat anti-mouse IgG, 1:500; Molecular Probes, USA) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, 0.5% PBS tween20. The sections were rinsed, then mounted on gelatine-bubbed slides they were cover-slipped using Fluorosheild Mounting Medium with DAPI (Abcam, UK) for nuclear counterstaining.

The expression of the DREADDs was assessed using a Fluorescence Microscope (Olympus BX41). Slices containing the NAcb-Shell were identified using the *NeuroLucida* Software (mbf Bioscience) from coronal sections of the NAcc located at the level of the apparition of the lateral ventricles, approximately at +2,52 and +2,28mm relative to Bregma (Section 1 and Section 2 respectively) according to Paxinos and Watson (2009). Within each section microphotographs at 20X magnification were captured using the blue (DAPI) and red (mCherry) filters for the NAcc-Shell that covered the entire extension of all the nuclei.

5.6. Drugs and substances.

Dopamine D1/D5 receptors antagonist SCH-23390 (R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride. Sigma Aldrich, Spain) was dissolved in Phosphate Buffered Saline (PBS; pH=7.4) at a concentration of 0.5µg/µl in a volume of 0.1ml/100 g of body weight. Dopamine D1DRs agonist SFK-81297 ((±)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide. Sigma Aldrich, Spain) was dissolved in PBS at a concentration of5mg/kg of body weight. All *Saline* animals received injections of sterile saline solution (0.9%).

The 3% cider vinegar solution was prepared daily in distilled water, ensuring that the taste and smell was the same across days.

6-Hydroxydopamine 6-OHDA (2-(2,4,5-Trihydroxyphenyl) ethylamine hydrochloride - Sigma Aldrich, Cat: H4381-100MG, Spain) was dissolved at a concentration of 12µg/µl in PBS + 0.01% L-ascorbic acid (L-Ascorbic Acid - Sigma Aldrich. Cat: A4403-100MG, Spain).

The novel taste used in the experiment consisted on a cider vinegar solution (5 grades of acidity) dissolved at 3% in tap water. All solutions were daily prepared by the experimenters ten minutes before the drinking session in order to prevent the loss of taste and smell properties.

The exogenous ligand used in Experiment 5.5. to activate the hM4D receptor expressed by the DREADD was Clozapine N-Oxide Dihydrochloride (8-Chloro-11-(4-methyl-4-oxido-1-piperazinyl)-5*H*-dibenzo[b,e][1,4]diazepine dihydrochloride; Tocris: #6329) and was dissolved in distilled H_2O at a concentration of 2mg/ml. We used this dose as it has been previously used elsewhere (Boender et al., 2014; Garner et al., 2012; Zhu et al., 2014).

6. Statistical analyses

Experiment 5.1.

Water consumption: baseline.

We began by testing whether the background auditory noise that can provide contextual information, affects water consumption. A global Mixed 5 x 2 x 2 x 2 x 2 (*Day* X *Solution* X *Injection* X *Context* X *Counterbalance*

Order) Repeated-Measures ANOVA comparing the water intake of allgroups on the five days of the baseline period only revealed a significant effect of *Day* [F(4, 200)=39.73 ; $p<.001$] and did not reveal any other significant effect or interaction (all *p's* >.2). Further analyses of the main factor *Day* revealed that all groups drank more water on Day 2 compared to Day 1 ($p<.001$) and more water on Days 3, 4 and 5 compared to Days 1 and 2 (both *p´*s <.001, respectively) and no differences in water consumption were observed from Day 3 to Day 5 (all *p´*s =1) indicating stabilized consumption. This also indicates that the auditory background used did not itself alter drinking and put us in a position to test the effect of changing the auditory background on taste neophobia.

Vinegar consumption: Phases I and II

We started with a global Mixed 5 X 2 X 2 X 2 (*Day* X *Injection* X *Context* X *Counterbalance Order*) Repeated Measures ANOVA that compared the intake of vinegar amongst the groups on the five days after the baseline period. There was a significant effect of the main factors *Day* [F(4,128)=147.74; $p<.001$] and the interactions *Day* X *Injection* [F(4,128)=8.77; $p<.001$], *Day* X *Context* [F(4,128)=7.97; $p<.001$] and *Day* X *Injection* X *Context* [F(4,128)=6.92; $p<.001$].

In order to understand the interactions, additional 4 x 2 (*Day* X *Context*) Repeated Measures ANOVAs were performed for the mice that

were injected with *Saline* and *SCH-23390* separately. The analyses performed for the *Saline* groups confirmed a significant effect of the main factors *Day* [F(4, 64)=122.51; $p<.001$] as well as the *Day* X *Context* interaction [F(4, 64)=23.05; $p<.001$]. Analysis of the interaction by Repeated Measures ANOVAs of the vinegar consumption was performed on the factor *Day* for each of the *Context* groups separately. The analyses confirmed a significant effect of *Day* in the *Saline-Same Context* [F(4, 32=86.40; $p<.001$] and *Saline-Different Context* [F(4, 32)=64.92; $p<.001$] groups. Further comparisons using Bonferroni-corrected tests identified significantly less vinegar consumed on Day 1 compared to Days 2, 3, 4 and 5 (all *p's*<.001) in the *Saline-Same Context* group, and this confirms that the neophobic response to the vinegar taste was completely attenuated on Day 2 and its consumption remained stable across the rest of days. In contrast, the same analysis performed in the *Saline-Different Context* group identified that the amount of vinegar consumed on Days 1 and 2 were indistinguishable ($p=1$) and less than consumption of vinegar on Days 3, 4 and 5 (*p's*<.001). Thus unlike the mice that did not experience a change of auditory background, the animals that experienced the change maintained the neophobic response for one more day; the attenuation of taste neophobia occurred on Day 3, when the novel auditory background became familiar (see **Figure 1A**).

We repeated the above analysis for the groups injected with either SCH-23390 or SFK-81297groups. The global repeated measures ANOVA 2 x 2 x 5 (*Injection* X *Context* X *Day*) revealed a significant effect of the factors *Injection* [$F(1, 28)=271.32$; $p<.001$], *Context* [$F(1, 28)=6.21$; $p=.018$], *Day* [$F(4, 112)=151.17$; $p<.001$] and also the interactions *Day* X *Injection* [$F(4, 112)=17.36$; $p<.001$], *Day* X *Context* [$F(4, 112)=5.70$; $p<.001$] and the third order interaction *Day* X *Injection* X *Context* [$F(4, 112)=3.90$; $p=.0052$]. In order to understand the third order interaction, separate repeated measures ANOVAs were performed for each of the *Injection* groups.

The analysis performed for the groups injected with SCH-23390 revealed a significant effect of *Day* [$F(4, 72)=59.16$; $p<.001$] and no other effect or interaction was found significant (all $p´s>.33$). Further post-hoc analyses using Bonferroni-corrected tests confirmed that the amount of vinegar consumed on Days 1 and 2 were indistinguishable ($p=1$) and less than vinegar consumed on Days 3, 4 and 5 ($p's<.001$). No other comparisons were significant, indicating that AN occurred on Day 3, regardless the auditory background changed or not.

The analysis performed for the groups injected with SFK-81297 revealed a significant effect of *Day* [$F(4, 40)=51.41$; $p<.001$] and no other effect or interaction was found significant (all $p´s>.45$). Further post-hoc analyses using Bonferroni-corrected tests confirmed that the amount of

vinegar consumed on vinegar Day 1 was less than the vinegar consumed on Days 2, 3, 4 and 5 (all *p´s*<.001). No other comparison was significant, indicating that AN occurred on Day 2, regardless that the auditory background changed or not.

A 5 X 2 (*Day* X *Drug Infused*) Repeated Measures ANOVA was used to observe differences in water consumption after the injection of SCH-23390 or SFK-81297. This analysis of water consumption not reveal any significant effect or interaction (all *p´*s >.4). This indicates that the injection received did not alter water consumption and this allows us to discard unspecific effects of SCH-23390 over the drinking behavior, motor activity or general motivation to drink, as these animals were subjected to the same water restrictions (**Figure 3B**).

Experiment 5.2.

A global repeated measures ANOVA (*Drug Infusion* X *Day*) comparing the amounts of vinegar intake during the five days revealed a significant effect of *Drug Injection* [F(2, 21)=29.96; *p*<.001], *Day* [F(4, 84)=97,05; *p*<.001] and the interaction *Drug Infusion* X *Day* [F(8, 84)=11.81; *p*<.001]. In order to understand this interaction, separate repeated measures ANOVAs were performed for each of the *Infusion Drug* groups.

The analysis performed for the Saline group revealed a significant effect of the factor *Day* [$F(4, 36)=44.36$; $p<.001$] and further post-hoc analyses using Bonferroni corrected tests revealed no differences in vinegar consumption between Days 1 and 2 ($p=1$) and less vinegar consumed on Days 1 and 2 compared to Days 3, 4 and 5 (all $p´s<.001$).

The analysis performed for the group injected with SFK-81297 revealed a significant effect of the factor *Day* [$F(4, 20)=124.21$; $p<.001$] and further post-hoc analyses using Bonferroni corrected tests revealed no differences in vinegar consumption between Days 1 and 2 ($p=1$) and less vinegar consumed on Days 1 and 2 compared to Days 3, 4 and 5 (all $p´s<.001$).

The analysis performed for the group injected with SCH-23390 revealed a significant effect of the factor *Day* [$F(4, 28)=11.16$; $p<.001$] and further post-hoc analyses using Bonferroni corrected tests revealed no differences in vinegar consumption between Days 1, 2 and 3 ($p=1$) and less vinegar consumed on Days 1, 2 and 3 compared to Days 4 and 5 (all $p´s<.001$), indicating that AN did not occurred until Day 4.

Experiment 5.3.

A global repeated measures ANOVA (*Drug Infusion* X *Day*) comparing the amounts of vinegar intake throughout the five days between the two injection groups revealed a significant effect of the factors *Drug*

Infusion [F(1, 11)=25.47; $p<.001$], *Day* [F(4, 44)=54.88; $p<.001$] and the interaction *Drug Infusion* X *Day* [F(4, 44)=27.52; $p<.001$]. In order to understand this interaction, repeated measures ANOVAs were performed for each of the *Drug Infusion* groups separately.

The analysis performed for the group injected with NaCl revealed a significant effect of *Day* [F(4, 20)=20.87; $p<.001$] and further post-hoc analyses using Bonferroni corrected tests revealed no differences in consumption of vinegar between Days 1 and 2 ($p=1$) and lesser amounts of vinegar consumed on Days 1 and 2 compared to Days 3, 4 and 5 (all $p´s<.001$).

The analysis performed for the group injected with SCH-23390 revealed a significant effect of *Day* [F(4, 24)=76.08; $p<.001$] and post-hoc analyses using Bonferroni corrected tests revealed no differences in consumption of vinegar between Days 1, 2, 3 and 4 (all $p´s=1$) and a higher consumption of vinegar on Day 5 compared to the rest of Days (all $p´s<.001$).

Experiment 5.4.

Baseline: Water consumption.

A global Mixed 4 x 2 x 2 x 2 (*Day* X *Injection* X *Context Change*) repeated measures ANOVA comparing the amount of water intake between all the groups during the four days of baseline (BL) revealed only a

significant effect of *Day* [F(3,84)=33.36; p<.001]. No other effect or interaction was significant (all *p's*>.5). Further analyses of the main effect *Day* using Bonferroni-corrected tests revealed that all groups consumed less amounts of water on BL Day 1 compared to BL Days 2, 3 and 4 (p<.001).

Vinegar consumption: Autitory context change and 6-OHDA lesions.

We confirmed these impressions starting with a global Mixed 4 X 2 X 2 (*Day* X *Injection* X *Context Change*) Repeated Measures ANOVA that compared the intake of vinegar amongst the groups on the four days after the baseline period. There was a significant effect of the main factors *Day* [F(3,72)=126.72; p<.001], the interactions *Day* X *Context Change* [F(3,72)=7.69; p<.001] and *Day* X *Injection* X *Context Change* [F(3,72)=3.589; p=.018].

To analyze the interactions, additional 4 X 2 (*Day* X *Context Change*) repeated measures ANOVAs were performed for the *Sham* and *6OHDA* groups separately. The analysis performed for the *Sham* groups confirmed a significant effect of the main factors *Day* [F(3,36)=51.87; p<.001] as well as the *Day* X *Context Change* interaction [F(3,36)=8.25; p<.001]. Analysis of the interaction by additional repeated measures ANOVAs of the vinegar consumption was performed on the factor *Day* for each of the *Context Change* groups separately. The analyses confirmed a

significant effect of *Day* in the *Sham-Vinegar-Same Context* group [F(3,18)=25.73; $p<.001$] as well as the *Sham-Vinegar-Different Context* [F(3,18)=35.18; $p<.001$], thus indicating AN. Further comparisons using Bonferroni-corrected tests identified significantly less vinegar was consumed on Day 1 compared to Days 2, 3 and 4 (all *p's*<.01) in the *Sham-Vinegar-Same Context* group, and this confirms that the neophobic response to the vinegar taste was completely attenuated on Day 2 and its consumption remained stable across the rest of days. In contrast, the same analysis performed in the *Sham-Vinegar-Different Context* group identified that the amount of vinegar consumed on Days 1 and 2 were indistinguishable ($p=1$) and less than on Days 3 and 4 (*p's*≤.007). Thus unlike the rats that did not experience a change of auditory background, the animals that experienced the change maintained the neophobic response for one more day; the attenuation of taste neophobia occurred on Day 3, when the novel auditory background became familiar.

We repeated the above analysis for the *6OHDA* groups. There was a significant effect of *Day* [F(3,36)=83.85; $p<.001$] and no other effect or interaction (all *p's*>.2). Post-hoc analysis of the effect of *Day* using Bonferroni-corrected t tests confirmed less vinegar intake on Day 1 compared Days 2, 3 and 4 (all *p's*<.005) but no other comparisons were significant. This indicates that unlike the sham rats, the animals injected with 6-OHDA attenuated the neophobic response to the vinegar taste on

Day 2, regardless of whether the background tone was or was not changed (see **Figure 4A**).

Inmunofluorescence analyses.

Multiple independen samples T-tests comparing the number of DAPI stained cells and TH+ fibers between the Sham and 6-OHDA groups for the three regions of interest (dCA1, vCA1 and VTA) revealed only significant differences between groups in the TH+ fibers of vCA1 (t=-12.06; p=.001) and dCA1 (t=-3.591; p<.05) and no differences were found between groups in TH+ fibers of VTA and the number of DAPI stained cells of the three regions (all p>.05).

Experiment 5.5.

A global global repeated measures ANOVA (*DREADD* X *Context Change* X *CNO Injection* X *Day*) comparing the amounts of vinegar intake between all groups of animals revealed a significant effect of *DREADD* [F(1, 31)=8.40; p=.006], *Context Change* [F(1, 31)=33.02; p<.001], *Day* [F(4, 124)=259.71; p<.001], the interactions *Context Change* X *CNO Injection* [F(1, 31)=7.96; p=.008], *DREADD* X *Day* [F(4, 124)=6.60; p<.001], *Context Change* X *Day* [F(4, 124)=29.52; p<.001], *CNO Injection* X *Day* [F(4, 124)=8.31; p<.001], the interactions *DREADD* X *Context Change* X *Day* [F(4, 124)=10.87; p<.001], *DREADD* X *CNO Day* X *Day*

[F(4, 124)=6.11; *p*<.001], *Context Change* X *CNO Injection* X *Day* [F(4, 124)=5.88; *p*<.001] and the interaction *DREADD* X *Context Change* X *CNO Injection* X *Day* [F(4, 124)=4.16; *p*=.003]. In order to understand the fourth-order interaction separate repeated measures ANOVAs were performed with the factors *DREADD, Context Change* and *Day* for the two injection days of CNO.

-Analyses of the groups injected with CNO before Day 2.

The analysis performed for the animals injected with CNO-dHCL before Day 2 showed a significant effect of *DREADD* [F(1, 15)=4.61; *p*=.048], *Context Change* [F(1, 15)=6.33; *p*=.023], *Day* [F(4, 60)=129; *p*<.001], and the interactions *DREADD* X *Day* [F(4, 60)=3.23; *p*=.018], *Context Change* X *Day* [F(4, 60)=7.41; *p*<.001] and the third order interaction *DREADD* X *Context Change* X *Day* [F(4, 60)=8.25; *p*<.001]. In order to understand this third order interaction, repeated measures ANOVAs were performed for both *Context Change* groups separately.

The analysis performed for the *Same Context* group revealed a significant effect of *DREADD* [F(1, 8)=5.39; *p*=.048], *Day* [F(4, 32)=52.97; *p*<.001] and the interaction *DREADD* X *Day* [F(4, 32)=11.40; *p*<.001]. We repeated the analyses for both *DREADD* groups. The analysis performed for the *hM4D(Gi)* group revealed a significant effect of the factor *Day* [F(4, 12)=33.73; *p*<.001] and further post-hoc analyses using

Bonferroni corrected test revealed significant less vinegar consumption of vinegar on Days 1 and 2 compared to Days 3, 4 and 5 (all *p´s*<.001). The analysis performed for the control *No-DREADD* group revealed a significant effect of the factor *Day* [F(4, 12)=33.72; *p*<.001] and further post-hoc analyses using Bonferroni corrected test revealed significant less vinegar consumption of vinegar on Day 1 compared to Days 2, 3, 4 and 5 (all *p´s*<.001). Additionally, there was a significant difference in consumption of vinegar on Day 2 between the DREADD groups on Day 2 (*t-value*=8.05; *p*<.001).

The analysis performed for the *Different Context* group revealed a significant effect of *Day* [F(4, 60)=82.22; *p*<.001] and no other effect was found significant. Further post-hoc analyses of this factor using Bonferroni-corrected tests revealed that there were no differences in consumption of vinegar between Days 1 and 2 (*p*=1) and there was increased consumption of vinegar on Days 3, 4 and 5 compared to Days 1 and 2 (*p*<.001).

-Analyses of the groups injected with CNO before Day 3.

The global analysis revealed a significant effect of the factors *Context Change* [F(1, 15)=28.40; *p*<.001], *Day* [F(4, 60)=138.40; *p*<.001] the interactions *DREADD* X *Day* [F(4, 60)=10.40; *p*<.001], *Context Change* X *Day* [F(4, 60)=31.00; *p*<.001] and the third order interaction *DREADD* X *Context Change* X *Day* [F(4, 60)=6.40; *p*<.001]. In order to

understand this third order interaction, repeated measures ANOVAs were performed for both *Context Change* groups separately.

The analysis performed for the *Same Context* group revealed a significant effect of *Day* [F(4, 32)=55.36; $p<.001$] and no other effect or interaction was found significant. Further post-hoc analyses of this factor using Bonferroni-corrected tests revealed significant less vinegar consumption of vinegar on Day 1 compared to Days 2, 3, 4 and 5 (all *p´s*<.001).

The analysis performed for the *Different Context* group revealed a significant effect of *DREADD* [F(1, 8)=12.50; $p=.008$], *Day* [F(4, 32)=131.90; $p<.001$] and the interaction *DREADD* X *Day* [F(4, 32)=19.40; $p<.001$].]. We repeated the analyses for both *DREADD* groups. The analysis performed for the *hM4D(Gi)* group revealed a significant effect of the factor *Day* [F(4, 12)=96.29; $p<.001$] and further post-hoc analyses using Bonferroni corrected test revealed no differences in vinegar consumption between vinegar Days 1,2 and 3 (all *p´s*=1) and significant less vinegar consumption of vinegar on Days 1, 2 and 3 compared to Days 4 and 5 (all *p´s*<.001). The analysis performed for the control *No-DREADD* group revealed a significant effect of the factor *Day* [F(4, 12)=63.57; $p<.001$] and further post-hoc analyses using Bonferroni corrected test revealed no differences in vinegar consumption between Days 1 and 2 ($p=1$) significant less vinegar consumption of vinegar on Days 1 and 2

compared to Days 3, 4 and 5 (all *p´s*<.001). Additionally, there was a significant difference in consumption of vinegar on Day 2 between the DREADD groups on Day 3 (*t-value*=9.20; *p*<.001).

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Capítulo 8

Discusión General

Esta tesis doctoral está basada en un planteamiento propio de la Psicobiología dirigido al estudio de los mecanismos cerebrales responsables de la memoria de reconocimiento empleando como modelo la atenuación de la neofobia (AN) producida por un sabor novedoso y su dependencia del contexto. La comprensión de la relación sistema nervioso-ambiente que posibilita el reconocimiento de sabores familiares seguros, es decir, que no produjeron malestar visceral en ocasiones anteriores, requiere aplicar distintos niveles de análisis, lo que conlleva el empleo de una variedad de técnicas desarrolladas en diversas disciplinas biomédicas. Junto al análisis de la conducta, se ha aplicado el nivel de análisis de circuito mediante bien la lesión selectiva de vías catecolaminérgicas por 6 hidroxidopamina (6-OHDA), bien la inactivación farmacogenética de vías mediante DREADDs. La lesión neurotóxica mediante NMDA y el registro de la actividad neural empleando inmunohistoquímica de la proteína c-Fos han permitido un nivel de análisis celular. Por su parte, la intervención farmacológica mediante agonistas y antagonistas de los receptores dopaminérgicos D1 ha posibilitado aplicar un nivel de análisis molecular. Además, se ha aplicado una aproximación de desarrollo mediante estudios transversales con sujetos de edades avanzadas.

La combinación de técnicas y estrategias (lesión/inactivación, estimulación y registro) ha permitido identificar la participación en AN de áreas cerebrales que, sin formar parte del sistema sensorial gustativo ni del

circuito responsable de la memoria de reconocimiento, parecen jugar un papel crítico en el componente emocional del proceso de familiarización con el sabor y su interacción con el contexto exteroceptivo. Por una parte, el sistema olfativo, cuya participación en el reconocimiento de sabores había sido previamente propuesta (Ekstrand et al., 2001; Maier et al., 2012; Small et al., 2013), dando especial relevancia a la contribución del componente olfativo en la experiencia de sabor. Por otra parte, el sistema dopaminérgico mesocortical o sistema de recompensa, que se encuentra relacionado con el procesamiento del valor hedónico de los sabores, así como el sistema hipocampal, que se encuentra fundamentalmente relacionado con el componente cognitivo y con fenómenos complejos asociados a la memoria del sabor, tal como la relación con el contexto de aprendizaje. Además, la exploración de las proyecciones dopaminérgicas, mediadas receptores D1 (D1DR), que permiten la interacción de dichas áreas entre sí, y con el sistema sensorial gustativo, ha aportado una concepción más amplia y compleja del circuito cerebral subyacente a la memoria de reconocimiento de sabores.

Mejorar la comprensión del funcionamiento de los circuitos involucrados en la adquisición de este tipo de memoria adquiere especial relevancia con vistas a desarrollar estrategias de intervención en aquellas situaciones en las que resulta deteriorada, tales como el envejecimiento normal o los trastornos neurodegenerativos asociados. Así, en el transcurso

de este trabajo se ha iniciado un abordaje del estudio del envejecimiento como modelo de alteraciones fisiológicas que, por un lado, permite explorar la reorganización de los circuitos de memoria a lo largo de la vida y, por otro, representa una situación ideal para poner a pruebas estrategias que mejoren la capacidades de memoria.

De esta forma, en el **Experimento 1 (Capítulo 3)** se ha evaluado la actividad de la corteza piriforme durante el proceso de atenuación de la neofobia al sabor, así como el efecto del envejecimiento no patológico sobre su actividad. La corteza piriforme forma parte de la corteza olfativa secundaria en el cerebro de los roedores y puede ser dividida en dos regiones siguiendo criterios tanto anatómicos como funcionales: la corteza piriforme anterior (aPirCx) y la corteza piriforme posterior (pPirCx). La aPirCx se caracteriza por sus conexiones directas y bidireccionales con otras áreas de la corteza olfativa primaria, tales como los bulbos olfatorios. Su actividad ha sido relacionada con la detección y discriminación de olores, es decir con actividad puramente sensorial. Por el contrario, la pPirCx se caracteriza por sus conexiones con otras estructuras relacionadas con un procesamiento de la información de mayor nivel, tales como la corteza prefrontal o determinados núcleos de la amígdala, así como con otras estructuras relacionadas con el procesamiento de sabores tal como la corteza gustativa primaria situada en la corteza insular. Estudios con registros electrofisiológicos han descrito neuronas que responden de forma

diferencial bien a la modalidad sensorial olfativa, bien a la gustativa o a ambas. Su actividad se ha relacionado con cambios plásticos al asociar un olor con un reforzador concreto (Ekstrand et al., 2001; Haberly & Price, 1978; Johnson et al., 2000; Verhagen & Engelen, 2006).

Por ello, en el**Experimento 1 (Capítulo 3)** de esta tesis se utilizó la identificación inmunohistoquímica de la proteína c-Fos como índice de actividad neural en la corteza piriforme tanto anterior como posterior de ratas macho tanto adultas como envejecidas. Los animales fueron expuestos a una solución de vinagre de sidra de manzana (3%) por primera vez (sabor novedoso), dos veces (sabor ligeramente familiar) y seis veces (sabor muy familiar). Los resultados mostraron un incremento en el número de células c-Fos positivas en la región rostral de pPirCx en el caso de ratas adultas expuestas al sabor muy familiar. No se encontraron diferencias entre los grupos de familiaridad en aPirCx. Esto indica la existencia de cambios plásticos relacionados con la familiaridad de un sabor que se pueden detectar en la región de la corteza piriforme implicada en procesos de aprendizaje, más que en la región relacionada con la percepción del sentido del olfato. Es importante destacar que dichos cambios en la actividad cerebral no parecen ser atribuibles aalteraciones sensoriales olfativas de forma general, ya que, en ese caso se registrarían cambios en la actividad de todo el PirCx y no exclusivamente en la región rostral asociada en estudios previos selectivamente con la modalidad sensorial

gustativa (Maier et al. 2012, Small et al. 2010). En el caso de las ratas envejecidas, además de la actividad cerebral alterada de la corteza piriforme, se produjo un retraso en la atenuación de neofobia, que requirió dos sesiones de exposición al sabor en vez de la única sesión necesaria en ratas adultas. Se observó un incremento de la actividad global en pPirCx, pero no en aPirCx, sin cambios relacionados con la familiaridad del sabor. Estos datos podrían explicarse por la hiperexcitabilidad de neuronas glutamatérgicas, propuesta como un marcador temprano de declive cognitivo. Ello concordaría con la explicación de la AN retrasada a edades avanzadas como consecuencia de un déficit de *reconocimiento* del sabor, más que un déficit sensorial (Dardou et al., 2008, 2010).

En el**Experimento 2 (Capítulo 4)** se aplicó la misma estrategia que en la serie anterior empleando la identificación inmunohistoquímica de la proteína c-Fos para explorar la participación del sistema de recompensa dopaminérgico mesocorticolímibico en la atenuación de neofobia de sabores, así como el papel del envejecimiento no patológico en la actividad de estos circuitos. El sistema dopaminérgico mesocorticolímibico ha sido propuesto como un sistema clave en el procesamiento del valor hedónico del sabor. El valor hedónico resulta clave a la hora de seleccionar la dieta y no es una propiedad estable en el tiempo, sino que se ve modificada por la experiencia (Bassareo et al., 2002; S. Fenu et al., 2001). El núcleo accumbens, situado en el estriado ventral, es un componente fundamental

del sistema de recompensa. En relación con el valor hedónico del sabor, se ha propuesto su papel crítico en los cambios de palatabilidad gracias a sus conexiones con áreas del sistema gustativo, tales como la amígdala o la corteza insular gustativa (Takeuchi et al., 2016; Yamamoto, 2006). La actividad del núcleo accumbens parece ser fundamental en el desarrollo de preferencias condicionadas y el aprendizaje aversivo gustativo.

Los resultados de el**Experimento 2** de esta tesis mostraron un incremento en el número de células c-Fos positivas en la región del núcleo accumbens shell (NAcb-Shell) el segundo día de exposición a la solución de vinagre. Cabe destacar que el incremento en actividad de c-Fos coincidió con la atenuación de neofobia al sabor que en ratas adultas tiene lugar el día 2 de exposición. En el caso de las ratas envejecidas, se observó un patrón alterado en la actividad de c-Fos, produciéndose un incremento del número de células c-Fos positivas en la sexta exposición al sabor. Esto concuerda con el retraso de la atenuación de neofobia descrito y refuerza la teoría del deterioro de memoria de reconocimiento gustativa en roedores envejecidos. Es importante destacar que las diferencias en la actividad de c-Fos entre animales adultos y envejecidos no pueden explicarse en términos de una pérdida global o inespecífica de neuronas en la zona del estriado ventral como consecuencia del envejecimiento, sino que parecen obedecer a una reorganización funcional del sistema.

En la **tercera serie experimental (Capítulo 5)** se profundizó en el análisis de la actividad del núcleo accumbens durante la atenuación de neofobia así como su interacción con otros núcleos implicados en la adquisición de la memoria gustativa, tal como la amígdala basolateral (BLA). Así, en el Experimento 3.1. se evaluó la actividad c-Fos de cuatro regiones cerebrales (núcleo accumbens core, núcleo accumbens shell, amígdala basolateral y amígdala central) y se correlacionó con la conducta de cada animal. Se halló una correlación negativa entre la actividad de c-Fos en el NAcb-Shell y BLA, de manera que en la segunda exposición al sabor, se produjo un incremento de actividad en NAcb-Shell que coincide con un decremento de la actividad de BLA. Los resultados indicaron, además, que mientras la actividad de BLA correlaciona positivamente con la respuesta neofóbica, la actividad de NAcb-Shell correlaciona con la atenuación de la neofobia, existiendo correlación negativa entre la actividad de ambas estructuras. Ello sugiere una relación de tipo inhibitorio entre estos dos núcleos que está directamente relacionada con la formación de una memoria gustativa segura el día 2 de exposición cuando el sabor comienza a ser familiar.

Dada la relevancia de las proyecciones dopaminérgicas en la interacción entre el núcleo accumbens y el resto de áreas cerebrales, en el Experimento 3.2. se exploró el papel que desempeñan los receptores dopaminérgicos D1 (D1DR) de NAcb-Shell y BLA en la atenuación de

neofobia gustativa. Para ello, ratas macho adultas fueron sometidas a una operación quirúrgica en la que se implantaron cánulas guía bien en NAcb-Shell o en BLA con el objetivo de aplicar inyecciones intracerebrales *in vivo* del antagonista D1DR SCH-23390, el agonista D1DR SFK-81297, o bien tampón fosfato (PBS) como control, en momentos temporales específicos. La inyección intracerebral se aplicó, bien antes de la primera exposición al sabor, antes de la segunda exposición al sabor o bien después de la segunda exposición al sabor. Los resultados mostraron que la administración del antagonista D1DR tanto en NAcb-Shell como en BLA antes de la segunda exposición al sabor impidió la atenuación de neofobia en esa sesión, pero sus consecuencias a largo plazo fueron opuestas. Mientras que la administración del antagonista D1DR en NAcb-Shell facilitó AN el día 3, su administración en BLA impidió AN el día 3. Ello sugiere una disociación funcional entre ambas estructuras durante la formación de la memoria gustativa responsable de AN. Por su parte, la administración del agonista D1DR también produjo efectos opuestos dependiendo de la región en la que se administró. En NAcb-Shell facilitó AN mientras que en BLA la impidió resultando en un descenso del consumo de la solución de vinagre al día siguiente. Esto concuerda con la implicación de la amígdala en la respuesta neofóbica así como con su papel en la formación de memorias aversivas de sabores.

En el Experimento 3.3. se exploró la direccionalidad de la relación inhibitoria entre NAcb-Shell y BLA mediante la aplicación de técnicas de farmacogenética. Para ello, a ratas Wistar macho adultas no transgénicas se les administró una combinación de vectores de tipo vírico: un caninovirus de tipo 2 CAV-cre, que se absorbe de forma retrógrada e induce la expresión de la recombinasa cre en el núcleo celular, y un adenovirus asociado (AAV) que posee el material genético de un receptor de membrana del receptor muscarínico humano (Roelofs et al., 2017). Dependiendo del tipo de actividad asociada al receptor de membrana expresado, se utilizaron dos vectores: el vector que expresa el receptor hM4D, asociado a proteína Gi (de tipo inhibitorio) y el vector que expresa el receptor hM3D, asociado a proteína Gq (de tipo excitatorio). Estos receptores de membrana pueden ser activados de forma artificial utilizando un ligando exógeno: la clozapina N-Oxidasa (CNO) mediante administración i.p. (intraperitoneal) 1,5 horas antes de la sesión experimental correspondiente. Ello permitió inducir de manera reversible durante el día 2 de exposición al sabor selectivamente bien la actividad de las neuronas BLA que proyectan al núcleo accumbens o bien la inhibición de las neuronas del NAcb-Shell que proyectan a BLA.

Los resultados mostraron que la inhibición farmacogenética de la vía NAcb-Shell–BLA en el día 2 de exposición al sabor resultó en un retraso de AN, que no ocurrió hasta el día siguiente, sugiriendo que la actividad del

núcleo accumbens se encuentra directamente relacionada con el incremento en consumo observado en la atenuación de neofobia. Por otro lado, la activación farmacogenética de la vía BLA-NAcb-Shell resultó en el desarrollo de una respuesta de aversión al sabor, de manera que no hubo atenuación de neofobia el día de la inhibición ni en los días siguientes, manteniéndose el consumo reducido. Es importante destacar que la manipulación de la actividad de la vía en ambas direcciones no tuvo ningún efecto general o inespecífico sobre la conducta consumatoria, dado que no se encontraron diferencias en los grupos controles expuestos a agua.

Para completar la caracterización neuroquímica de las proyecciones NAcb-Shell–BLA, en el Experimento 3.4. se combinó la inhibición de dicha vía neural con la administración de agonistas y antagonistas D1DRs en la amígdala. Como resultado, el retraso de AN observado tras la inhibición de las neuronas del NAcb-Shell que proyectan sobre BLA el día 2 de exposición fue revertido con la administración del agonista D1DR SFK-81297 en la amígdala. Ello sugiere que en condiciones fisiológicas normales la actividad excitatoria del accumbens inhibela de la amígdala que es necesaria para que se produzca el incremento en consumo del sabor propio de AN. El hecho de que la ausencia de AN se mantenga cuando, además de inhibir la vía el segundo día, se administra el agonista SFK-81297 en la amígdala al día siguiente, confirma la relevancia de la inhibición amigdalina.

Además, en este experimento también se combinó la inhibición del núcleo accumbens con la administración del agonista D1DR en la amígdala *después* de la segunda exposición al sabor. Los resultados mostraron que, a diferencia de la administración *previa* a la sesión experimental que revirtió el efecto de la inhibición de NAcb-Shell, los animales que recibieron la administración post-sesión disminuyeron el consumo del sabor al día siguiente, indicando ausencia de AN. Estos datos señalan la existencia de un patrón temporal de actividad en la amígdala, de manera que si los receptores D1DR son activados *durante* la exposición al sabor, estos receptores producirían una inhibición de BLA que promovería la formación de una memoria de sabor segura. Sin embargo, si los receptores D1DR de la amígdala son activados *después* de la exposición al sabor, se promueve una formación de memoria de sabor aversiva. Esto es congruente con el hecho de que BLA recibe proyecciones viscerales directas a través del nervio vago que envían información sobre las consecuencias viscerales del sabor ingerido. Esta información requiere más tiempo para alcanzar la BLA y, por tanto, la activaría minutos – incluso horas – después de la exposición al sabor y serían indicadores de malestar gástrico, que serían asociados directamente al sabor consumido. Nuestros resultados concuerdan con el reciente descubrimiento de dos poblaciones neuronales diferenciadas en distintos núcleos de la amígdala, incluyendo BLA. Una de ellas está

relacionada con la formación de memorias de tipo apetitivo y la otra con la formación de memorias de tipo aversivo (Kim et al., 2016, 2017).

En el**Experimento 4(Capítulo 6)** se estudió el papel del contexto auditivo en el que se realiza la atenuación de neofobia y el efecto de lesiones hipocampales. Estudios previos han demostrado que la memoria de los sabores está asociada tanto al contexto espacial (Moreno-Castilla et al. 2017;De la Casa, 2013; Morón et al., 2002) como temporal (Manrique et al., 2004, 2009a) y que dicha relación con el contexto requiere la integridad del hipocampo (Molero et al., 2005). Con respecto a la AN, la dependencia del contexto espacial queda demostrada por el hecho de que un cambio del lugar en el que se presenta un sabor familiar interrumpe la AN (De la Casa, 2011). Sin embargo, no existen datos sobre la capacidad de otras modalidades sensoriales para actuar como contexto.

En primer lugar, se evaluó si la AN se ve modulada por cambios en el contexto auditivo en el que se presenta el sabor. Para ello, se estudio el fenómeno denominado dependencia contextual de la AN mediante la manipulación del sonido ambiental durante la presentación del sabor de tal manera que o bien permanecía constante durante todas las fases del experimento (grupo "mismo contexto"), o bien cambiaba en alguna fase del experimento (grupo "diferente contexto"). Los resultados confirmaron la dependencia contextual de la AN empleando la modalidad auditiva. Aquellos gruposque experimentaron un cambio de contexto auditivo en la

segunda exposición al sabor no mostraron el incremento en consumo característico de la AN. Concretamente, ese incremento se produjo al día siguiente, cuando el segundo contexto se convirtió también en familiar. Cabe destacar que el cambio de contexto por sí mismo no tuvo efectos sobre el consumo de agua. Así, el fenómeno solo se observó cuando se combinó un sabor con el cambio contextual, sugiriendo que la memoria segura del sabor incluye información del contexto auditivo.

En segundo lugar, se evaluó el efecto de lesiones neurotóxicas del hipocampo sobre la dependencia contextual de la AN empleando la modalidad auditiva. La evidencia actual indica que la función del hipocampo es esencial en fenómenos complejos de aprendizaje de sabores tales como el fenómeno de bloqueo (Gallo & Cándido, 1995; Moron et al., 2002), o los que dependen del contexto en el que se produce el aprendizaje (Gallo & Cándido, 1995; Gallo et al., 1999). Las lesiones electrolíticas del hipocampo dorsal impiden tanto las aversiones condicionadas al contexto como el efecto del bloqueo del contexto en aprendizaje aversivo gustativo (Aguado et al., 1998). La dependencia del contexto de la extinción de la aversión condicionada también es deteriorada por lesiones electrolíticas del hipocampo dorsal (Fujiwara et al., 2012), así como la propia extinción que se considera dependiente de contexto (Garcia-Delatorre et al., 2010). La dependencia contextual de la inhibición latente de la aversión gustativa condicionada también es hipocampo dependientes (Manrique, Gámiz,

et al., 2009; Molero et al., 2005). Sin embargo, no existen datos previos sobre la posible participación del hipocampo en la modulación contextual de la AN. La única evidencia reportada hasta el momento demuestra que la modulación por parte del contexto espacial de la AN parece depender de la actividad del sistema dopaminérgico, pues dicha modulación desaparece por la administración sistémica del antagonista D1DR SCH-23390 (De la Casa & Díaz, 2013; Quintero et al., 2011). El hecho de que el hipocampo posea múltiples conexiones bidireccionales con áreas cerebrales involucradas en la AN, tales como la amígdala, el estriado ventral (entre ellos el núcleo accumbens), la corteza insular o la corteza perirrinal (Balderas et al., 2012; Garcia-Delatorre et al., 2010; Miranda et al., 2003) permite hipotetizar que su integridad sea necesaria para la dependencia contextual de la AN a pesar de no emplear información espacial.

Los resultados mostraron que el grupo de ratones sometido a una lesión excitotóxica de la región CA1 del hipocampo dorsal mediante ácido de N-Methyl D´Aspartato (NMDA) mostró AN a la solución de vinagre en la segunda exposición, independientemente de si el contexto auditivo había cambiado o no. Por tanto, la modulación de la AN por parte del contexto auditivo exhibida por el grupo control sham (falso operado) no aparece en el caso de los animales con daño hipocampal. Estos resultados indican que mientras la atenuación de neofobia no es dependiente de hipocampo, su

dependencia del contexto auditivo requiere la integridad de la región CA1 hipocampal.

Finalmente en la **serie experimental 5 (Capítulo 7)** se profundizó en el estudio de los mecanismos neurales responsables de la modulación contextual de la AN empleando el mismo procedimiento conductual del Experimento 4 tanto en ratones como en ratas, siendo los animales adultos en todos los casos. Esta serie se centró en la exploración de potenciales vías neurales subyacentes a la interacción entre el sistema hipocampal y el núcleo accumbens cuya participación en la AN quedó demostrada en el Experimento 2 y la Serie Experimental 3.

La investigación se dirigió en primer lugar al papel de la neurotransmisión dopaminérgica a través de los receptores D1DR en dicha modulación contextual (experimentos 5.1., 5.2. y 5.3.). La dopamina es un neurotransmisor asociado con procesos de aprendizaje y memoria. Se ha propuesto que la liberación de dopamina ante la presencia de estímulos novedosos y/o de gran saliencia supone la activación de los sistemas de modulación de memoria, que actuarían sobre otras áreas cerebrales, entre ellas el hipocampo, para reforzar los procesos de aprendizaje y memoria (Takeuchi et al., 2016; Yamasaki & Takeuchi, 2017a). Así, se ha demostrado que la liberación de dopamina participa en la modulación de la potenciación a largo plazo (LTP), uno de los mecanismos moleculares mejor conocidos que sustentan la plasticidad neural en la región CA1 del

hipocampo. En efecto, la liberación de dopamina promueve la transición de las fases de LTP tempranas (E-LTP) a las fases de LTP tardías (L-LTP) relacionadas con la memoria a largo plazo (Frey, Schroeder, & Matthies, 1990).

Por este motivo, en el experimento 5.1. se evaluó el efecto de la administración sistémica del antagonista D1DR SCH-23390 y del agonista D1DR SFK-81297 sobre la modulación contextual de la AN. Los resultados mostraron que SCH-23390 sustituyó al cambio contextual interrumpiendo la formación de la memoria segura del sabor el día en que se aplicó. Por su parte SFK-81297 revertió el efecto del cambio contextual sobre la AN. Estos datos sugieren que los mecanismos moleculares sobre los que actúa el cambio de contexto están relacionados con la actividad de los sistemas dopaminérgicos. Para estudiar la disociación entre los efectos del cambio de contexto y la intervención farmacológica se llevó a cabo el Experimento 5.2., en el que los animales fueron sometidos a un cambio contextual en la segunda exposición al sabor y recibieron los agentes dopaminérgicos en la tercera exposición al sabor. De nuevo, los resultados demostraron que tanto el cambio contextual como la administración del antagonista D1DR poseen ambos efectos similares sobre la atenuación de neofobia. Ello sugiere que el cambio de contexto actúa interfiriendo la neurotransmisión dopaminérgica mediada por D1DR para interrumpir la AN. Con el fin de descartar la posibilidad alternativa de que la interrupción

de AN por parte del antagonista dopaminérgico se deba a un efecto dependiente de estado (*state-dependent*) causado por un cambio en el estado fisiológico interno que represente un contexto novedoso, se realizó el Experimento 5.3. Los ratones recibieron inyecciones repetidas del antagonista D1DR durante la segunda, tercera y cuarta exposición al sabor. De esta manera, el contexto fisiológico interno convertido en familiar no debería afectar a la atenuación de neofobia si se tratara de un efecto dependiente de estado. Sin embargo, no se produjo AN y la respuesta neofóbica se mantuvo mientras perduró la intervención farmacológica sin que se incrementara el consumo del sabor. Ello permitió descartar la explicación alternativa en términos de dependencia del estado interno y apuntó a que la actividad dopaminérgica mediada por D1DR, necesaria para que se produzca AN, es interrumpida por el cambio de contexto auditivo.

En segundo lugar, dado que tanto la integridad de la región CA1 del hipocampo (Experimento 4) como la actividad dopaminérgica mediada por D1DR (Experimentos 5.1., 5.2., y 5.3.) son necesarias para que se produzca la modulación contextual de la AN, en el Experimento 5.4. se evaluó la necesidad de la neurotransmisión dopaminérgica hipocampal para dicho fenómeno. Para ello, se realizó una lesión neuroquímica selectiva de las terminales catecolaminérgicas en la región CA1 del hipocampo ventral (vCA1) mediante la administración intracerebral de 6-hidroxidopamina (6-

OHDA) en ratas macho adultas. Los análisis histológicos mediante técnicas de inmunofluorescencia confirmaron que la inyección intrahipocampal de 6-OHDA redujo selectivamente el número de fibras de tirosina hidroxilasa (precursor de la dopamina) sin que existieran diferencias en el número total de células teñidas con DAPI (marcador no selectivo de núcleos celulares) en la región.

Los resultados mostraron que el grupo control sham (falso operado) mostró la modulación contextual de la AN, consistente en la interrupción de la AN por un cambio del contexto auditivo en la segunda exposición al sabor. Sin embargo, la depleción catecolaminérgica hipocampal interfirió con este fenómeno, de manera que los animales incrementaron el consumo de vinagre en la segunda exposición al sabor, exhibiendo AN independientemente de si el contexto auditivo permaneció constante o cambió. Estos resultados amplían los resultados del Experimento 4, sugiriendo que la inervación dopaminérgica hipocampal es necesaria para la modulación contextual de la AN.

En tercer lugar, en el Experimento 5.5. se empleó de nuevo la farmacogenética de forma similar a la empleada en el Capítulo 5 usando DREADDs para estudiar la conectividad entre vCA1 y NAcb-Shell dada la relevancia de su actividad dopaminérgica en AN. Los mismos vectores virales fueron administrados intracerebralmente en ratas macho adultas, de manera que el caninovirus retrógrado de tipo 2 CAV-cre fue inyectado en

la región ventral de CA1 y el adenovirus asociado que expresaba el receptor inhibitorio hM4D(Gi) fue administrado en la región NAcb-Shell. De esta manera se inhibió de manera reversible selectivamente aquellas neuronas del NAcb-Shell que proyectan a vCA1 mediante la inyección i.p. de CNO en momentos temporales puntuales. Así, se combinó el cambio de contexto auditivo en la segunda exposición al sabor con la administración de CNO bien antes de la segunda o de la tercera exposición al sabor.

Los resultados mostraron que la inhibición de la vía NAcb-Shell-vCA1 el segundo día de exposición causó un efecto conductual similar al cambio de contexto auditivo interfiriendo con la AN que no se hizo evidente hasta el día 3 de exposición al sabor. Por su parte, la inhibición de la actividad de la vía NAcb-Shell-vCA1 durante la tercera exposición al sabor sólo interrumpió la AN en aquellos animales que habían sufrido previamente un cambio contextual en el día 2. Sin embargo, en aquellos animales que, manteniendo constante el contexto auditivo, mostraron AN el día 2 la inhibición de la vía NAcb-Shell-vCA1 en el día 3 no tuvo ningún efecto sobre la AN que se había producido el día anterior.

En conjunto los datos de la serie experimental 5 apoyan la teoría de que el contexto auditivo modula la formación de la memoria gustativa segura a través de vías neurales dopaminérgicas mediadas por D1DRs siendo la interacción entre el NAcb-Shell y el vCA1 crítica en la fase temprana del proceso. Estos resultados concuerdan con la teoría propuesta

por Lismann y Grace en 2005 por la que los estímulos novedosos y/o salientes producen la activación del denominado sistema hipocampo-ATV (área tegmental ventral), que resulta en la retroalimentación positiva hacia el hipocampo de fibras dopaminérgicas que refuerzan la consolidación de nuevos aprendizajes. Según esta propuesta el cambio contextual interferiría con la actividad de este sistema, impidiendo la adquisición de la memoria del sabor familiar y, por tanto, la AN.

En conclusión, la exposición repetida a un sabor novedoso resulta en un incremento del consumo de este sabor cuando no va asociado con consecuencias aversivas. Para que se produzca este incremento en el consumo, conocido como atenuación de la neofobia, se requiere la actividad de un complejo entramado de áreas cerebrales que pertenecen a distintos circuitos implicados en el procesamiento gustativo, olfativo, mnésico y emocional. Esta tesis doctoral contribuye a identificar la relevancia de vías dopaminérgicas mediadas por receptores D1 para la interacción del núcleo accumbens tanto con la amígdala basolateral como con la región CA1 del hipocampo ventral para el componente emocional de la atenuación de la neofobia y su dependencia contextual, respectivamente.

Capítulo 9

Conclusiones

1. La región rostral de la corteza piriforme posterior, que conforma la corteza olfativa secundaria en el cerebro de los roedores, muestra activación evaluada mediante la identificación de la proteína c-Fos que está asociada con el proceso de familiarización de un sabor en ratas adultas.

2. El núcleo accumbens shell, componente fundamental del sistema dopaminérgico mesocorticolímbico, incrementa su actividad, evaluada mediante la identificación de la proteína c-Fos, en la fase temprana de la atenuación de la respuesta neofóbica durante el segundo día de exposición al sabor en ratas adultas.

3. El envejecimiento no patológico modifica los patrones de actividad de c-Fos en el circuito cerebral que responde a la atenuación de la neofobia. Mientras que la región rostral de la corteza piriforme posterior se encuentra sobreactivada sin mostrar respuestas selectivas a la familiaridad del sabor, la activación de la región shell del núcleo accumbens asociada a la atenuación de la neofobia se retrasa a fases posteriores. Ello es congruente con el retraso en la atenuación de neofobia observado a edades avanzadas y con problemas de

reconocimiento, más que con diferencias entre ratas adultas y viejas en el procesamiento sensorial del sabor.

4. La actividad del núcleo accumbens shell muestra correlación positiva con la atenuación de neofobia durante la segunda exposición al sabor y correlación negativa con la actividad de la amígdala basolateral, área cuya actividad muestra correlación positiva con la respuesta neofóbica.

5. Durante la atenuación de la neofobia se produce la activación del núcleo accumbens shell y la inhibición de la amígdala basolateral. La actividad dopaminérgica mediada por receptores dopaminérgicos D1 juega un papel crítico en la interacción accumbens-amígdala, necesaria para que se produzca la atenuación de neofobia, de manera que si no se produce, se mantiene la respuesta neofóbica al sabor.

6. La activación farmacogenética reversible selectiva mediante DREADDs de las proyecciones entre el núcleo accumbens shell y la amígdala basolateral produce consecuencias contrapuestas dependiendo del momento temporal en que se produzca. Si se produce *durante* la segunda exposición del

sabor, promueve la inhibición de la amígdala y resulta en el incremento en el consumo del sabor, es decir, en atenuación de neofobia. Si, por el contrario, la activación de la vía ocurre *después* de la sesión de bebida, ello resulta en la adquisición de una aversión hacia ese sabor que se manifiesta en ulteriores encuentros.

7. La atenuación de la neofobia al sabor es modulada por el contexto auditivo en el que se produce, ya que queda interrumpida por un cambio de contexto. Esta dependencia contextual de la atenuación de neofobia requiere la integridad de la región CA1 hipocampal, de manera que las lesiones excitotóxicas mediante NMDA de esta zona eliminan el efecto.

8. Los mecanismos hipocampales responsables de la dependencia contextual de la atenuación de la neofobia requieren la integridad de la neurotransmisión dopaminérgica, ya que la depleción catecolaminérgica mediante inyección intracerebral de 6-hidroxidopamina (6-OHDA) interfiere con el fenómeno. Asimismo, el bloqueo farmacológico mediante agonistas de los receptores dopaminérgicos D1 reproduce el efecto del

cambio contextual interfiriendo con la atenuación de la neofobia. Dicho efecto no puede atribuirse a cambios de contexto interno (state-dependent effect) puesto que la repetición de la intervención farmacológica no lo elimina.

9. La inactivación farmacogenética reversible selectiva mediante DREADDs de las proyecciones entre el núcleo accumbens shell y la región CA1 hipocampal impide la formación de la memoria gustativa segura durante la fase temprana pero no la interrumpe en fases posteriores una vez que se ha producido la atenuación de neofobia.

10. Junto a las áreas cerebrales previamente descritas que forman parte del sistema gustativo y de los circuitos de memoria de reconocimiento compartidos con otras modalidades sensoriales, en esta tesis se identifican circuitos y mecanismos adicionales críticos para el componente hedónico y emocional propio de la memoria gustativa y su relación con fenómenos complejos asociados. Su conocimiento adquiere especial relevancia para comprender los cambios que la edad induce en los sistemas de memoria y recompensa asociados a la conducta

de ingestión y selección de la dieta, lo que permite diseñar estrategias de intervención.

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ÍNDICE DE ABREVIATURAS

µl	Microlitro
µm	Micrómetro
6-OHDA	6-hidroxidopamina
AAV	Vector de tipo Adenoviros asociado
AMY	Amígdala
AN	Atenuación de neofobia
AP	Anteroposterior
aPirCx	Corteza piriforme anterior
ATN / AN	Attenuation of Taste Neophobia
b.w.	body weight
BL	Baseline
BLA	Amígdala basolateral
CA1	Región de CA1 del hipocampo.
CAV-cre	Caninovirus tipo 2
CeA	Amígdala central
c-Fos EC	c-Fos Estimated Change
CNO-DHL	Clozapine N-Oxide dihydrochloride
CTA	Condicionamiento aversivo gustativo
Ctxt.	Contexto
D1DR	Receptores de dopamina de tipo 1
DA	Dopamina
DREADD	Designed Receptors Especifically Activated by Designed Drugs
DV	Dorsoventral
EC	Estímulo condicionado
EI	Estímulo incondicionado
Eq.	Equation (Ecuación)
Exp.	Experimento
F-I	Familiar 1
F-II	Familiar-2
H	Hour
HC	Hipocampo

Hz	Hertzio
i.c.	Intracerebral.
i.p.	Intraperitoneal.
ISI	Inter Stimulus Interval
LH	Hipotálamo lateral
LiCl	Cloruro de litio
M	Molar
mg/kg	miligramos/kilogramo
min	Minutes
mL	Mililitros
ML	Medial lateral
mm	Milímetros
NAcb	Núcleo Accumbens
NAcb-Core	Nucleo accumbens región Core
NAcb-Sh	Nucleo accumbens región shell
Neo	Neofobia
NMDA	N-metil-D-aspartato
PBS	Phosphate Buffered Saline
PirCx	Corteza piriforme
pp	Partículas
pPirCx	Corteza piriforme posterior
PT	Pure Tone
S	Segundo
Vin	Vinagre de sidra (3%)
vCA1	Región ventral de CA1 del hipocampo
Vg/mL	Copias genómicas de vector por mililitro
VTA	Área tegmental ventral
WN	White noise

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

ANEXO I

Table 1

Mean (± SEM) consumption (ml.)

	Group	Water	Vin 1 (Novel)	Vin 2 (F-I)	Vin 3	Vin 4	Vin 5	Vin 6(F-II)
Adult	Novel	10,42 (±1,46)	6,32 (±0,53)					
	F-I	10,78 (±1,43)	5,59 (±0,95)#	9,64 (±0,87)*				
	F-II	11,85 (±0,94)	5,72 (±0,77)#+	9,20 (±0,80)*	10,89 (±1,43)*	12,16 (±1,22)*	10,99 (±1,46)*	11,73 (±0,78)*
Aged	Novel	13,42 (±0,95)	5,65 (±0,8)#					
	F-I	14,75 (±0,67)	6,18 (±1,79)#	7,01 (±0,8)#				
	F-II	13,00 (±1,21)	5,44 (±0,96)#+	7,43 (±1,21)#+	8,65 (±0,64)#*	9,26 (±0,53)#*	10,57 (±0,8)*	11,51 (±0,79)*

Water (last day of Baseline); Vin= 3% cider vinegar solution; #=*p*<.05 compared to Water of the same age group; +=*p*<.05 compared to Vin6 of the same age group; *=*p*<.05 compared to Vin1 of the same group; # =p<.05 compared to Water of the same group.

Table 1. Correlations between c-Fos+cells and behavioral parameters.

			Pearson r	R^2	*P*
Novel group					
Directconsumption (mL)	vs.	NAcb-Sh c-Fos cells	0,185	0,034225	0,692
Directconsumption (mL)	vs.	NAcb-Core c-Fos cells	-0,203	0,041209	0,663
Directconsumption (mL)	vs.	BLA c-Fos cells	**0,83**	0,6889	0,021*
Directconsumption (mL)	vs.	CeA c-Fos cells	0,293	0,085849	0,524
F-1 group					
Directconsumption (mL)	vs.	NAcb-Sh c-Fos cells	-0,515	0,265225	0,236
Directconsumption (mL)	vs.	NAcb-Core c-Fos cells	-0,269	0,072361	0,56
Directconsumption	vs.	BLA c-Fos cells	-0,568	0,322624	0,183

(mL)					
Directconsumption (mL)	vs.	CeA c-Fos cells	0,416	0,173056	0,35
Neo Index	vs.	NAcb-Sh c-Fos cells	0,061	0,003721	0,897
Neo Index	vs.	NAcb-Core c-Fos cells	-0,402	0,161604	0,371
Neo Index	vs.	BLAc-Fos cells	**0,789**	0,622521	0,035*
Neo Index	vs.	CeAc-Fos cells	0,137	0,018769	0,77
AN Index	vs.	NAcb-Sh c-Fos cells	**0,805**	0,648025	0,029*
AN Index	vs.	NAcb-Core c-Fos cells	0,452	0,204304	0,308
AN Index	vs.	BLAc-Fos cells	**-0,775**	0,600625	0,041*
AN Index	vs.	CeAc-Fos cells	0,21	0,0441	0,651
Novel group (between-nuclei correlations)					
NAcb-Sh c-Fos cells	vs.	NAcb-Core c-Fos cells	0,368	0,135424	0,417
NAcb-Sh c-Fos cells	vs.	BLAc-Fos cells	-0,003	0,000009	0,995
NAcb-Sh c-Fos cells	vs.	CeAc-Fos cells	0,123	0,015129	0,792
NAcb-Core c-Fos cells	vs.	BLAc-Fos cells	-0,669	0,447561	0,1
NAcb-Core c-Fos cells	vs.	CeAc-Fos cells	0,699	0,488601	0,081
BLAc-Fos cells	vs.	CeAc-Fos cells	-0,197	0,038809	0,672
		.			
F-1 group (between-nuclei correlations)					
NAcb-Sh c-Fos cells	vs.	NAcb-Core c-Fos cells	0,243	0,059049	0,599
NAcb-Sh c-Fos cells	vs.	BLAc-Fos cells	**-0,814**	0,662596	0,026*
NAcb-Sh c-Fos cells	vs.	CeAc-Fos cells	0,082	0,006724	0,862
NAcb-Core c-Fos cells	vs.	BLAc-Fos cells	-0,193	0,037249	0,679
NAcb-Core c-Fos cells	vs.	CeAc-Fos cells	-0,303	0,091809	0,509
BLAc-Fos cells	vs.	CeAc-Fos cells	0,271	0,073441	0,556
c-Fos Estimated Change (EC)					
NAcb-Sh EC	vs.	NAcb-Core EC	0,271	0,073441	0,556
NAcb-ShvEC	vs.	BLA EC	**-0,862**	0,743044	0,027*
NAcb-Sh EC	vs.	CeA EC	0,216	0,046656	0,681
NAcb-Core EC	vs.	BLA EC	0,099	0,009801	0,853
NAcb-Core EC	vs.	CeA EC	-0,165	0,027225	0,755
BLA EC	vs.	CeA EC	-0,041	0,001681	0,939