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Papel de los circuitos cerebrales amígdalo-corticales en la respuesta a la novedad en ratas adolescentes

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"The brain is a monstrous, beautiful mess. Its billions of nerve cells lie in a tangled web that displays cognitive powers far exceeding any of the silicon machines we have built to mimic it."

William F. Allman

PAPEL DE LOS CIRCUITOS CEREBRALES AMÍGDALO-CORTICALES EN LA RESPUESTA A LA NOVEDAD EN RATAS ADOLESCENTES

AMYGDALO-CORTICAL BRAIN CIRCUITS ON THE RESPONSE TO NOVELTY IN ADOLESCENT RATS

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Resumen

La neofobia gustativa y su atenuación (AN) se han revelado de gran valor para investigar los mecanismos cerebrales responsables de la respuesta a la novedad a lo largo del desarrollo en modelos animales. Sin embargo, los conocimientos actuales sobre el componente afectivo de la respuesta a la novedad de los sabores y su familiarización, así como los circuitos neurales responsables, son escasos. Los resultados han sido contradictorios y parecen depender de los procedimientos y pruebas empleadas. Del mismo modo, la evidencia existente sobre la actividad de la amígdala basolateral (BLA) en ratas adultas, basada en la aplicación de técnicas de microdiálisis e inmunohistoquímica de c-Fos, la relaciona tanto con la neofobia gustativa como con la AN, siendo en ocasiones contradictoria. Por otra parte, de acuerdo con el incremento en la reactividad a estímulos potencialmente dañinos propio de la adolescencia, se ha descrito un retraso de la AN que depende de la palatabilidad del sabor en ratas adolescentes. Por ello, se ha propuesto que las diferencias madurativas entre la BLA y la corteza prefrontal medial (mPFC) puedan jugar un papel importante ya que la mPFC muestra aumento de actividad durante la primera fase de la AN en ratas adultas, pero no se ha explorado previamente en ratas adolescentes.

Esta tesis doctoral está formada por tres series experimentales. La primera se dirige a investigar en ratas adultas de ambos sexos los procesos afectivos involucrados en neofobia a una solución de vinagre de sidra y su atenuación. Para ello, además de la cantidad ingerida, se combina en el mismo estudio el registro de las respuestas orofaciales mediante la prueba de reactividad al sabor (*Taste Reactivity Test*, TRT) y el microanálisis de la estructura del lamido (*Microanalysis of Licking Structure*, MLS). Los resultados con el MLS, pero no la TRT, indican un efecto del sexo en la reactividad al sabor y permiten extender resultados previos que indican incremento de la palatabilidad del sabor durante la familiarización al sabor ácido.

La segunda serie experimental explora la participación de la BLA en los procesos afectivos implicados en la AN evaluando la actividad metabólica de la zona mediante la cuantificación de los niveles de la enzima citocromo oxidasa. Los resultados muestran un incremento selectivo de la actividad metabólica en la BLA durante la segunda presentación del sabor lo que apoya su participación en el proceso de familiarización del sabor.

Por último, la tercera serie experimental emplea inmunohistoquímica de c-Fos para registrar la actividad en distintas regiones de la mPFC durante la neofobia gustativa y la AN en ratas adolescentes y adultas. Los adolescentes muestran una reducida actividad de las áreas ventrales de la mPFC en comparación con los adultos durante la AN a una solución de vinagre, reducción que no se encuentra en las áreas dorsales. Ello puede relacionarse con la necesidad de más exposiciones descrita en esta etapa del desarrollo y sugiere que la maduración de la mPFC contribuye a acelerar el proceso.

En conjunto, los resultados que integran esta tesis doctoral aportan nueva evidencia sobre el papel de los procesos afectivos y motivacionales implicados en la neofobia gustativa y su atenuación, poniendo de manifiesto la participación de una compleja red neural formada por circuitos emocionales y de recompensa cuya investigación se beneficia de una aproximación de desarrollo y de la combinación de una variedad de técnicas para el registro de la conducta y de la actividad neural.

Abstract

Taste neophobia and its attenuation (AN) have proven to be of great value for studying the brain mechanisms involved in responding to novelty along development in animal models. The present knowledge on the emotional component of the response to flavour novelty and the familiarization process is nevertheless scarce. Moreover, the results have been contradictory depending on the procedures and tests applied. Regarding the underlying brain circuit, the available evidence in adult rats is based on microdialysis and c-Fos immunohistochemistry. Although the results point to the involvement of the basolateral amygdala (BLA) activity both in taste neophobia and AN, they are often inconsistent. Besides, adolescent rats exhibit delayed AN depending on taste palatability in accordance with the increased reactivity to potentially harmful stimuli described in this developmental stage. It has been proposed that maturational differences between BLA and the medial prefrontal cortex (mPFC) might play a role in this effect since adult rats exhibit increased mPFC activity during the initial AN process. There is no evidence, however, in adolescent rats.

This doctoral thesis contains three experimental series aimed at applying a developmental approach to study the amygdalo-cortical interaction involved in flavour neophobia and AN.

The first experimental series explored in male and female adult rats the emotional processes involved in flavour neophobia and AN using a cider vinegar solution. Both the taste reactivity test (TRT) and the microanalysis of the licking structure (MLS) were combined in addition to the assessment of the amount ingested. MLS results, but not TRT, yielded a sex effect on taste reactivity and they allowed us to extent previous findings supporting increased palatability during acid flavour familiarization.

The second experimental series explored the BLA participation in the emotional processes involved in AN by assessing the metabolic activity of the area using quantification of the cytochrome oxidase levels. The results evidenced a selective increase of BLA enzymatic activity during the second flavour exposure. This supports BLA involvement in the flavour familiarization process.

Finally, the third experimental series applied c-Fos immunohistochemistry in order to assess in adolescent rats the activity of the different mPFC subregions during taste neophobia and AN. Using a cider vinegar solution, adolescents showed reduced mPFC activity in comparison to adults during AN. This can be related to the fact that more flavour exposures are required for completing AN at this age than in adulthood. These findings support that the activity of mPFC contributes to accelerate the AN process.

Taken together, the results included in this doctoral thesis, taking advantage of a developmental approach, provide new evidence on the contribution of the emotional processes to taste neophobia and its attenuation revealing the participation of a complex amygdalo-cortical circuit.

Capítulo 1

Introducción

1.1. Novedad y aprendizaje gustativo

La novedad de un estímulo o de una situación representa una valiosa fuente de información para la adaptación al medio ya que puede implicar tanto una ventaja como un peligro potencial para la supervivencia. Respuestas reflejas atencionales, tales como las que integran el reflejo de orientación, contribuyen a la evaluación inicial del estímulo desconocido. El resultado de la evaluación desencadenará conductas de aproximación, evitación o rechazo, favoreciendo diversos procesos de aprendizaje. Si el estímulo se presenta de forma repetida o sostenida en el tiempo sin consecuencias, las respuestas de orientación desaparecerán, gracias a un proceso de aprendizaje llamado habituación (Gallo, 2018).

La evaluación de elementos novedosos comestibles o bebibles es crucial para la selección de la dieta, especialmente en especies omnívoras, tales como el ser humano o los roedores. En este sentido, la modalidad gustativa es crítica para identificar los alimentos y bebidas ingeridas, aunque el olor y la textura contribuyan a la percepción del sabor. Degustar un sabor requiere una conducta de ingesta que lo diferencia de la percepción de otras modalidades estimulares. Tal y como exponía uno de los investigadores pioneros en este campo, Michael Domjan (2018): “comer o beber algo supone una de las formas más íntimas de interacción entre el organismo y el entorno”.

El estudio de la respuesta ante la novedad gustativa se ha beneficiado de modelos animales, siendo la rata y el ratón lo más utilizados. Ante un sabor desconocido los animales se mostrarán reticentes a consumir ese alimento, fenómeno denominado neofobia gustativa. El animal sólo consumirá una pequeña cantidad del alimento en este primer encuentro. Esta reticencia desaparecerá si el sabor no es seguido de consecuencias viscerales negativas gracias a un proceso de aprendizaje denominado atenuación de la

neofobia (AN; *Attenuation of Neophobia*). Así, el sabor será reconocido en ulteriores ocasiones como seguro y se incrementará su consumo. Por el contrario, si se producen consecuencias viscerales negativas, tendrá lugar un condicionamiento aversivo gustativo (CTA; *Conditioned Taste Aversion*) y el sabor se rechazará en posteriores presentaciones (Garcia & Koelling, 1966). Este último tipo de aprendizaje fue descubierto por John García en 1955 (Garcia et al., 1955) y sus peculiares características representaron una revolución que contribuyó a reevaluar las teorías del aprendizaje de la época (Garcia et al., 1972; Garcia & Koelling, 1966). Entre las peculiaridades que presenta el CTA destacan la adquisición en ensayo único, larga dilación entre los estímulos a asociar y selectividad de la asociación entre estímulos gustativos e interoceptivos. Efectivamente, un solo ensayo es suficiente para que el sabor sea asociado a los síntomas de malestar visceral, nausea y vómito en el caso de especies eméticas. Dicha asociación se produce con dilación de minutos e incluso horas entre el sabor y sus consecuencias viscerales (Burešová & Bureš, 1980). Se trata de una rápida asociación selectiva entre estímulos interoceptivos que no se produce cuando se trata de asociar el sabor o el malestar con estímulos exteroceptivos, tales como un shock en las patas o la textura del alimento (Garcia et al., 1968). Otra de las características del CTA es la naturaleza de la respuesta condicionada. García y colaboradores (1972) propusieron que no sólo el sabor es evitado, sino que su valor hedónico disminuye. La evidencia obtenida con diversas técnicas indica que lo que se condiciona es una aversión que reduce la palatabilidad del sabor (Dwyer, 2009; Grill & Norgren, 1978a). Asimismo, los fenómenos de aprendizaje que modulan el CTA modifican también el valor hedónico. Es el caso de la inhibición latente por la exposición previa, que previene o retrasa el CTA y evita la disminución del valor hedónico (Dwyer et al., 2013; Gasalla et al., 2016; López et al., 2010), o el de la extinción del CTA, que parece producir una recuperación del valor hedónico del sabor previo a la

asociación (Cantora et al., 2006; Dwyer, 2009, 2012). Sin duda, la novedad del sabor es crucial para que se pongan de manifiesto las peculiaridades del CTA, lo que representa gran valor adaptativo para evitar la ingestión de venenos potenciales que amenacen la supervivencia del organismo.

Por tanto, el CTA y la AN podrían considerarse los dos posibles resultados de la misma situación: una experiencia con una comida o bebida novedosa. Si bien el CTA podría considerarse un mecanismo que excluye alimentos de la dieta, en el otro extremo se encuentra la AN, el mecanismo por el cual se introducen nuevos alimentos. De este modo, la evolución ha proporcionado mecanismos, simples pero eficaces, para seleccionar una dieta segura. Tal es su utilidad, que se pueden encontrar en casi todas las especies (Domjan, 2018; Reilly, 2018).

1.2. Atenuación a la neofobia

La identificación de nuevos alimentos y bebidas que puedan incluirse en la dieta se produce gracias al fenómeno que conocemos como la AN gustativa. Si la ingestión de un alimento novedoso no es seguida de consecuencias viscerales negativas en subsecuentes exposiciones, el consumo incrementará (Domjan, 1976; Domjan & Gillan, 1976). La denominación surge de los primeros estudios de Michael Domjan al exponer a varios grupos de ratas de forma repetida a una novedosa solución de sacarina sin consecuencias negativas y observar un aumento en el consumo con respecto a la primera presentación (Domjan, 1976). Teniendo en cuenta el procedimiento empleado, este incremento en el consumo no se podía atribuir a que las ratas asociasen el sabor con una reducción de la sed. Domjan propuso que la mera exposición reduce la neofobia innata de los animales a sabores novedosos, por lo que se refirió a ella como la AN.

Inicialmente, la AN podría interpretarse como un proceso de habituación, es decir un tipo de aprendizaje no asociativo por el que se deja de responder a un estímulo sin consecuencias. Sin embargo, existen interpretaciones alternativas. Así, Rozin y Kalat propusieron la "hipótesis de la seguridad aprendida" (Rozin & Kalat, 1971), según la cual la AN no sería producto simplemente de la habituación, sino que el sabor de un alimento o bebida se asociaría con las consecuencias positivas de su ingestión convirtiéndose en un aprendizaje seguro. Desde este punto de vista, la AN se trata de un aprendizaje asociativo y, por tanto, está sujeto a los principios de este tipo de aprendizaje, así como las peculiaridades de los aprendizajes gustativos, tal como sería el caso del CTA. Si ello fuera así, es de esperar que la AN y el CTA compartan algunas características tales como ensayo único y cambios en el valor hedónico del sabor. Efectivamente, se ha demostrado en repetidas ocasiones que una exposición es suficiente para que se produzca la AN, pero la evidencia sobre cambios en palatabilidad inducidos por la AN es escasa y los resultados difieren dependiendo del tipo de procedimiento empleado para medir las respuestas hedónicas.

1.3. Palatabilidad en la atenuación a la neofobia

Palatabilidad y consumo, aunque resultan congruentes en la mayor parte de las ocasiones, son procesos diferentes que no siempre van en la misma dirección. Un sabor preferido puede ser potencialmente peligroso mientras que otro menos palatable puede ser beneficioso. Por tanto, el valor hedónico y el consumo de un sabor reflejan procesos independientes, pero complementarios. Clásicamente, se ha relacionado el consumo con el concepto de querer (*wanting*), haciendo referencia a factores motivacionales (por ejemplo, hambre y sed); mientras que para la palatabilidad se ha utilizado el concepto de gustar (*liking*), en relación con el placer o el desagrado en saborearlo, lo cual lo

relacionaría con factores emocionales (Berridge, 1996; Blundell & Rogers, 1989; Castro & Berridge, 2014; Neath et al., 2010).

Existen protocolos experimentales que permiten disociar wanting y liking (Berridge, 1996; Castro & Berridge, 2014). De hecho, se han desarrollado dos pruebas específicas para evaluar el valor hedónico del sabor. Una de ellas es el microanálisis de la estructura del lamido (MLS; *Microanalysis of Licking Structure*) (Davis & Levine, 1977; Davis & Smith, 1992; Dwyer, 2009, 2012; Riordan & Dwyer, 2019). Para ello, se analiza el patrón de lametazos evaluando su número y frecuencia, así como su agrupación en clusters de diferente tamaño (LCS; *Licking Cluster Size*). Otra de las principales pruebas utilizadas en la evaluación del valor hedónico de sabores es el test de reactividad al sabor (TRT; *Taste Reactivity Test*) que evalúa las reacciones orofaciales apetitivas, aversivas o neutras del animal mientras consume el sabor (Dwyer, 2012; Grill & Norgren, 1978a, 1978b; López et al., 2010; Neath et al., 2010). Esta última prueba se puede aplicar sin necesidad de que exista ingestión voluntaria gracias al empleo de cánulas intraorales para administrar el sabor. Ello permite eliminar buena parte del componente motor que acompaña a la ingestión voluntaria favoreciendo en parte la disociación entre consumo y palatabilidad. Ambas técnicas han sido repetidamente aplicadas para estudiar los cambios hedónicos asociados al CTA, pero solo existen cuatro publicaciones en las que se apliquen a la AN. Solo una de ellas utiliza la TRT y tres aplican el MLS.

Neath y sus colaboradores fueron los primeros en abordar esta cuestión utilizando la TRT (Neath et al., 2010). Se exponía brevemente (10 segundos) a agua o a una de tres soluciones diferentes de sacarina (0.1%, 0.25% o 0.5%), durante 5 días consecutivos. Tras esto, se privaba a las ratas de agua a lo largo de la noche, y, a la mañana siguiente se llevaba a cabo una prueba de consumo voluntario de una solución de sacarina. Los resultados de TRT no indicaron cambios en palatabilidad asociados a la familiaridad del

sabor, aunque todos los grupos expuestos a sacarina presentaron mayor número de reacciones apetitivas en comparación con los que fueron expuestos a agua. La prueba de consumo voluntario posterior confirmó la existencia de AN en los grupos expuestos, mientras que el grupo control no expuesto mostró neofobia. No obstante, durante este experimento, los registros obtenidos mediante la TRT fueron muy breves (10 segundos en cada sesión), mientras que las pruebas de consumo voluntario fueron de 5 minutos y hasta 24 horas. Por tanto, sería posible que el motivo por el cual no se encontraron diferencias en la TRT, pero sí en el consumo voluntario, radicara en que durante la TRT el tiempo no fuese suficiente para detectar los posibles cambios relacionados con la familiaridad. Para abordar este problema, los autores llevaron a cabo un segundo experimento con una sesión más larga de TRT (5 minutos) que arrojó resultados similares al anterior. Por ello, los autores llegaron a la conclusión de que la atenuación de la neofobia no implica un cambio en la palatabilidad de sabor, sino en la renuencia a acercarse a la botella.

Posteriormente, Lin y colaboradores (Lin, Amodeo, et al., 2012) abordaron esta problemática utilizando una aproximación diferente, mediante el MLS. Para ello, sometieron a distintos grupos de ratas a 4 exposiciones de 3 sabores distintos: sacarina, quinina y policosa, sustancia de alto valor calórico y que no provoca reacción neofóbica. En los tres grupos se observó un incremento en el LCS indicando que la familiaridad del sabor aumenta su palatabilidad independientemente de la existencia de neofobia.

Los dos estudios posteriores que han utilizado medidas basadas en el MLS han confirmado estos resultados (Austen et al., 2016; Monk et al., 2014). Por un lado, Monk y colaboradores (2014) diseñaron un procedimiento consistente en una prueba de acceso breve (BAT; *Brief Access Task*) para medir el consumo de una solución de sacarina sódica o agua en ensayos muy breves (15s) durante bloques de 4 ensayos a lo largo de varias

sesiones de 10 bloques cada una. En primer lugar, validaron que el consumo se correspondía con el número total de lametazos y pudieron observar cómo la AN sucedía en tiempo real dentro de la misma sesión. En segundo lugar, midiendo tanto la AN a corto plazo (5 minutos) como a largo plazo (24 horas), encontraron un mayor LCS para el sabor familiar en comparación con el novedoso. Por otro lado, Austen y colaboradores (2016), empleando sabores de cereza o uva mezclados con sacarosa, observaron que los sabores familiares producían un mayor LCS que los novedosos, aunque el consumo total no variaba. Ello fue evidente tanto en tests realizados a intervalos cortos de 15 minutos como largos de 24 horas. Ello apunta a la disociación entre consumo y palatabilidad, estando asociada la familiaridad con incremento del valor hedónico.

Aunque escasos, estos estudios ponen de manifiesto la complejidad de la cuestión, así como la relevancia del procedimiento empleado para estudiar el papel de la palatabilidad tanto en la neofobia como en su atenuación. Los resultados parecen indicar que la TRT no detecta cambios en palatabilidad asociados a la familiarización del sabor mientras que el MLS muestra cambios asociados a la familiaridad que no siempre están relacionados con los cambios en consumo. Sin embargo, tanto los sabores empleados como los parámetros temporales de la exposición y la prueba, así como el empleo de ratas o ratones, varían entre los estudios. No existen estudios en los que se hayan combinado la TRT y el MLS siguiendo un procedimiento similar de administración del sabor en la misma especie.

1.4. Neuroanatomía de la atenuación de la neofobia

1.4.1. Organización del sistema gustativo

Los receptores gustativos se encuentran agrupados en las papilas gustativas situadas en la cavidad oral, principalmente en la lengua, paladar y laringe (Matsumoto, 2013; Norgren,

1983; Schier & Spector, 2019). Pueden distinguirse receptores gustativos acoplados a proteínas G (T1R y T2R) y receptores asociados a canales iónicos (T3R). Los T1R median los sabores dulce y umami, los T2R, el amargo y los T3R responden a sabores salados y ácidos. También puede encontrarse este tipo de receptores en otras zonas del sistema gastrointestinal e incluso otras no relacionadas con la ingesta cuya función aún necesita ser determinada (Schier & Spector, 2019). Las fibras nerviosas que transmiten información gustativa son los pares craneales facial, glosofaríngeo y la rama timpánica del vago (VII, IX y X, respectivamente). La información de estos nervios llega directamente al primer relevo gustativo, el núcleo del tracto solitario (NTS), estructura situada en el bulbo raquídeo. La región más rostral del NTS (rNTS) recibe aferencias de las papilas mayormente provenientes de la lengua, mientras que la parte más caudales del NTS, reciben información, principalmente, de la laringe y faringe (King, 2006; Matsumoto, 2013; Schier & Spector, 2019). Las primeras evidencias del procesamiento de la palatabilidad se encuentran en el rNTS que, además de contener grupos de neuronas que responden a cada sabor, contiene grupos de neuronas que muestran un patrón de activación diferente según las propiedades hedónicas del sabor (King, 2006). Chang y Scott (1984) observaron que, tras someter un sabor dulce, como el de la sacarina, a un CTA, el patrón de neuronas que lo codificaban en el rNTS cambiaba, y aparecía uno más parecido al obtenido típicamente con un sabor amargo, como por ejemplo, la quinina (Berridge, 1996; Chang & Scott, 1984).

En roedores, la principal vía gustativa ascendente a partir del rNTS se compone de fibras eferentes al núcleo parabraquial (PBN), situado en la protuberancia del tronco cerebral. En este núcleo confluyen las aferencias gustativas con las viscerales que son transmitidas tanto por vía vagal como humoral a través del área postrema. Así, el PBN juega un papel importante en la capacidad de asociar un sabor con malestar visceral para

inducir una aversión condicionada. Estudios con lesiones reversibles, que no interfieren con la función de la zona durante la asociación, han propuesto que representa el locus asociativo de este (Ivanova & Bureš, 1990b, 1990a). Asimismo, lesiones permanentes bilaterales severas interfieren con los cambios hedónicos asociados al CTA (Spector, 1995; Spector et al., 1993) y atenúan, aunque no eliminan, las respuestas hedónicas a sabores no condicionados (Schier & Spector, 2019).

El PBN envía la información a través de dos vías principalmente: por un lado, la vía límbica, denominada así porque la información llega a estructuras del prosencéfalo ventral, como el hipotálamo lateral (LH), amígdala central (CeA) y basolateral (BLA), el núcleo del lecho de la estría terminal, la substantia innominata y el área tegmental ventral; y por otro lado, la vía talamocortical, por la cual se envía información al relevo gustativo talámico, situado en una región del tálamo ventroposteromedial (VPMpc), y una porción de la corteza insular (IC) denominada corteza gustativa (GC), debido a su relevancia en el procesamiento gustativo (Berridge, 1996; King, 2006; Schier & Spector, 2019). Se han descrito proyecciones recíprocas monosinápticas entre la GC y el PBN implicadas en el mantenimiento de la memoria del sabor relevantes para la adquisición del CTA (Gallo & Bureš, 1991). No es de extrañar que la vía límbica haya sido el foco de atención en la investigación con relación al valor hedónico de los sabores, dado que mantiene conexiones directas con estructuras implicadas en la regulación emocional y el sistema de recompensa. Por su parte, la segunda vía se ha relacionado con reflejos fisiológicos y los componentes oromotores en relación al sabor (Schier & Spector, 2019).

En el PBN, el procesamiento hedónico se lleva a cabo a partir de información gustativa muy básica proveniente de las vías gustativas ascendentes (Berridge, 2009; Grill & Norgren, 1978b; Steiner, 1973). Estudios en ratas descerebradas ya mostraban reacciones aversivas a sabores amargos, como la quinina (Grill & Norgren, 1978b) y

reacciones apetitivas a sabores dulces, como el azúcar (Berridge, 1988). No obstante, estas ratas no eran capaces de aprender cambios en el valor hedónico de un sabor inducidos por CTA. Los datos indican que solo es necesaria la participación cortical durante el procesamiento del sabor para que se produzca el CTA (Gallo & Bureš, 1991), pero no para que se establezca la asociación. Una aversión condicionada puede ser adquirida aun cuando tras consumir el sabor el animal esté anestesiado o decorticado funcionalmente por depresión cortical propagada (Burešová & Bureš, 1980). Sin embargo, estos procedimientos impiden la AN, apuntando a la existencia de circuitos neurales disociables.

Además, se ha propuesto que el cambio en el valor hedónico inducido por la experiencia requiere la participación de áreas tales como el núcleo accumbens (NAcb), el pálido ventral (VP), y la corteza prefrontal (PFC) así como la región posterior de la IC (Morales & Berridge, 2020). En estas áreas se han identificado zonas de muy pequeño volumen (alrededor de $1mm^3$) denominadas "hotspots" que, al ser estimuladas con determinados neurotransmisores, tales como endocannabinoides y orexina, hacen que se amplifiquen las respuestas apetitivas mientras se consume un alimento palatable. En contrapartida, existen "coldspots" que al ser estimulados provocan la supresión de las reacciones apetitivas (Berridge, 2009; Castro & Berridge, 2014; Morales & Berridge, 2020).

1.4.2. Amígdala y atenuación a la neofobia

La amígdala es una estructura con forma de almendra situada en el lóbulo temporal que está subdividida en distintos núcleos (Swanson & Petrovich, 1998). Debido a la heterogeneidad de su población neuronal, existe un debate sobre qué criterio elegir a la hora de realizar estas subdivisiones (LeDoux, 2007; Swanson & Petrovich, 1998). No

obstante, parece bastante aceptado que se pueden diferenciar al menos dos regiones: una centromedial, y otra basolateral. La amígdala basolateral (BLA) está compuesta por la porción lateral de la amígdala, la porción basal y la porción basomedial. La mayor parte de las aferencias del NTS llegan a la porción lateral, la cual proyecta a la BLA y de ahí a estructuras de la región centromedial de la amígdala (CeA). Asimismo, la BLA mantiene conexiones recíprocas con distintas estructuras de todo el cerebro, a destacar, con la PFC y el HC, así como varias áreas sensoriales de asociación (Janak & Tye, 2015).

La amígdala, y, en particular BLA, ha sido identificada como una estructura de gran importancia en relación con el procesamiento de la familiaridad de los estímulos gustativos. Por una parte, varios estudios se han centrado en registrar su actividad durante la neofobia y su atenuación. Empleando inmunohistoquímica de c-Fos como índice de actividad, varios estudios han descrito un incremento de células c-Fos positivas en la BLA durante la exposición a sabores novedosos, tales como sacarina (Lin, Roman, et al., 2012; Wiaderkiewicz & Reilly, 2023) o vinagre (Grau-Perales et al., 2020), mientras que no se ha encontrado una activación significativa en la segunda exposición (Grau-Perales et al., 2020). Con ambos sabores, vuelve a encontrarse un incremento de células c-Fos positivas tras varias exposiciones al estímulo gustativo, cuando se considera que la memoria del sabor se encuentra bien consolidada, sugiriendo que esta estructura participa tanto en la respuesta a la novedad como en el reconocimiento de sabores familiares (Grau-Perales et al., 2020; Lin, Roman, et al., 2012; Wiaderkiewicz & Reilly, 2023). Asimismo, utilizando microdiálisis *in vivo* se ha descrito un aumento de los niveles de catecolaminas en la BLA durante la exposición a una solución novedosa de sacarina (Guzmán-Ramos et al., 2012), aumento que no se encuentra durante la AN (Osorio-Gómez et al., 2017). También estudios que miden expresión génica encuentran cambios específicos en la BLA durante la AN de una solución de sacarina (Gómez-Chacón et al., 2016).

Por otra parte, estudios con lesiones apoyan la participación de la BLA en el procesamiento de la novedad y familiaridad del sabor. Lesiones de la BLA aumentan el consumo en la primera exposición a soluciones dulces de sacarina, sacarosa, y leche condensada (Lin et al., 2009; Nachman & Ashe, 1974; Rollins et al., 2001) y a una solución de vinagre de sidra (Gómez-Chacón et al., 2012). Inactivaciones reversibles mediante la manipulación de la neurotransmisión normal indican que la respuesta neofóbica disminuye o, incluso, se suprime al bloquear los receptores catecolaminérgicos (Roozendaal & Cools, 1994) y al administrar agonistas GABAérgicos (Lin et al., 2018; Shinohara & Yasoshima, 2019). En este sentido, se ha observado que la administración directa de catecolaminas, aumenta la respuesta neofóbica. Sin embargo, inyecciones de ketamina (Aguado et al., 1994) o infusiones en la BLA de MK-801, antagonista no competitivo de receptores glutamatérgicos (Figueroa-Guzmán & Reilly, 2008), no parecen tener efecto sobre la respuesta neofóbica. Con respecto a la AN, lesiones excitotóxicas de la BLA provocan un retraso en su aparición cuando las ratas son expuestas a una solución de vinagre de sidra (Gómez-Chacón et al., 2012). Del mismo modo, el uso de antagonistas glutamatérgicos no competitivos, si bien no parece tener efecto alguno sobre la respuesta neofóbica, retrasa la AN (Aguado et al., 1994; Figueroa-Guzmán & Reilly, 2008), mientras que el uso de agonistas GABAérgicos parece arrojar resultados contradictorios con respecto a la AN utilizando una solución de sacarina (Lin et al., 2018; Shinohara & Yasoshima, 2019). La infusión en la BLA de SCH-23390, antagonista de los receptores dopaminérgicos D1, previa a la segunda exposición a una solución de vinagre de sidra impide la AN (Grau-Perales et al., 2020). Cabe destacar que, en el mismo estudio, la inyección en la BLA de SKF-81297, agonista de los receptores dopaminérgicos D1, antes de la segunda exposición al sabor, incrementa el consumo, siendo este uno de los pocos datos disponibles sobre cómo la BLA puede facilitar la AN.

En resumen, parece que la BLA sostiene un papel importante tanto en la neofobia gustativa como en su atenuación, ya sea de forma directa, o de forma indirecta por su interacción con otras áreas cerebrales. Los registros de la actividad cerebral apuntan a un incremento de actividad en la BLA durante la exposición a un sabor novedoso, y las manipulaciones que impiden el funcionamiento de la BLA, como lesiones o inactivación, resultan generalmente en una reducción de la respuesta neofóbica, y, en ocasiones, en una supresión total de la misma. Además, la BLA también parece ser relevante en la AN, aunque los hallazgos son escasos e inconsistentes, y parecen depender de la técnica utilizada.

1.4.3. Corteza prefrontal medial

La PFC, situada en la parte más anterior del telencéfalo, destaca por integrar información de distintos sistemas y modular la respuesta de estos (Uylings et al., 2003). Recibe aferencias somatosensoriales, visuales, gustativas y olfativas en ratas y mantiene conexiones recíprocas con el cuerpo estriado ventromedial, distintos núcleos talámicos como el núcleo mediodorsal del tálamo, el NAcb, caudado y putamen (Cpu). Asimismo, está conectada con prácticamente todas las estructuras temporales que conforman el sistema límbico: distintas áreas de la amígdala (tanto la CeA como la BLA), hipotálamo, subículo y CA1 del HC, corteza entorrinal y la PRh (Öngür & Price, 2000).

En primates, según su conectividad anatómica y estructura citoarquitectónica, se divide en PFC orbitofrontal, PFC ventrolateral, PFC dorsolateral, PFC rostrolateral, y PFC medial (mPFC) (Barbas & García-Cabezas, 2016). Cada una de estas áreas parece estar especializada en diferentes funciones ejecutivas (Uylings et al., 2003; Werchan & Amso, 2017). Así, las regiones dorsomedial y ventromedial se relacionan con la toma de decisiones: la primera, en situaciones de riesgo e incertidumbre; la segunda, cuando se

trata de obtener el máximo beneficio o minimizar el coste en una situación determinada. Las regiones laterales, tendrían un rol clave en la conducta dirigida a meta (Werchan & Amso, 2017).

En roedores, la PFC se encuentra mucho menos diferenciada. La citoarquitectura de esta estructura destaca, en primer lugar, por su composición exclusivamente agranular (Öngür & Price, 2000), en contraste con humanos y primates no-humanos, de los que se encuentran capas tanto granulares como agranulares (Öngür & Price, 2000; Uylings et al., 2003). Es en parte por esto que en un principio se consideró que las ratas no tenían una PFC comparable a la de los primates, y que se trataba de una extensión del córtex cingulado (Öngür & Price, 2000). No ha sido hasta hace relativamente poco que ha habido cierto consenso dentro de la comunidad científica sobre que los roedores poseen una PFC diferenciada, de forma similar a como ocurre en mamíferos superiores. Efectivamente, atendiendo a los estudios más recientes basados en técnicas de neuroimagen, de conectividad funcional y de lesión, parece obvio que la PFC en las ratas se encuentra diferenciada y especializada topográficamente (Kolb & Gibb, 2015; Öngür & Price, 2000; Paxinos & Watson, 2013; Uylings et al., 2003). En ratas, la PFC puede dividirse en tres áreas: PFC lateral, PFC orbital y mPFC (Akhter et al., 2014; Heidbreder & Groenewegen, 2003). A su vez, la mPFC puede subdividirse en una región dorsal (dmPFC) y otra ventral (vmPFC). La región dorsal está compuesta por la corteza precentral, la corteza cingulada anterior (ACC) y la parte dorsal de la extensa corteza prelímbica (PrL). Por su parte, la región ventral la forman la zona ventral de la PrL, la corteza infralímbica (IL) y la corteza dorsal peduncular (DP). Aunque la DP hasta hace poco se consideraba como la parte ventral de la IL, recientes estudios la han identificado como un área de la mPFC independiente, debido a sus conexiones con el núcleo mediodorsal del tálamo y sus

funciones disociables de las de la IL (Akhter et al., 2014; Expósito et al., 2020; Paxinos & Watson, 2013; Smith et al., 2019).

Estudios de lesión han puesto de manifiesto diversas funciones de la mPFC incluyendo memoria de trabajo, atención, elaboración de estrategias, secuenciación motora, comportamiento conflictivo, aprendizaje operante, extinción del miedo y habituación, entre otros (Uylings et al., 2003). La investigación sobre su participación en la memoria de reconocimiento se ha centrado en la modalidad visual (para una revisión, ver Morici et al., 2015). También se ha relacionado con el aprendizaje gustativo. La mayor parte de los estudios apuntan a que la mPFC juega un papel en la consolidación y formación del CTA (González et al., 2015), su recuperación (Reyes-López et al., 2010), su retención (Caynas-Rojas et al., 2019; Zhou et al., 2013) y su extinción (Akirav et al., 2006; González et al., 2014, 2015; Mickley et al., 2005; Xin et al., 2014), así como en el fenómeno de inhibición latente (Caynas-Rojas et al., 2019).

Sin embargo, en lo referido a la relación entre la neofobia gustativa y su atenuación, la evidencia es escasa. Empleando inmunohistoquímica de c-Fos se ha descrito que el consumo de sabores novedosos, tales como aceite de maíz, fructosa y glucosa, provoca un incremento en la actividad de la mPFC. No obstante, este incremento no se ha observado al utilizar sacarina que carece de valor calórico (Dela Cruz et al., 2016). Atendiendo a esta información, se podría interpretar que la mPFC no posee un papel relevante en el procesamiento de la novedad del sabor en sí, sino, más bien, en la detección de una nueva fuente de calorías. A pesar de lo expuesto, un estudio reciente midiendo la actividad neuronal mediante fosforilación de la quinasa regulada extracelular (pERK) y la conectividad entre áreas, describió que la mPFC, la BLA y la GC interactúan entre sí formando un circuito encargado de procesar la novedad de un estímulo gustativo, y que la interacción mPFC-GC es necesaria para reconocer un sabor como familiar y

seguro (Kayyal et al., 2021). En el mismo sentido, Expósito y colaboradores utilizaron inmunohistoquímica de c-Fos para comparar la actividad de la mPFC durante un procedimiento de AN en ratas adultas y envejecidas (Expósito et al., 2020). Las ratas adultas mostraron una actividad significativamente mayor en la vmPFC atribuible a la DP durante la segunda exposición a una solución de vinagre de sidra con respecto a la primera o sexta exposición, mientras que no encontraron diferencias en la dmPFC. Ello apunta a la participación selectiva de la zona en el proceso de AN frente al procesamiento de la novedad o de la memoria consolidada del sabor familiar.

En conjunto, los resultados que relacionan la actividad de la mPFC con la neofobia gustativa y su atenuación son escasos y en ocasiones contradictorios, dependiendo del sabor empleado. Por ello, se hace necesaria más investigación en este campo.

1.5. Papel de la respuesta neofóbica y su atenuación a lo largo del desarrollo

Identificar la novedad de un sabor y reconocer los sabores seguros que en el pasado no fueron seguidos de consecuencias negativas es esencial para la supervivencia a lo largo de la vida. Las condiciones y exigencias de cada etapa del desarrollo moldean la respuesta neofóbica y su atenuación introduciendo modificaciones que se corresponden con la maduración y desarrollo de los circuitos cerebrales involucrados.

Ya en etapas embrionarias, los roedores exhiben movimientos peculiares ante la novedad del sabor y muestran AN con la presentación repetida (para una revisión, ver Gallo, 2018). Este aprendizaje se mantiene postnatalmente y representa un mecanismo clave en los mamíferos recién nacidos para el reconocimiento de la zona mamaria como segura y el inicio del primer episodio de lactancia, esencial para su supervivencia.

Por su parte, a edades avanzadas la respuesta neofóbica está modulada por la naturaleza de las experiencias previas con los sabores. Se ha descrito un aumento de la

respuesta neofóbica en ratas envejecidas en comparación con adultas exclusivamente cuando han adquirido previamente CTAs a otros sabores (Morón & Gallo, 2007). Sin embargo, en ausencia de experiencia previa la respuesta neofóbica de las ratas envejecidas no difiere de la exhibida por ratas adultas (Bond et al., 1989; Gómez-Chacón et al., 2015; Pelleymounter & Cullen, 1993). Sin embargo, la AN de una solución de vinagre parece ser más lenta en ratas envejecidas que en adultas (Gómez-Chacón et al., 2015), lo que puede interpretarse como mayor cautela cuando se trata de reconocer como seguros sabores con reducida palatabilidad inicial. Ambas características son congruentes con la acumulación de experiencias previas a lo largo de la vida y la desventaja en relación con la capacidad de responder a la ingestión de un veneno en la que con frecuencia se encuentra un organismo envejecido (Gallo, 2018; Gámiz & Gallo, 2011).

En el curso del desarrollo, la adolescencia representa una etapa crucial para la transición de la infancia hacia la vida adulta. En los mamíferos esta etapa se ve marcada por una serie de cambios conductuales que les preparan para la supervivencia como individuos independientes fuera de la protección parental. Entre estos cambios, destacan una mayor búsqueda de la novedad en comparación con los adultos, combinada con una alta reactividad emocional que regula los riesgos que implica el exponerse a situaciones novedosas (Lynn & Brown, 2010; Spear, 2000). En relación con los alimentos y bebidas, una rata adolescente que abandona el nido materno se enfrenta a la necesidad de explorar nuevos ambientes e incluir nuevos sabores en su dieta, más allá de aquellos a los que ha sido expuesto durante su infancia. Sin embargo, los resultados obtenidos en el laboratorio sobre la neofobia gustativa son contradictorios. Mientras que algunos autores describen que las ratas adolescentes beben más que los adultos en la primera presentación de una solución dulce (Vaidya et al., 2004), otros estudios no encuentran diferencias entre

adultos y adolescentes utilizando soluciones dulces (Dannenhoffer & Spear, 2016) o ácidas (Expósito et al., 2023).

El procedimiento experimental llevado a cabo en el laboratorio que implica privación de agua, podría contribuir a crear un efecto techo en animales sedientos, que impidiera detectar un mayor consumo del sabor novedoso en adolescentes, tal como podría esperarse de acuerdo con la búsqueda de novedad descrita en otras modalidades sensoriales. Sin embargo, la presentación repetida del sabor para estudiar la AN ha arrojado resultados que no apoyan este planteamiento y ponen de manifiesto la complejidad del comportamiento adolescente. Mientras no se han encontrado diferencias entre ratas adolescentes y adultas cuando se trata de una solución de sacarina, las ratas adolescentes necesitan mayor número de ensayos que las adultas para reconocer como familiar y segura una solución de vinagre (Expósito et al., 2023). Aunque son necesarios más estudios al respecto, parece que los resultados obtenidos en el laboratorio cuando se trata de sabores no confirman la búsqueda de la novedad observada durante la adolescencia en tareas conductuales relativas a otras modalidades sensoriales. Parece que los adolescentes son incluso más cautos a la hora de incluir ciertos alimentos en su dieta cuando se trata de sabores poco palatables. Ello es consistente con la mayor reactividad ante estímulos potencialmente peligrosos en esta etapa de la vida. Es concebible que, aunque la búsqueda de nuevos alimentos sea necesaria, el riesgo de ingerir una gran cantidad de un alimento potencialmente venenoso obligue a los adolescentes a actuar con más cautela, especialmente en especies que carecen de capacidad de emesis.

Las características peculiares del comportamiento adolescente se corresponden con la peculiar organización cerebral durante esta etapa (para una revisión, ver Spear, 2000). Siendo la PFC una de las áreas cerebrales de maduración más tardía, se ha descrito una disminución de su volumen durante la adolescencia, lo que se atribuye a que durante

esta etapa se refinan infinidad de conexiones neuronales mediante poda sináptica (Van Eden et al., 1991). Sin embargo, no existen datos sobre la activación neuronal en ratas adolescentes en relación con la AN y su interacción con el valor hedónico del sabor.

Capítulo 2

Justificación, objetivos e hipótesis

La neofobia gustativa y su atenuación se han revelado de gran valor para investigar los mecanismos cerebrales responsables de la respuesta a la novedad a lo largo del desarrollo en roedores. Debido a que la reducción en la ingestión de soluciones novedosas y su posterior incremento según el sabor se convierte en familiar ha sido la medida más ampliamente utilizada, los resultados sobre la existencia de cambios hedónicos paralelos han sido escasos y contradictorios dependiendo de la prueba de registro empleada. En estudios independientes se ha informado, bien de reducción de la palatabilidad ante un sabor desconocido e incremento asociado a la AN como indica el microanálisis de la estructura del lamido (MLS) (Lin, Amodeo, et al., 2012), bien de ausencia de cambios cuando se emplea el registro de las reacciones orofaciales (TRT) (Neath et al., 2010). Además, ambas técnicas han sido aplicadas exclusivamente en ratas macho, por lo que se requiere dirigir la investigación a ambos sexos. Dado que la determinación del papel que juegan los circuitos cerebrales emocionales en la neofobia gustativa y su atenuación requiere conocer previamente con precisión los parámetros comportamentales que permitan su evaluación, así como los factores influyentes, resulta pertinente integrar en el mismo estudio ambas medidas de palatabilidad (MLS y TRT) para facilitar comparaciones.

Con respecto a la actividad neural implicada, cabe plantear que la existencia de un componente afectivo en la neofobia gustativa y su atenuación implicaría a la amígdala como parte del circuito cerebral modulador de las respuestas emocionales. Efectivamente, la mayor parte de los resultados de estudios empleando técnicas de lesión e inactivación (Gómez-Chacón et al., 2012; Lin et al., 2009; Nachman & Ashe, 1974; Rollins et al., 2001; Roozendaal & Cools, 1994), así como de registro de la actividad (Grau-Perales et al., 2020; Guzmán-Ramos et al., 2012; Lin, Roman, et al., 2012; Wiaderkiewicz & Reilly, 2023) indican que BLA está implicada en la respuesta neofóbica, aunque existen también

resultados negativos (Aguado et al., 1994; Figueroa-Guzmán & Reilly, 2008). BLA también ha sido relacionada con el curso de la AN ya que su lesión permanente (Gómez-Chacón et al., 2012) o reversible (Aguado et al., 1994; Figueroa-Guzmán & Reilly, 2008; Grau-Perales et al., 2020) parece retrasarla. Asimismo, BLA muestra cambios en la expresión de genes asociados a plasticidad neural durante la AN (Gómez-Chacón et al., 2016). No obstante, los resultados son escasos y se requiere más investigación.

Con respecto a la investigación existente, es de destacar que el registro de la actividad de la zona se ha realizado exclusivamente mediante técnicas de microdiálisis (Guzmán-Ramos et al., 2012; Osorio-Gómez et al., 2017) e inmunohistoquímica de c-Fos (Grau-Perales et al., 2020; Lin, Roman, et al., 2012; Wiaderkiewicz & Reilly, 2023). Ambas permiten obtener información de distinta naturaleza. Para registrar con precisión el número y localización de las neuronas activadas es adecuado emplear inmunohistoquímica de c-Fos. Sin embargo, esta técnica refleja con retraso los cambios asociados a la actividad, no detecta parte de la actividad inhibitoria, ni aporta información sobre los procesos subyacentes. Es por esta razón, que es aconsejable la combinación de distintas técnicas de registro para mejorar la calidad de la información que podemos obtener de un mismo proceso. En este sentido, la histoquímica de citocromo C oxidasa (CCO) empleando densitometría óptica (Gonzalez-Lima & Cada, 1994), que evalúa directamente el metabolismo oxidativo de las células, ha sido utilizada en distintos procedimientos conductuales (Banqueri et al., 2018; Conejo, González-Pardo, et al., 2007; Conejo, Pardo, et al., 2007; Méndez-Couz et al., 2016; Méndez et al., 2009) incluyendo aprendizaje gustativo (Gasalla et al., 2016), pero nunca se ha explorado directamente la AN. Es previsible que la aplicación de CCO para investigar la participación de BLA en la neofobia gustativa y su atenuación pueda aportar información adicional y resolver algunas de las discrepancias existentes en el campo.

Por último, dado que el desarrollo cerebral se extiende hasta la adolescencia, esta etapa representa una valiosa oportunidad para disociar la participación de áreas cerebrales de maduración tardía, tal como la corteza prefrontal, en la modulación del comportamiento. La evidencia existente sobre la relación entre la actividad de esta zona y el procesamiento de la novedad gustativa en ratas adultas es escasa y parece depender del componente calórico de la solución utilizada. Empleando inmunohistoquímica de c-Fos para evaluar las diversas regiones que conforman la mPFC se ha descrito aumento de actividad en vmPFC, especialmente en DP, durante la segunda exposición a una solución de vinagre de sidra (Expósito et al., 2020). Ello respalda un posible papel de la zona en la primera fase de la AN cuando se emplea un sabor ácido de reducida palatabilidad inicial.

Por su parte, el papel modulador de la palatabilidad del sabor en la AN puede explicar el comportamiento de las ratas adolescentes que muestran retraso de la AN a una solución ácida de vinagre de sidra, pero no a una solución dulce de sacarina (Expósito et al., 2023). Ello puede relacionarse con la mayor reactividad a estímulos potencialmente aversivos en esta etapa del desarrollo que se ha asociado a hiperreactividad de BLA (Spear, 2000). Es concebible que la vmPFC juegue un papel importante en la modulación de la respuesta afectiva ante el sabor ácido. Dicha modulación podría estar alterada o ausente durante la adolescencia debido al curso de maduración retrasado. Dado que no existen datos sobre la actividad de la mPFC durante la exposición a un sabor novedoso y su atenuación en ratas adolescentes, sería especialmente valioso aplicar inmunohistoquímica de c-Fos durante la exposición repetida a una solución de vinagre de sidra similar a la empleada en ratas adultas (Expósito et al., 2020) a fin de establecer comparaciones.

En este sentido, con la intención de investigar los cambios afectivos asociados a la atenuación de la neofobia gustativa, los circuitos cerebrales responsables y su contribución al comportamiento adolescente, se plantea:

1. Explorar la evolución de la palatabilidad en relación con la neofobia gustativa y su atenuación, a fin de observar cambios emocionales aplicando tanto el microanálisis de la estructura del lamido (MLS) como el registro de las reacciones orofaciales (TRT). Si existen cambios emocionales durante este proceso, se reflejarán en ambas pruebas indicando una baja palatabilidad asociada a la neofobia, que aumentará una vez que el sabor haya sido atenuado.

2. Si existen cambios emocionales durante la neofobia gustativa y su atenuación, explorar la actividad de los circuitos cerebrales subyacentes poniendo el foco en el circuito afectivo-emocional, con especial interés en la amígdala basolateral, considerada una estructura clave del sistema límbico. Si los cambios en palatabilidad durante la atenuación a la neofobia están mediados por los circuitos emocionales, se encontrarán cambios en la actividad neuronal de la amígdala basolateral durante la neofobia y/o su atenuación.

3. Si se encuentran cambios en la actividad de los circuitos emocionales asociados a la neofobia y su atenuación, explorar cómo este proceso puede diferir durante las etapas tempranas del desarrollo poniendo el foco en aquellas áreas que maduran tardíamente, tal como la corteza prefrontal medial (mPFC). Es previsible que el retraso en la atenuación de la neofobia descrito en la etapa adolescente en comparación con adulto se vea reflejado en una reducida actividad de la región ventral de la corteza prefrontal medial, correspondiente al núcleo peduncular dorsal.

Capítulo 3

Palatability changes during attenuation of flavour neophobia assessed with licking microstructure and taste reactivity

Abstract

Taste neophobia is the reluctance of an animal to consume new flavours, a survival mechanism that prevents the intake of large amounts of potentially noxious foods. When this initial consumption is not followed by negative consequences, neophobia will attenuate and the intake of that flavour will increase. This phenomenon is called attenuation of neophobia. Although consumption alterations on attenuation of neophobia have been well reported, the possible changes in hedonic value have not been specially considered. In fact, hedonic value has been proven to decrease when a flavour is associated with some aversive stimulus, like gastrointestinal malaise, so it is necessary to explore how the pleasure for a novel flavour is modified when it becomes safe. In this regard, two experimental procedures were designed using the two main tests used to assess palatability along an attenuation of neophobia procedure using a 3% cider vinegar solution as a new flavour. In the first experiment, using microanalysis of the licking structure, animals were exposed to the novel flavour for six days and both consumption and licking parameters were measured. On the second one, using the taste reactivity test, orofacial responses to an intraorally administrated solution were recorded and evaluated. We found contradictory results: licking analysis showed attenuation of neophobia-related changes in hedonic value, while taste reactivity test data did not. Additionally, since sexual differences have been particularly neglected, both male and female animals have performed this study, allowing us to appreciate different behavioural responses from each sex. The main findings are the differential results obtained from each test while theoretically measuring the same parameters, and the evident sexual dimorphism on these tasks.

3.1. Introduction

Taste is the critical sense for identifying foods although it is generally accepted that retronasal olfaction and trigeminal stimulation contribute to the flavour perception for most foods (Spence, 2015). Flavour likes and dislikes modulate food choices thus determining diet selection which represents a major challenge for omnivorous species. It is widely accepted that evolutionary adaptations have led to the preference for sweet and salty tastes which are associated with nutrients and hydromineral balance while bitter and sour tastes related to rotten or poisonous edibles are rejected. However, flavour preferences are shaped by learning throughout life from prenatal stages to ageing (Gallo, 2018; Gámiz & Gallo, 2011). Therefore, learning about the consequences of liquid and food consumption is critical to adjust ingestive behaviour to the individual needs and conditions (Bermúdez-Rattoni, 2004). Various learning processes involved have been widely explored in rodents that are omnivorous and lack emetic reflexes, thus depending to a great extent on acquired responses to avoid ingestion of potential poisons threatening their life.

Rodents exhibit taste neophobia, that is, they are reluctant to ingest novel flavours. Thus, they consume reduced amounts in the first presentation in comparison with later encounters. If no aversive consequences occur following the ingestion, intake increases as the flavour becomes familiar in later exposures until reaching the asymptote. This is called attenuation of neophobia (AN). Alternatively, if consumption is followed by negative consequences involving visceral discomfort, the flavour will become aversive after one trial being avoided in later encounters (Garcia et al., 1955, 1968; Revusky & Bedarf, 1967). This learning process is known as conditioned taste aversion (CTA). Subsequent exposure to the aversive flavour without consequences leads to the extinction of the conditioned response. Although extinction leads to increased consumption, it has

been reported that not always recover to the baseline level (Rosas & Bouton, 1996). Likewise, conditioned flavour preferences (CFP) can be developed if consumption is followed by positive consequences as in the case of nutrients (Riordan & Dwyer, 2019). Thus, based on consumption it can be difficult to distinguish the processes involved in AN, CTA extinction and CFP. A confounding issue is that flavoured solutions are usually applied to water-deprived rodents in the AN experimental procedures, which prevents flavours from being considered as neutral stimuli. Even in the absence of negative consequences or caloric properties, consumption is necessarily followed by positive consequences in thirsty animals. Hence, the learning processes contributing to AN remain poorly known.

The changes in consumption induced by flavour learning might reflect the impact on different underlying processes. Affective processes play a critical role as likes and dislikes modulate approach and avoidance behaviours. However, the final ingestive behaviour is influenced by several factors including motivational and motor functions (Dwyer, 2012). It has been emphasized the relevance of distinguishing between "wanting" and "liking" (Berridge, 1996; Morales & Berridge, 2020). On the one hand, wanting refers to a motivational state related to incentive salience. On the other hand, liking refers to the hedonic value or palatability of the flavour. Thus, a distinction has been proposed between learning procedures that modify the rewarding properties of flavours and those altering the hedonic responses either appetitive or aversive. In fact, concerning learned aversive responses a distinction has been proposed between conditioned taste avoidance and conditioned taste aversions (Parker, 2003, 2014; Parker & Limebeer, 2006). Whilst both reduce consumption, only CTA seems to change the hedonic value of the flavour thus reducing palatability. Although there are alternative theoretical positions that interpret these changes in terms of quantitative differences

instead of qualitatively different types of learning (Lin et al., 2014; Lin, Arthurs, et al., 2012), there is evidence supporting this latter view. It has been demonstrated that pairing a taste with visceral distress induced by LiCl in rats, results in aversive responses while pairing the taste with visceral pain induced by NaCl induces immobility reflecting fear (Dwyer et al., 2017). Therefore, it has been suggested that to modify palatability, CTA requires the flavour to be associated with visceral illness that induces nausea and vomiting in emetic species (Garcia et al., 1968; Pelchat et al., 1983).

The evaluation of palatability requires more complex methods beyond consumption. Two widespread methods applied to assess palatability are the taste reactivity test (TRT) (Grill & Norgren, 1978b) and microanalysis of the licking pattern/structure (MLS) (Davis & Levine, 1977; Davis & Smith, 1992; Dwyer, 2012). TRT focuses mainly on assessing orofacial patterns reflecting affective reactions to a flavoured solution. The orofacial responses are classified as appetitive (mouth movements, tongue protrusions, and paw licking), aversive (head shake, forelimb flicking, chin rubbing, paw treading, and gaping) or neutral (passive dripping). Among them, gaping has been proposed as a selective measure of nausea in rats (Parker & Limebeer, 2006) as it is induced by emetic drugs and is associated selectively with CTA (Parker, 2014). In this paradigm, the solutions are usually infused via an intraoral cannula surgically implanted (Berridge, 2000; Dwyer, 2012; Neath et al., 2010). In this way, TRT provides a measure of taste palatability that reflects fewer motivational and motor factors as it does not require voluntary consumption, thus focusing on the hedonic qualities instead. In contrast, MLS assess the pattern of ingestive behaviour recording the frequency and number of licks a rodent gives when consuming a flavoured solution. Rodents drink in rapid rhythmic licking blocks called clusters, which last for a variable length of time depending on the palatability of the flavour (Ahmed et al., 2017; Dwyer,

2012). Rats will give more licks per cluster (Lick Cluster Size, LCS) if the flavour is liked (Davis & Smith, 1992; Lin, Amodeo, et al., 2012), while LCS will be lower if the flavour is disliked (Lin, Amodeo, et al., 2012). In this test, the animal voluntarily approaches the bottle and therefore both consumption and taste palatability data are obtained.

Using these methods, it has been demonstrated that taste learning induces palatability changes. However, some discrepancies have been reported. TRT seems to be more sensitive to flavour preferences since the increased hedonic value of the conditioned flavour has been reported but the effect found in licking microstructure analysis was smaller than in TRT (Riordan & Dwyer, 2019). Both consumption and palatability of a flavour decay dramatically after CTA independently of the method applied (Dwyer, 2012; Lin et al., 2014; Parker, 2003). During CTA extinction, however, consumption recovers more slowly than palatability measured either with TRT (Cantora et al., 2006) or LCS (Dwyer, 2009). While the hedonic value of the flavour returns to pre-conditioning levels, consumption not always reaches them (Rosas & Bouton, 1996). Contradictory results, however, have been obtained concerning AN depending on the method applied to assess the palatability shift as the flavour becomes familiar and safe. Lin et al. (2012) reported increased LCS after four exposures to saccharin or quinine solutions. In contrast, Neath et al. (2010) found no increase in appetitive reactions using TRT after five consecutive exposures to different concentrations of saccharin, even though a subsequent consumption test indicated that AN had taken place. The authors interpreted that AN does not increase taste palatability, but rather the tendency to approach the bottle, thus wanting but not liking would be involved. This is contradictory, however, with the results obtained regarding MLS.

No effect of sex has been reported in AN using either cider vinegar (3%) or sodium saccharin (0.1%) solutions in adolescent rats based on the assessment of consumption

(Expósito et al., 2023) while, to our knowledge, there are no data available using TRT or MLS. In fact, previous experiments were performed only in male rats. Nevertheless, sex differences in taste reactivity have been found using TRT (Clarke & Ossenkopp, 1998; Flynn et al., 1993) and licking rate (Curtis et al., 2004). Female rats exhibit more appetitive reactions than male rats in response to sucrose (0.3 M), quinine (0.0003 M) (Clarke & Ossenkopp, 1998) and low NaCl concentration (Flynn et al., 1993) solutions. Females also lick at higher rates than males in NaCl and sucrose solutions. These sex differences do not seem to be fully explained by hormonal differences (Curtis et al., 2004).

In order to explore the nature of palatability changes induced by AN in rats, we designed two experiments applying both TRT and MLS with repeated exposures to cider vinegar, a flavour previously used in our laboratory. In Experiment 1 we applied the MLS while in Experiment 2 the TRT method was used. In both experiments, consumption was also recorded either during the procedure (Experiment 1) or at the end (Experiment 2). We hypothesize that if the palatability of a novel taste increases as it is recorded as safe, it will be reflected in higher consumption, LCS and increased appetitive reactions while aversive reactions will decrease. Finally, given that previous data have been obtained in males and there are sex-dependent differences in the processing of flavours, we included both sexes in order to explore potential sex-dependent effects modifying the effect of AN on palatability.

3.2. Experiment 1: Microanalysis of the Licking Pattern

The results reported using microstructure analysis of licking during an AN procedure indicate increased LCS during AN (Lin, Amodeo, et al., 2012), although conflicting results have been obtained with TRT (Neath et al., 2010). In this experiment, we explored MLS during AN, exposing animals to a cider vinegar solution which is a neophobic

flavour previously used in our lab. Given that sex differences have been found in licking rates (Curtis et al., 2004) both groups of male and female rats are included. If the familiarity of the flavour increases palatability, it is expected an increased LCS and consumption as AN proceeds. No sex differences are expected as they have not previously been reported in AN using the same flavour (Expósito et al., 2023).

Additionally, we added two groups of male and female heterozygous Cacna1c +/- rats. CACNA1C is a gene that encodes the Cav 1.2 L-type voltage-gated calcium channels whose variations have been related to an increased risk of psychiatric disorders (Moon et al., 2018; Sykes et al., 2019). Previous unpublished data suggest that Cacna1c +/- rats exhibit reduced appetitive reactions to palatable solutions indicating anhedonia. It is anticipated lower LCS of heterozygous Cacna1c +/- rats in comparison with wild-type controls.

3.2.1. Method

Subjects. Forty-two adult male and female Cacna1c heterozygous ($Cacna1c^{+/-}$, HET) on a Sprague-Dawley background (TGR16930, Horizon, Sage Research Labs, USA) and wild type (WT) littermates were obtained and housed in mixed-genotype, no mixed-sex groups of 2-3 in standard cages (38 × 56 × 22cm) per cage. Of these, there were 27 males (±450g) and 15 females (260g), distributed in 4 groups: MaleHET (n = 9), MaleWT (n = 18), FemaleHET (n = 5) and FemaleWT (n = 10). The $Cacna1c^{+/-}$ model is a constitutive zinc finger nuclease knockout, resulting in an approximately 50% and 40% decrease in hippocampal mRNA and protein levels, respectively (Sykes et al., 2019). Hence, this model accords with altered brain expression in CACNA1C in patient cohorts. Rats were bred and housed at Cardiff University (United Kingdom) under a 12h/12h light/dark cycle, taking place all manipulations in the light cycle. Animals had ad libitum water and food until the experiment started, and were handled daily for a week prior to

the start of experimental procedures and during the acclimatization to water restrictions. Experiments were conducted in accordance with local ethic guidelines, the UK Animals (Scientific Procedures) Act 1986 and the European Communities Council Directive (1986/609/EEC).

Stimulus and apparatus. The rats were tested for both water and vinegar sessions in 15 drinking chambers (Med Associated Inc., United States). These chambers measured 32 × 15 × 12cm (L × W × H), with steel mesh flooring and white acrylic walls. The drinking chambers were located in a room separate from the home cages. Fluids were accessible through drinking spouts made of stainless steel, attached to 50-ml cylindrical bottles. These could be inserted on the left- or right-hand side of the lid (made of wire mesh). The distance between the holes for the bottles was 8 cm. Only the left-hand side was used for the present experiment. A contact-sensitive lickometer registered the time of each lick to the nearest 0.01 s. This was recorded by a computer using MED- PC software (Med Associates, Inc., St. Albans, VT). The amount of fluid consumed by each rat was measured by weighing the drinking bottle before and after each session. The stimuli were tap water or a solution of 3% (w/w) cider vinegar (Vin).

Procedure. These procedures were carried out in the Behavioural Neurosciences Laboratory (BNL) of the School of Psychology of Cardiff University. The animals were water-deprived the day before to start the experiment in the afternoon. The experimental sessions took place in the morning (at 11:00 am). They consisted of 15-min of free access to water (Baseline day) or 3% vinegar solution (Vin days) in the drinking chambers, while the licking patterns were being recorded. Consumption was measured by weighing the bottles before and after the session. Each day, animals were weighed and had a rehydration session in the afternoon (at 3:00 pm) in order to ensure their well-being. During the baseline, animals both habituated to the drinking chambers as well as provided

baseline data for water consumption and palatability. All animals received 6 consecutive sessions of Vin.

Data analysis. Due to technical problems, the fifth exposure to cider vinegar solution (Vin5) had to be excluded from the analyses. In addition to the consumption data, the mean cluster size for each rat was extracted from the record of licks for analysis. A cluster was defined as a set of licks, each separated by an inter-lick interval of no more than 0.5 s. This criterion was used by Dwyer (Dwyer, 2009; Dwyer et al., 2013; for a review, see Dwyer, 2012). Mixed Linear Models (MLM) were used to analyze the test data with the two between-group factors: Genotype (HET vs. WT), Sex (Males vs. Females) and Day as within-subject factor (Baseline, Vin 1, Vin2, Vin3, Vin4, Vin6). Independent MLM analyses 2 (Genotype) × 2 (Sex) × 6 (Day) were carried out for Consumption and LCS. All data were analyzed using SPSS (IBM, United States). All tests reported here used a criterion for significance of $p < .05$.

The MLM was used rather than ANOVA to deal with the fact that there were several missing datapoints. These missing points are related to unsystematic equipment failures (e.g. bottle blockages or leaks, lick recording failures). For this purpose, three variables were used as a control: consumption, volume/1000 licks and average inter-lick interval. Consumption's exclusion criteria left out those intake data less than 0.5g. Volume/1000 licks exclusion criteria removed those values less than 3g or greater than 10g. The average inter-lick interval's exclusion criteria left out those values less than 130ms or larger than 160ms.

3.2.2. Results.

Figure 1 shows the results obtained in Experiment 1. LSD post-hoc tests were used to explore the differences yielded by the MLM. Figures 1A and 1B display the results for

Consumption. A 2 (Genotype) × 2 (Sex) × 6 (Day) MLM analysis revealed a significant effect of Day [$F(5, 184.772) = 44.388$, $p < .001$] and the interaction Sex × Day [$F(5,184.772) = 3.113$, p = .010] but not the interaction Genotype × Day [F(5,184.772) = 1.182, p = .320] or Genotype × Sex × Day [$F(5,184.772) = .953$, p = .448]. Analysis of the Day effect revealed that water baseline intake was higher than any of the other days (Baseline vs. All: $p < .001$), which means that cider vinegar consumption never reached the previous water consumption levels. By contrast, Vin1 consumption was significantly lower than any other (Vin1 vs. All: $p < .001$). This indicates flavour neophobia. However, this neophobia could be considered partially attenuated in the second exposure (Vin2), since there were no differences in consumption between Vin2 and Vin3 ($p = 0.362$), but consumption increased gradually during the following exposures, with significant differences between Vin2 and Vin6 ($p = 0.012$). There were no differences in consumption between Vin3, Vin4 or Vin6 ($p > .05$), suggesting that neophobia was completely attenuated at the third exposure. However, the analysis of the interaction Sex × Day indicated that females exhibited complete AN but males did not. Males had significantly reduced consumption on Vin1 in comparison with Baseline (p < .001), indicating flavour neophobia. Indeed, AN was not complete since baseline consumption was significantly higher than any other day (p < .001). On the other hand, Vin1 consumption was lower than any other day (p < .001). A significantly higher consumption on Vin2 after the flavour neophobia can be interpreted as AN. Vin2, Vin3 and Vin4 did not yield significant differences between them, but Vin 2 and Vin 3 still maintained differences with Vin6 ($p = .041$ and $p = .018$, respectively), whilst Vin4 and Vin6 had no differences. In contrast, females exhibited complete AN as baseline consumption was higher than that of Vin1 (p < .001) and Vin2 (p = .008), indicating neophobia but it did not differ for the rest of the days. In addition, Vin1 consumption was significantly lower

than any other day ($p < .001$). Vin2 did not yield any other significant difference. The comparison between the consumption of males and females each day yielded significant differences only on Baseline, as the intake of males was higher than females.

Regarding LCS results, they are shown in Figures 1C and 1D. The 2 (Group) × 2 (Sex) × 6 (Day) MLM showed a significant effect of Day [$F(5,181.282) = 13.339$, $p < .001$] and Sex [$F(1,38.223) = 5.700$, $p = .022$]. No effects of Genotype [$F(1,38.223) = .002$, $p = .963$], Genotype*Sex [$F(1,38.223) = .532$, $p = .470$] or the interactions Genotype × Day [$F(5,181.282) = 1.222$, $p = .300$], Sex × Day [$F(5,181.282) = 1.131$, $p = .345$] or Genotype × Sex × Day [$F(5,181.282) = .517$, $p = .763$] were found. Females displayed higher LCS than Males presumably indicating increased palatability of the cider vinegar solution although other factors cannot be excluded. Analysis of the Day effect revealed higher LCS on the Baseline day than on Vin1 ($p < .001$) and lower LCS on Vin1 day than on any other day consistent with reduced palatability to the novel solution. Baseline LCS were also higher than Vin2 ($p < .001$) and Vin3 ($p = .004$), but there were no differences with Vin4 ($p < .094$) or Vin6 ($p < .537$). This indicated reduced palatability to a neophobic vinegar solution which recovers completely reaching the level of water baseline when the flavour becomes familiar and safe after 6 exposures. The process involves a progressive increase of LCS along the repeated exposures concerning Vin1 (Vin2: $p = .002$; Vin3, Vin4, Vin6: $p < .001$). In fact, animals displayed a significantly higher LCS on Vin2 than on Vin1, but Vin2 had no differences with Vin3 ($p = .332$). Nevertheless, Vin2 still being significantly lower than Vin4 ($p = .031$) and Vin6 ($p = .002$). In the same way, Vin3 had no differences with Vin4 ($p = .227$), but LCS on this day was lower than on Vin6 ($p = .028$). There were no differences between Vin4 and Vin6 ($p = .305$). Hence, this process parallels that of consumption if we take into

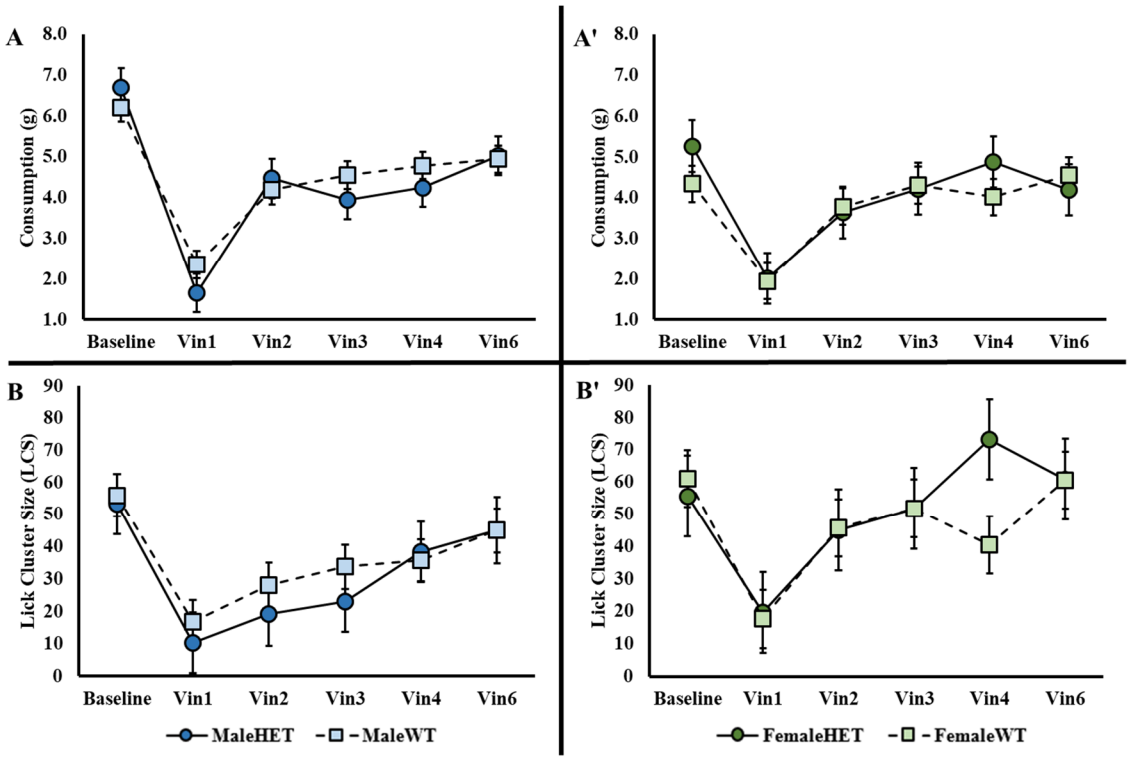

Figure 1. Mean consumption (± SEM; A and B) and Licks per Bout (± SEM; C and D) of each group.

account the analysis of the Day effect although it did not reach the previous baseline level in the case of males.

3.2.3. Discussion

The results of Experiment 1 indicate sex differences in AN both in consumption and palatability measured using LCS. Increased palatability in females in comparison with males is in accordance with previous reports using sucrose (Curtis et al., 2004), thus extending this finding to the acid vinegar taste which has not been tested before using this method. However, the sex differences in consumption found are not consistent with previous reports that did not find significant differences using the same flavoured solution (Expósito et al., 2023). The results of consumption reveal that repeated vinegar exposures induced complete AN in females but not in males while LCS results show that palatability returned progressively to the previous baseline level in both sexes. Nevertheless, it is interesting to emphasize the differences comparing LCS data and consumption. While

LCS in both sexes increased progressively along the exposures, consumption raised abruptly after one exposure reaching the asymptote even if only females exhibit complete AN. No differences were found between CACNA1C heterozygous and wild-type groups. Taken together, the results provide evidence indicating sex differences and support that AN induces not only increased consumption but increased palatability of the familiar flavour.

3.3. Experiment 2: Taste Reactivity Test and voluntary intake test

The results of Experiment 1 support that repeated exposure to a novel vinegar solution increased its palatability. However, prior findings with TRT have been inconsistent; although consumption increased suggesting AN, there were no observed increased appetitive responses following five exposures to saccharin (Neath et al., 2010). Given that Neath et al. (2010) used a sweet taste solution and that used in Experiment 1 was an acid solution with an olfactory component, in Experiment 2 we applied TRT using the same vinegar solution to assess potential palatability changes induced by AN. Male and female groups were included since sex differences in taste reactivity have been found using TRT (Clarke & Ossenkopp, 1998; Flynn et al., 1993). Also, control groups exposed to water were added in order to precisely assess the AN process.

3.3.1. Method

Subjects. Forty-seven adult Sprague-Dawley rats (ENVIGO, Netherlands) (Males: n = 23, ± 300g; Females: n = 24, ±210g) were used. They were housed in standard home cages (38 × 56 × 22cm) in groups of 3-4 animals per cage. After surgery, all animals were randomly assigned to a group depending on the solution infused during TRT either a 3% cider vinegar solution (Vin group) or plain water (Water group). So, there were a total of 4 groups: MaleVin (n = 12), MaleWater (n = 11), FemaleVin (n = 12) and FemaleWater

(n = 12). Rats were housed in the animal facilities located in the Biomedical Research Center (CIBM) of the University of Granada (Spain) under a 12h/12h light/dark cycle, taking place all manipulations during the light cycle. They had ad libitum water and food until the experiment started and received 3-min daily handling sessions for the previous 4 days to the surgery. The procedures were approved by the University of Granada Ethics Committee for Animal Research and by the Regional Ministry of Agriculture, Fisheries and Rural Development of Andalusia (1/06/2022/078).

Surgery. Animals were surgically implanted with an intraoral cannula. First, they were anaesthetized using ketamine (50mg/kg, i.p.) combined with medetomidine clorhydrate (0.15mg/kg, i.p.). In order to implant the cannula a thin-walled 14-gauge stainless steel needle was inserted at the back of the neck, directly subcutaneously around the ear and brought out behind the first molar inside the mouth. A length of polyethylene tubing with an inner diameter of 0.86 mm and an outer diameter of 1.27 mm was then run through the needle after which the needle was removed. The tubing was secured with a rubber disc placed over and drawn to the exposed skin at the back of the neck, to stabilize the cannula. The tubing was held secure in the oral cavity by an O-ring and a rubber stopper, which were sealed behind the tubing prior to the cannulation surgery. Finally, the animal received subcutaneously atipamezol (an anesthesia-reverser drug), buprenorphine (an anti-inflammatory drug), enrofloxacine (an antibiotic drug) and 5% glucose (nutrients reposition). The animals were returned to an individual home-cage (43 × 27 × 19cm) and the solid food was removed until the next day. Then, they were recovering for 3 days more, receiving each day analgesic and antibiotic subcutaneous injections and cleaning the cannulae with plain water.

Equipment. The test was carried out in a dark room, lit with two manipulable spotlights that allowed control of the light conditions for recording. Two transparent

methacrylate equipment were used for video recording TRT. Each of these was formed by a methacrylate holder structure (28 × 24 × 55cm) and a methacrylate box (27 × 24 × 21cm) with a pierced removable lid. A webcam plugged into a laptop was under the box, recording it from the bottom. For fluid infusion, the cannula was connected to the infusion pump by slipping the tubing of the cannula inside a second polyethylene tubing (inner diameter 1.19 mm; outer diameter 1.70 mm) attached to a model 55-2226 infusion pump (Harvard Apparatus, United States).

Procedure. The procedures were carried out in the Animal Behaviour Analysis Unit of the Biomedical Research Center (CIBM) of the University of Granada. Three days after surgery, and upon full recovery of animals from surgery, testing began. Table 1 describes the behavioural procedure applied. On Day 1 (Baseline day) habituation to the TRT chamber and infusion procedure took place. All groups received a 5 ml/min intraoral infusion of water for 5 min (Pre-Water). From Day 2 to Day 7, the rats were infused at the same rate (5ml/min, for 5min) with 3% cider vinegar solution or tap water depending on the group they belonged to. On Day 8, all groups received a water session again (Post-water). In the afternoon they were water-deprived to start voluntary access in their own individual homecages during the following daily 15 min morning sessions (11:00 am) and they had free access to water in the afternoon (4:00 pm) for 30 min (rehydration session). Solutions were available in pre-weighed bottles weighed again after the session. After establishing the baseline for three days in which water was available during the experimental sessions animals had access to a 3% cider vinegar solution instead of water for the following 3 days.

	D1 TRT	D2 TRT	D3 TRT	D4 TRT	D5 TRT	D6 TRT	D8 TRT	D9-11 Water Deprivation	D12 VI	D13 VI	D14 VI
Vin Group	Wat	Vin1	Vin2	Vin3	Vin4	Vin5	Vin6	Wat	Vin7	Vin8	Vin9
Water Group	Wat	Wat	Wat	Wat	Wat	Wat	Wat	Wat	Vin1	Vin2	Vin3

Table 1. Experimental design of Experiment 2. Shadowed cells highlights the days when the groups had vinegar exposures. D = Day; TRT = Taste Reactivity Test; VI = Voluntary Intake; Wat = Water; Vin = Vinegar.

Data analysis. TRT videos were analyzed using BORIS software (Friard & Gamba, 2016). Behaviours were coded and a researcher assessed each video individually. Behaviours were coded and a researcher assessed each video individually. A researcher blind to the experimental procedures analyzed some of the videos to confirm that the criteria used were accurate. Once all videos were analyzed, data were organized and analyzed. Mixed analyses of variance (ANOVAs) were applied to the results obtained with TRT and consumption with the between-group factors Group (Vin vs. Water) and Sex (Male vs. Female) and Day (PreWater, Day1-Day6, PostWater) as within-subject factor. Thus, were carried out three 2 (Group) × 2 (Sex) × 8 (Day) mixed ANOVAs for analyzing each TRT behavioural category (aversive, appetitive, and passive dripping) and a 2 (Group) × 2 (Sex) × 6 (Day) mixed ANOVA for analyzing consumption. All analyses were carried out using SPSS (IBM, United States). All tests reported used a criterion of significance of $p < .05$.

3.3.2. Results

Figure 2 shows the results obtained in Experiment 2. When the assumption of sphericity was violated (Mauchly's sphericity test: $p < .05$), Greenhouse-Geisser corrections were used. LSD post-hoc tests based on the marginal means were used to explore the differences yielded by the mixed ANOVAs.

Concerning the TRT results, Figures 2A and 2A' present the mean number of aversive reactions of each group in the daily 5-min TRT session for Males and Females, respectively. A mixed 2 (Group) × 2 (Sex) × 8 (Day) ANOVA yielded significant effects of Day [$F(7, 301) = 5.136$, $p < .001$, $\eta_p^2 = .107$] and Day × Sex [$F(7,301) = 2.216$, $p = .033$, $\eta_p^2 = .049$], but not Group [$F(1, 43) = 3.200$, $p = .081$, $\eta_p^2 = .069$], Sex [$F(1, 43) = 1.842$, $p = .182$, $\eta_p^2 = .041$], Group × Sex [$F(1, 43) = 1.174$, $p = .285$, $\eta_p^2 = .027$], Day × Group [$F(7, 301) = .904$, $p = .503$, $\eta_p^2 = .021$] or Day × Group × Sex [$F(7, 301) = .785$, $p = .600$, $\eta_p^2 = .018$]. When the interaction Day × Sex effect was analyzed, it was confirmed a significant Day effect ($p < .001$) in Females but not in Males ($p = .053$). Females increased the number of aversive reactions when exposed to the novel vinegar flavour (PreWater vs. Vin1: $p = .011$) that decreased as the flavour became familiar to reach the level of response to water (Vin1 vs. Vin4-6 and PostWater: $p < .001$; Vin2 vs. Vin 4-6 and PostWater: $p < 0.016$; Vin3 vs. Vin5-6: $p < 0.045$). No effect of novelty or familiarity was observed in Males. Attending each day, Females displayed a significantly higher number of aversive reactions on PreWater ($p = 0.023$) and on Vin1 ($p = .032$).

Figures 2B and 2B' show the mean number of appetitive reactions. A mixed 2 (Group) × 2 (Sex) × 8 (Day) ANOVA was carried out. There were significant effects of Group [$F(1, 43) = 4.149$, $p = .048$, $\eta_p^2 = .088$] as the group exposed to the vinegar solution displayed a higher number of appetitive reactions than the group exposed to water and Sex, [$F(1, 43) = 13.747$, $p = .001$, $\eta_p^2 = .242$], since males displayed a higher number of appetitive reactions than females. There were no significant effects of Day [$F(5.108, 219.652) = .831$, $p = .531$, $\eta_p^2 = .019$], Day × Group [$F(5.108, 219.652) = 2.046$, $p = .072$, $\eta_p^2 = .045$], Day × Sex [$F(5.108, 219.652) = 1.026$, $p = .404$, $\eta_p^2 = .023$] or Day × Group × Sex [$F(5.108, 219.652) = .644$, $p = .669$, $\eta_p^2 = .015$]. Thus no effect of novelty or familiarity was found on appetitive responses.

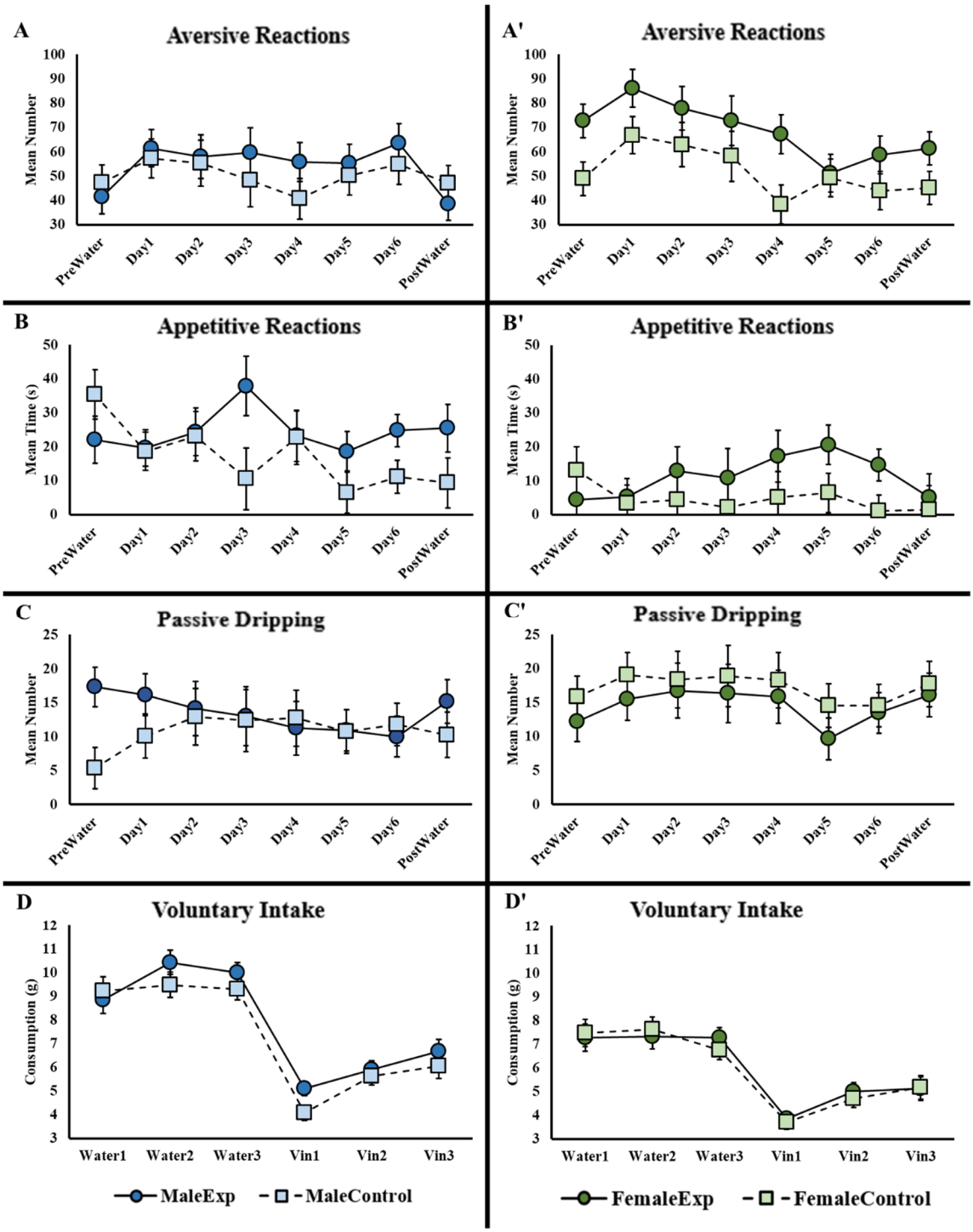

Figure 2. Mean number (± SEM) of aversive (A, A'), appetitive (B, B') and passive dripping (C, C') reactions along TRT for each group; mean consumption (± SEM; D, D') during the baseline (Water1-3) and experimental (Vin1-Vin3) sessions during Voluntary Intake test for each group. Graphs use different scales.

Figures 2C and 2C' present the mean number of neutral passive dripping reactions of each group. The 2 (Group) × 2 (Sex) × 8 (Day) mixed ANOVA did not yield

significant effects including Group [$F(1, 42) < .001$, $p = .995$, $\eta p2 < .001$], Sex [$F(1, 42) = 1.839$, Day [$F(4.773, 200.480) = 1.558$, $p = .177$, $\eta p2 < .036$], Day × Group [$F(4.773, 200.480) = .790$, $p = .553$, $\eta p2 = .018$], Day × Sex [$F(4.773, 200.480) = .263$, $p = .927$, $\eta p2 = .006$], Day × Group × Sex [$F(4.773, 200.480) = 1.127$, $p = .347$, $\eta p2 = .026$]; $p = .182$, $\eta p2 = .042$], Group × Sex [$F(1, 42) = .990$, $p = .325$, $\eta p2 = .023$]. This confirms the neutral nature of passive dripping.

Finally, Figures 2D and 2D' show the consumption of each group during the voluntary intake test carried out after the TRT sessions including 3 water days and 3 vinegar exposures. A 2 (Group) × 2 (Sex) × 6 (Day) mixed ANOVA yielded significant effects of Day [$F(5, 215) = 88.956$, $p < .001$, $\eta p2 = .674$], Sex [$F(1, 43) = 33.971$, $p < .001$, $\eta p2 = .441$] and the interaction Day × Sex [$F(5, 215) = 3.753$, $p = .003$, $\eta p2 = .080$]. There were no significant effects of Group [$F(1, 43) = 1.172$, $p = .285$, $\eta p2 = .027$], Day × Group [$F(5, 215) = .638$, $p = .671$, $\eta p2 = .015$], Group × Sex [$F(1, 43) = .700$, $p = .407$, $\eta p2 = .016$] or Day × Group × Sex [$F(5, 215) = .492$, $p = .782$, $\eta p2 = .011$]. The Sex effects are due to the fact that Males had an average consumption higher than Females ($p < .001$). The analysis of the interaction Day × Sex yielded a Day effect for both Males ($p < .001$) and Females ($p < .001$). For Males, Water3 consumption was significantly higher than any of the Vinegar days (Water3 vs. Vin1-3: $p < .001$). A higher consumption on Water3 than in Vin1 means flavour neophobia. On Vin2, consumption was significantly higher than on Vin1, indicating AN, whilst there were no differences between Vin2 and Vin3. In the case of the Females, similar results to Males are obtained on Water3 consumption: higher consumption than in Vinegar days (vs. Vin1-3: $p < .001$), and Vin1 had a significantly lower consumption than the following days (vs. Vin2-3: $p < .001$), so there was flavour neophobia; a greater consumption on Vin2 reflects AN, whilst there

were no differences with Vin3. Comparing Males and Females on each Day, Males had a significantly higher consumption on all days ($p < .022$).

3.3.3. Discussion

The results of Experiment 2 using intraoral infusions and TRT in addition to consumption indicated that the intraoral infusion did not reduce flavour neophobia. Both the groups exposed to vinegar and those exposed to water during the previous TRT sessions decreased vinegar intake during the first voluntary drinking session. This suggests that voluntary intake might have a relevant role in identifying and processing flavours. In addition, the results provided evidence indicating sex differences in palatability indexes. While both males and females reduced consumption of the novel vinegar solution which was enhanced as the flavour became familiar, no TRT changes selectively associated with neophobia or AN were found. Females exhibited increased aversive reactions associated with drinking either water or vinegar solution. These aversive reactions decreased as they were habituated to the drinking sessions over the days. We did not find, however, changes in the number of aversive reactions related to AN in males. This is consistent with previous studies performed only in male rats that reported no TRT changes to saccharin solutions across repeated exposures (Neath et al., 2010). Higher reactivity in females in comparison with males has also been previously found (Clarke & Ossenkopp, 1998; Flynn et al., 1993). Our results point to non-selective reactivity to the procedure in females that it does not allow us to confirm our hypothesis regarding palatability changes associated with AN.

With respect to the appetitive response, our hypothesis has to be rejected because the changes in appetitive responses do not seem to reflect vinegar neophobia and its attenuation whatever the sex. Despite this, the fact that vinegar triggers a higher number of responses than water suggests that flavour exposure is appetitive. Besides,

females displayed a lower number of appetitive responses than males which is also opposite to previous reports using sucrose, NaCl and quinine (Clarke & Ossenkopp, 1998; Flynn et al., 1993). Finally, as expected, there were no changes in passive dripping indicating that it is a neutral control measure.

3.4. General discussion

Two main findings should be highlighted. First, MLS but not TRT was sensitive to changes induced by taste neophobia and its attenuation. Second, sex differences should be taken into account for assessing the effects of taste novelty and familiarity.

Concerning palatability, the results obtained using MLS are consistent with previous reports using different tastes. In accordance with the results of Lin, Amodeo et al. (2012) who reported increased LCS in male rats after four saccharin and quinine exposures, we found reduced LCS in response to the novel vinegar solution which increases after four exposures reaching the asymptote both in males and females. Thus, our results extend those of Lin, Amodeo et al. (2012) to acid tastes and both sexes. Regarding TRT, our results are also consistent with the negative results reported by Neath et al. (2010) in male rats using saccharin solutions as we did not find hedonic changes induced by vinegar novelty or familiarity in males even though consumption indicated neophobia and AN. In fact, no effects were found in appetitive or aversive orofacial reactions, thus extending Neath et al. (2010) findings to the acid taste in male rats. In accordance, female rats did not show hedonic changes selectively associated with flavour neophobia and AN. The results, however, are more complex than those of males. Female rats increased TRT aversive reactions to both vinegar solution and water. These were reduced progressively presumably as they habituated to the infusion procedure. They also exhibited lower appetitive responses than males. It is conceivable that TRT results reflect that females are more sensitive than males to aversive situations such as intraoral infusion.

This would be also consistent with the higher reactivity described in females (Clarke & Ossenkopp, 1998; Flynn et al., 1993).

It is interesting the discrepancy in the sensitivity of the methods to assess palatability using different behavioural procedures in male rats. We found MLS to be more sensitive than TRT to assess the potential hedonic changes involved in AN while TRT has been reported to be more sensitive to assess the hedonic changes involved in flavour preferences (Riordan & Dwyer, 2019). It cannot be discarded as a contribution of the voluntary consumption effects. In fact, in flavour preferences intake is high potentially leading to ceiling effects for MLS. Alternatively, the processes involved could be different. It can be proposed that exposures without consequences do not involve learning flavour preferences but learning that the flavour is safe. Thus, a novel flavour might have mild aversive properties that disappear as it becomes familiar but preferences associated with appetitive responses are not necessarily learned. This would be congruent with the fact that our results indicated that only aversive but not appetitive responses were influenced by novelty and AN in females. Also, the absence of differences between the results of CACNA1C and wild-type rats stands against the appetitive nature of the changes induced by AN. Accordingly, CACNA1C has been related selectively to appetitive reactions to palatable solutions but not to aversive responses.

In spite of the fact that AN shares with CTA extinction increased consumption along the trials, the pattern of results of Experiment 1 do not seem to parallel those obtained during extinction. In our experiment consumption was abruptly recovered and reached the asymptote in the third day being AN complete in females at the end of the procedure. LCS, however, increased gradually reaching the baseline levels both in males and females. This pattern is different to that described during CTA extinction. Dwyer (2009) reported a gradual increase in consumption never reaching the asymptote,

while LCS changes were faster and larger (Dwyer, 2009; Dwyer et al., 2013). The different pattern of consumption together with the fact that the procedure selectively changed aversive TRT responses while extinction did affect both aversive and appetitive reactions point to different hedonic processes involved. It is conceivable that during CTA extinction a change in flavour labelling from unsafe to safe took place while AN would turn the flavour from unknown to safe. The first would require overcoming previous aversive experiences while the second only would require assessing the effect of a non-familiar flavour.

An additional issue concerns the relevance of voluntary consumption in order to induce AN in Experiment 2. Despite six previous forced exposures throughout the intraoral cannula during TRT assessment, both males and females exhibited a similar vinegar neophobia in the first consumption test in comparison with non-exposed groups. This contrasts with the results of Neath et al. (2010) using saccharin solutions that found increased consumption in the groups previously exposed during TRT in comparison with those non-exposed. It cannot be discarded that this discrepancy could be due to differences in the tastes and procedures used. Besides the saccharin solution, they used shorter TRT sessions (10s) and no water baseline stabilization period while we applied longer TRT sessions (5min) and 3 days of habituation to the water deprivation schedule before the voluntary consumption assessment. Nonetheless, our results seem to suggest that voluntary drinking plays a crucial role in evaluating the consequences of ingesting novel tastes. Thus, voluntary drinking would be required for AN to take place. Considering previous results obtained with latent inhibition and CTA (Dwyer et al., 2013; López et al., 2010) it can be conceived that the current procedure did not support generalization between intraoral infusion and voluntary consumption. The described aversive effect of intraoral infusion can have been relevant. In addition, the role of the

context change from the intraoral infusion phase to the voluntary consumption in their homecages cannot be discarded. It has been previously demonstrated that a context shift can modulate neophobia and its attenuation (De la Casa & Díaz, 2013; Grau-Perales, Levy, et al., 2019).

A second critical issue is the relevance of sex as a relevant variable in our study. This is to our knowledge the first assessment of palatability changes associated with AN in female rats. Females showed higher reactivity than males in palatability tests, both for appetitive and aversive reactions. They exhibit greater LCS than males in response to the familiar vinegar solution in Experiment 1. In Experiment 2 they exhibited changes in the aversive reactions during the TRT sessions while males did not. High female reactivity to sweet, salty and sour tastes has been previously found in assessing the licking rate (Curtis et al., 2004) and TRT (Clarke & Ossenkopp, 1998; Flynn et al., 1993). Our results extend these findings to the acid taste.

A discrepancy between previous TRT reports and our results, however, merits discussion. While it has been reported that increased female appetitive orofacial responses to sucrose, quinine (Clarke & Ossenkopp, 1998) and low NaCl concentration (Flynn et al., 1993) solutions, in the Experiment 2 females displayed less appetitive reactions than males. They were, however, not related to flavour neophobia or AN in any sex. It cannot be discarded that the appetitive reactions could have been overshadowed in females by the high number of aversive reactions. Further research is needed at this point, in order to determine the processes involved in AN and the impact of sex differences.

Moreover, sex differences in consumption are found in Experiment 1 since AN after six vinegar exposures is complete in females but not in males. The difference can be attributed to differences in consumption during the baseline since the consumption pattern during the flavour solution exposure is similar in both sexes. Previous results using the

same vinegar solution and a similar procedure did not find sex differences (Expósito et al., 2023). The reason might lie in the number of subjects in each sex group which was higher in the present study. This could unveil unnoticed sex differences.

In all, the present study attempts to provide a comprehensive view of the processes involved in taste novelty and its attenuation including both consummatory and hedonic analyses of behaviour. Particularly, the combined assessment of the licking microstructure and taste orofacial reactions contributes to solving discrepancies that arise using only one palatability test. Understanding the nature of wanting and liking, and how these processes can be studied in the laboratory, is fundamental in both non-pathological and pathological contexts. This is particularly relevant for facing pathological disorders such as drug addiction or mental disorders that lead to anhedonia.

Capítulo 4

Increased basolateral amygdala metabolic activity during flavour familiarization

Este capítulo corresponde al artículo publicado *Menchén-Márquez S, Banqueri M, Gómez-Chacón B, Arias JL, Gallo M. (2023) Increased basolateral amygdala metabolic activity during flavor familiarization: an experimental study. Behav Brain Funct 1;19(1):1–6. DOI: 10.1186/s12993-023-00206-x*

Abstract

Novel flavors elicit a cautious neophobic response which is attenuated as the flavor becomes familiar and safe. The attenuation of neophobia reveals the formation of a safe memory. Previous lesion studies in rats have reported that basolateral amygdala integrity is required for taste neophobia, but not neophobia to flavor, i.e., taste linked to an odorous component. Accordingly, immunohistochemical analyses show that novel tastes induced higher basolateral amygdala activity when compared to familiar ones. However, a different role of the basolateral amygdala in flavour attenuation of neophobia is suggested by lesion studies using a vinegar solution. Studies assessing basolateral amygdala activity during flavour attenuation of neophobia are lacking. Thus, we quantified cytochrome oxidase as an index of basolateral amygdala activity along the first and second vinegar exposures to assess flavour neophobia and attenuation of neophobia. We exposed adult male Wistar rats either once or twice to a 3% cider vinegar solution or water and compared the basolateral amygdala, piriform cortex and caudate putamen brain metabolic activity using cytochrome c-oxidase histochemistry. We found increased flavour intake and cytochrome c-oxidase histochemistry activity during the second exposure in the basolateral amygdala, but not in the piriform cortex and caudate/putamen. The main finding of the study is that basolateral amygdala metabolic activity was higher in the group exposed to a familiar vinegar solution than in the groups exposed to either water or a novel vinegar solution.

4.1. Introduction.

The ability to recognize familiar flavours which have been safe in previous encounters is critical for diet selection and survival. A novel flavour induces a neophobic response that is attenuated in a second exposure to the flavour without negative consequences, thus leading to increased consumption. The attenuation of neophobia (AN) requires the integrity of the perirhinal cortex (PRh), a brain area involved in taste and object recognition memory. Accordingly, PRh excitotoxic (Morillas et al., 2017) and lidocaine-reversible lesions (Ramos, 2020b) interfere with the increased consumption during the second presentation of vinegar and saccharin solutions, respectively. We have also previously reported a similar impairment induced by basolateral amygdala (BLA) excitotoxic lesions. These lesions disrupt the pattern of PRh activity associated with AN during the second exposure to the vinegar solution (Gómez-Chacón et al., 2012). Nonetheless, the critical mechanism disrupted is not yet known.

There is evidence supporting the BLA involvement in taste novelty detection. In fact, BLA permanent and reversible lesions have been found to interfere with neophobia to a 0.5% sodium saccharin solution (Lin et al., 2009, 2018) and c-Fos expression induced in BLA by a novel saccharin solution is higher than that induced by a familiar one (Lin, Roman, et al., 2012). However, Lin and colleagues (2009) did not find any effect of BLA lesions on odour neophobia using a 0.1% amyl acetate solution. This is a relevant issue since taste is rarely found isolated under natural conditions, but it is associated with other components, such as odour, becoming more appropriate to use the term "flavour". Indeed, using a cider vinegar solution, a flavour which contains an acetic acid odorous component, we have reported increased activity during the second exposure in the nucleus accumbens shell (Grau-Perales, Gómez-Chacón, & Gallo, 2019) and the medial

prefrontal cortex (Expósito et al., 2020). However, there is no data about the role of BLA in the vinegar familiarization process.

Cytochrome c oxidase (CCO) is a marker of neural activity that indicates ATP increases and decreases according to the oxidative metabolic activity requirements. Thus, unlike other markers, CCO allows us to detect not only increases but also decreases in brain activity. This technique has been previously applied to assess changes in the brain metabolic activity associated with taste familiarity on aversive conditioning using a latent inhibition procedure (Gasalla et al., 2016). Likewise, CCO histochemistry has proven to be of great value in mapping functional brain networks involved in spatial learning acquisition (Conejo et al., 2010; Méndez-Couz et al., 2016), retrieval (Conejo et al., 2013) and extinction (Méndez-Couz et al., 2016). It is also sensitive to the effect of various treatments on spatial memory, such as social isolation (Zorzo et al., 2019), maternal separation (Banqueri et al., 2018), exercise (Sampedro-Piquero et al., 2013) and pharmacological interventions (Hescham et al., 2014).

Hence, CCO quantification during the first and second vinegar exposures would allow us to assess the potential BLA involvement either in the detection of flavour novelty or flavour familiarization. Increases in the BLA metabolic activity during the first flavour exposure would support a role in novelty detection. This would be consistent with the results found using a pure taste solution. On the other hand, increases in the BLA metabolic activity during the second flavour exposure would point to a role in flavour familiarization. This would be in accordance with the reported AN disruption by BLA lesions. Given the odorous component of the flavour used, the metabolic activity of the olfactory piriform cortex (Pir), as well as caudate/putamen (Cpu) as additional control areas, was assessed. No sex differences have been reported either in vinegar neophobia or AN using the present behavioural procedure (Expósito et al., 2023). Hence, male rats

were used to allow comparison with previous results on brain activity, thus reducing the number of animals used. Additionally, the use of males avoids mildly invasive procedures required for assessing the estrus cycle.

4.2. Method

4.2.1. Behavioural procedure

Twenty-one naive adult male Wistar rats were individually housed and maintained in a 12-hour-light-dark cycle (8:00-20:00 h). Food was available *ad libitum* but access to water was restricted to the daily experimental 15-min drinking sessions at 10:00 h and to a daily additional 20-min rehydration session at 16:00 h. Water Baseline (BL) was recorded during the morning sessions in which the rats were handled for 3-5 minutes after BL1, BL3 and BL5 in order to avoid stress. After the water intake baseline, the animals were randomly assigned to one of the following groups: Novel (n = 8), Familiar (n =5) and Control (n = 8). A cider vinegar solution (3%) was available during one (Novel) or two (Familiar) experimental sessions. The control group drank water throughout the experiment.

Ninety minutes after the experimental session each animal was sacrificed, the brain was removed and quickly frozen in isopentane (2-methylbutane; Sigma-Aldrich, Germany) to be stored at -80 ºC. Brain coronal sections (20 µm) were cut in a cryostat (Microm, HM505E, Germany). From each brain, sixty sections were taken in order to assess CCO in BLA, Cpu and Pir. The stereotaxic coordinates of the brain areas assessed according to the Paxinos and Watson atlas (Paxinos & Watson, 2013) are shown in Figure 1.

The procedures were approved by the University of Granada Ethics Committee for Animal Research and by the Regional Ministry of Agriculture, Fisheries and Rural

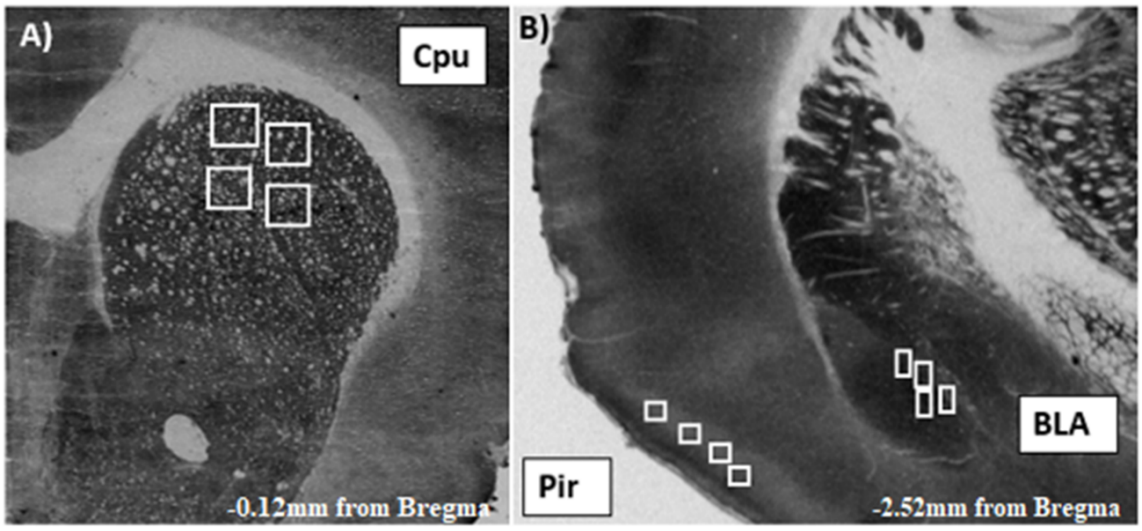

Figure 1. Anatomical location of Regions of Interest. BLA = Basolateral amygdala (-2.52 mm), Cpu = Caudate/Putamen (-0.12 mm), Pir = Piriform cortex (-2.52 mm). Anteroposterior coordinates according to bregma (Paxinos & Watson, 2013). All coordinates were taken from coronal brain slices.

Development of Andalusia (17-02-15-195), following the ARRIVE guidelines and in accordance with the EU Directive 2010/63/EU for animal experiments.

4.2.2. CCO histochemistry

The procedure for quantitative CCO histochemistry has been described elsewhere(Gonzalez-Lima & Cada, 1994; Zorzo et al., 2019). In brief, after obtaining sets of tissue homogenate standards from the Wistar rat at different thicknesses (10, 30, 50 and 70 µm) in order to quantify the enzymatic activity, both sections and standards were incubated for 5 minutes in 0.1 M phosphate buffer (7.6 pH) with 10% sucrose (w/v) and 5% glutaraldehyde (v/v) (Merck, Germany). The slides were then rinsed 3x in a 0.01 M phosphate buffer (7.6 pH) with 10% sucrose (w/v) and 0.05 M Tris buffer (7.6 pH) with 275 mg/L hexahydrated cobalt chloride, 10% sucrose (w/v), and 0.5% dimethylsulfoxide (v/v) for 10min. This was followed by 1x0.01 M phosphate buffer (7.6 pH). Then, the sections and standards were incubated in 0.0075% cytochrome-c (w/v), 0.002% catalase (v/v), 5% sucrose (w/v), 0.25% dimethylsulfoxide (v/v) and 0.05% diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Madrid, Spain). Both sections and standards were

incubated for 5 minutes in 0.1 phosphate buffer (7.6 pH), at 37 ºC for 1 hour. The reaction was stopped by fixing the tissue in buffered 4% (v/v) formalin for 30 min. Finally, the slides were dehydrated, cleared with xylol and cover-slipped with Entellan (Merck, Germany). The intensity of the CCO staining was quantified through an optic densitometry analysis using a computerized image analysis system (MCID Elite, Interfocus Imaging Ltd., United Kingdom). The mean optical density (OD) of each region was measured in the right hemisphere using three consecutive sections. In each section, four non-overlapping readings were taken using a square-shaped sampling window that was adjusted for each region size, taking a total of 12 measurements per region and subject. These regions were averaged to obtain one mean per region for each animal. Then, OD values were converted to CCO activity units determined through the enzymatic activity of the standards measured spectrometrically.

4.2.3. Experimental design and statistical analyses.

Flavour neophobia and AN were assessed using a two-factor mixed ANOVA design that includes a between-group factor *Group* with 2 levels (*Control* group drinking water; *Familiar* group drinking vinegar twice) and a within-subjects factor *Days* with 3 levels (Water baseline, first vinegar exposure, second vinegar exposure), being the rat ID the random factor (animals were randomly assigned to groups). Post-hoc Bonferroni tests were applied.

Given the need to sacrifice the animals to assess brain CCO activity, an additional Novel group was required. Thus, a two-factor mixed ANOVA design was applied including a between-groups factor *Group* with 3 levels (Control group drinking water; Novel group drinking vinegar once, Familiar group drinking vinegar twice) and a within-subjects factor *Region of Interest (ROI)* with 3 levels (BLA, Cpu, Pir). Again, since

animals were randomly assigned to the groups, rat ID was the random factor. Post-hoc comparisons were performed with post-hoc Bonferroni tests.

4.3. Results

Figure 2 shows the mean (± SEM) intake of the Familiar group drinking the 3% cider vinegar solution that evidences the neophobic response and its attenuation on the second presentation in comparison with the Control group drinking water. A mixed 2 (Group) × 3 (Days) ANOVA analysis showed a within-subject Day effect [$F(2, 18) = 41.121$, $p = .001$, $\eta_p^2 = .820$] and interaction of Group × Day [$F(2, 18) = 61.407$, $p < .001$, $\eta_p^2 = .872$]. A Group effect was found too [$F(1, 9) = 23.982$, $p < .002$, $\eta_p^2 = .727$]. Levene's test showed homogeneity of variances for all within-subject variables. Post-hoc Bonferroni tests indicated that Group interaction is due to the Familiar group drank a lower amount of the novel vinegar solution on Day1 than both the water (Baseline) ($p < .001$) and the familiar vinegar solution on Day2 ($p < .03$). No differences were found in the Control group ($p > .05$).

Regarding the CCO results, Figure 3 shows the mean (± SEM) CCO units in each region of interest of the three groups (*Control, Novel and Familiar*). A mixed 3 (Group) × 3 (ROI) ANOVA analysis was carried out. Levene's tests showed homogeneity of ROI, [$F(2,36) = 38.858$, $p < .001$, $\eta_p^2 = .179$]. Neither the interaction Group × ROI [$F(4, 36) = 1.964$, $p > .05$, $\eta_p^2 = .683$] nor the main factor Group [$F(2, 18) = 3.235$, $p > .05$, $\eta_p^2 = .264$] were significant. Post-hoc Bonferroni tests showed higher metabolic activity in BLA than Pir ($p < .001$) and Cpu ($p < .01$), being Pir activity higher than Cpu ($p < .005$). Although the interaction was not significant, one-way ANOVA analyses were applied to each ROI because testing our hypotheses required gaining knowledge about the difference between groups in BLA. They indicated significant differences only in BLA [$F(2,18) = 5.365$, $p < .05$, $\eta_p^2 = .373$], but not in Pir [$F(2,18) = 1.374$, $p > .05$, $\eta_p^2 = .132$]

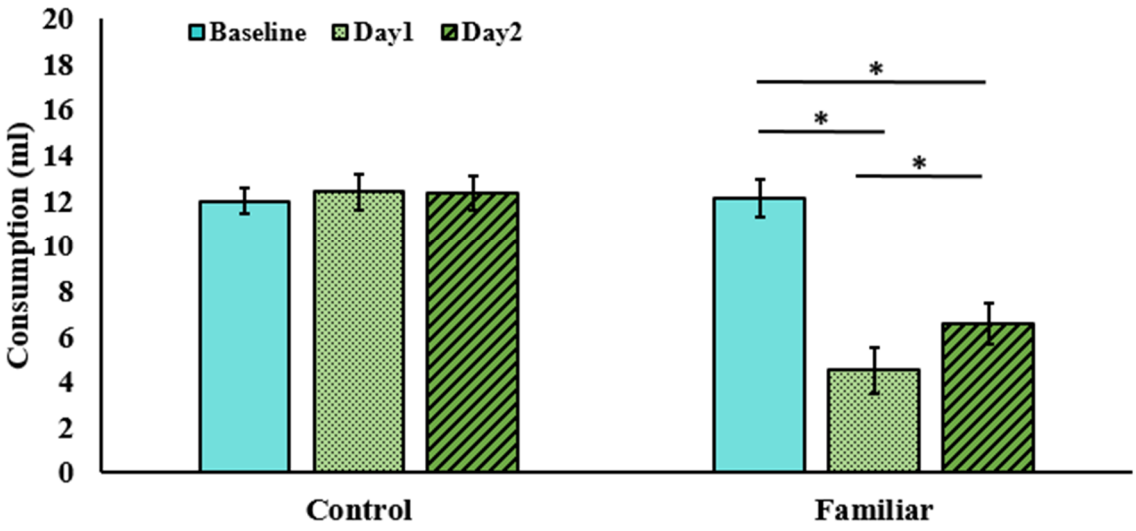

Figure 2. Mean (± SEM) consumption of each group during the water baseline, the first and the second exposure to water (Control) or cider vinegar solution (Familiar). * = $p < .05$.

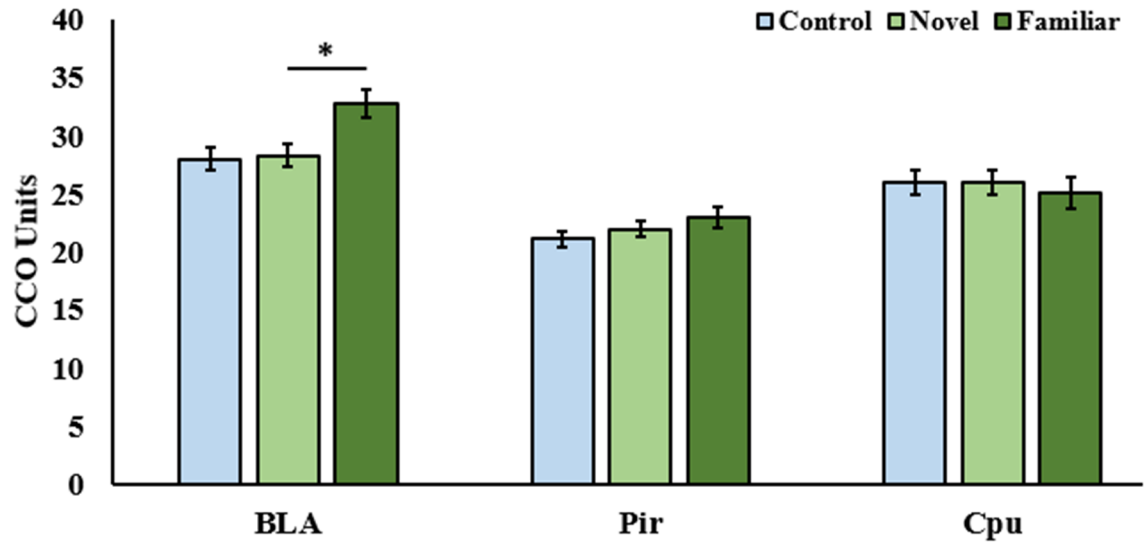

Figure 3. Mean (± SEM) brain metabolic activity of each region of interest (BLA, Pir, Cpu) when exposed to water (Control) or to a cider vinegar solution either once (Novel) or twice (Familiar). * = $p <$ .05.

variances for all within-subject variables. The analysis revealed a significant effect of or Cpu [$F(2,18) = .148$, $p > .05$, $\eta_p^2 = .016$]. Post-hoc Bonferroni tests showed that the Familiar group exhibited higher metabolic BLA activity than both the Novel group drinking vinegar for the first time ($p < .05$) and Control group drinking water ($p < .05$). There were no differences between the Novel and Control groups ($p > .05$).

4.4. Discussion and conclusions

The behavioural results are consistent with those previously reported (Gómez-Chacón et al., 2012). Flavor neophobia and AN are evidenced by reduced intake of the novel vinegar solution which significantly increases on the second exposure as the flavor becomes familiar. The results indicate higher BLA metabolic activity in the group exposed twice to the vinegar solution than that exposed once. This supports a selective involvement of the area in the processes leading to the formation of safe taste memory but not novelty detection. Our results do not allow us to draw conclusions regarding the specific process associated with BLA activity. A potential role of BLA in the retrieval of the flavour memory formed during the first exposure is conceivable. Both retrieval and stabilization of the safe memory should be taking place in the second flavour exposure. Hence, it is feasible an association between the reported increase in BLA activity and memory retrieval. In fact, memory formation should have been initiated during the first exposure but BLA activity changes were not found at this stage. Also, the increased BLA activity during the second vinegar exposure could be associated with a selective stabilization process.

Moreover, since no significant effects were found in Pir, this undercuts the hypothesis that the increased energy expenditure in BLA could be due to enhanced olfactory processing during the second exposure. This is consistent with our previous findings using c-Fos immunohistochemistry (Grau-Perales, Gómez-Chacón, Morillas, et al., 2019). We did not find significant differences between the activity induced by one and two vinegar exposures in neither the anterior nor the posterior Pir regions evaluated. In fact, we have previously reported increased activity induced by drinking a well-familiarized vinegar solution after six exposures, but not after two, only in the rostral part of the posterior Pir (Grau-Perales, Gómez-Chacón, Morillas, et al., 2019). This region

corresponds to the level assessed in the present study. Thus, the absence of differences after two exposures confirms no role of Pir during the familiarization process. Furthermore, the fact that no effect of flavour exposures was found in Cpu corroborates a selective BLA involvement in flavour familiarization.

These findings suggest that neophobia and AN might be independent processes that rely on dissociable brain areas. Accordingly, there was a selective increase of BLA activity in those animals drinking the familiar vinegar solution in comparison with those drinking water while there were no changes in those drinking the novel vinegar solution. We have previously reported similar results in the nucleus accumbens shell (Grau-Perales, Gómez-Chacón, & Gallo, 2019) and the medial prefrontal cortex (Expósito et al., 2020). Although our results do not allow us to draw a circuit approach, it is conceivable that BLA would form a functional network with these areas. Hence, we cannot discard that the reported BLA activity changes during AN could be driven by top-down control from areas such as the prefrontal cortex. Previous reports indicated higher BLA activity during drinking a novel saccharin solution (Lin, Roman, et al., 2012) but no effect of BLA lesions on odour neophobia using a 0.1% amyl acetate solution (Lin et al., 2009). The present findings would suggest the involvement of BLA in flavour familiarization but not novelty detection. Thus, those flavours composed of an odorous component would involve BLA activity during the formation of the flavor-safe memory.

Our results confirm the value of the CCO technique to explore changes associated with learning and memory. Hence, flavour recognition memory is added to taste and spatial learning in which CCO has proven to be a useful technique for identifying the brain areas involved. In fact, previous reports indicated increased CCO activity in BLA during the first day of training in a spatial learning task (Mendez-Couz et al., 2015).

Taken together, our results would support a relevant role of the amygdala either in retrieval and/or the stabilization process leading to a safe flavour memory.

As a conclusion, the main finding of the study is that BLA metabolic activity was higher in the group exposed to a familiar vinegar solution than in the groups exposed to either water or a novel vinegar solution. These results are consistent with the results of lesion studies and support the basolateral amygdala involvement in those processes leading to the attenuation of flavour neophobia.

Capítulo 5

Taste familiarization and medial prefrontal cortex activity in adolescent rats

Abstract

It has been previously reported changes of the ventromedial prefrontal cortex activity during the neophobia and its attenuation in adult and aged rats. These are particularly relevant functions for survival as they prevent overeating of potentially noxious foods and determine diet selection. Adolescence is a developmental period associated with the exploration of novel food sources and delayed attenuation of neophobia. Given that the prefrontal cortex is a late developing area, it can be envisaged altered ventromedial prefrontal cortex activity patterns related to the peculiar adolescent requirements. In this regard, c-Fos immunohistochemistry was performed as an index of neural activity in adolescent (PND28) and adult male Wistar rats (PND 150) during the first, second and sixth exposure to a cider vinegar solution. Both the dorsal and ventral medial prefrontal cortex, as well as the different medial prefrontal cortex regions (anterior cingulate cortex, prelimbic cortex, infralimbic cortex and dorsal peduncular cortex) were compared. The results showed reduced c-Fos expression selectively in the adolescent ventromedial prefrontal cortex in comparison with adults, which was attributable mainly to reduced dorsal peduncular cortex activity. This is consistent with a delayed attenuation of neophobia while no differences between the age groups were found during the first or the sixth exposure. These findings are discussed in terms of a proposed dorsal peduncular cortex modulatory function relevant for contributing to a longer taste evaluation during the first stages of attenuation of neophobia. More research is needed on the role of this region in taste evaluation and its interaction with the brain circuit involved in the taste familiarization process.

5.1. Introduction

Adolescence is a transition period from childhood to adulthood associated with the exploration of novel peers, environments and food sources. Since novelty seeking is often risky, careful assessment of potentially dangerous consequences may be critical for adolescent survival away from parental care (Spear, 2000). One of the challenges that adolescents face is diet selection in novel environments. This requires both learned and unconditioned behavioural responses. Novel tastes induce unconditioned neophobic responses consisting of reduced consumption that protects the organism from potential life-threatening toxic effects (Bermúdez-Rattoni, 2004). This is especially intense for acid and sour tastes which can be associated with potentially harmful edibles. If there are no negative post-ingestive consequences, a learning process called attenuation of neophobia (AN) will take place so that the taste will be labelled as safe and larger amounts of it will be ingested in later exposures (Bermúdez-Rattoni, 2004).

We have previously reported that adolescent rats might require a longer assessment to evaluate acid-tasting novel foods as safe. Adolescent and adult rats exhibited similar taste neophobia but adolescents required longer exposure without consequences for completing the AN to a vinegar solution. While adults exhibited AN after a single exposure, adolescents required two exposures (Expósito et al., 2023). A similar delayed AN was found in aged rats requiring one more vinegar exposure to reach the asymptotic consumption (Gómez-Chacón et al., 2015).

Previous research using c-Fos immunohistochemistry has shown that the delayed attenuation of neophobia found in aged rats corresponds with changes in the pattern of the medial prefrontal cortex (mPFC) activity (Expósito et al., 2020) as well as other brain areas such as perirhinal cortex (PRh) (Gómez-Chacón et al., 2015), piriform cortex (Pir) (Grau-Perales, Gómez-Chacón, Morillas, et al., 2019) and nucleus accumbens (NAcb)

(Grau-Perales, Gómez-Chacón, & Gallo, 2019). Thus, it can be envisaged that an altered pattern of brain activity is also associated with the delayed AN reported in adolescents. This is feasible given that adolescence is a late developmental stage marked by pronounced reorganization of the brain networks. During this period in which the maturational processes continue sculpting the brain formation, the prefrontal cortex is one of the later developing areas. As a matter of fact, the different course of the cortical and subcortical brain areas maturation induces great changes in the brain networks organization. It has been proposed that the delayed maturation of the modulatory prefrontal descendent projections over the subcortical brain areas involved in the reward and emotional system is responsible for peculiar adolescent behaviour (Spear, 2000).

The mPFC has been commonly associated with flexibility and decision-making processes. This is in accordance with its proposed role in shifting behaviours relevant to diet selection such as AN (Expósito et al., 2020) as well as learning and extinction of taste aversions and preferences (González et al., 2015; Malkusz et al., 2012; Shehadi & Maroun, 2013; Touzani et al., 2010). In the rat brain mPFC is a heterogeneous region that can be subdivided into different subdomains depending on the criteria used. Based on cytoarchitectonic criteria, mPFC can be divided into anterior cingulate (ACC), prelimbic (PrL), infralimbic (IL) and dorsal peduncular DP cortices (Öngür & Price, 2000; Paxinos & Watson, 2013). Taking into account anatomy and connectivity, it has been proposed a subdivision into a dorsal component (dmPFC) that includes ACC and the dorsal region of PrL (dPrL), versus a ventral component (vmPFC) that includes the ventral region of PrL (vPrL), IL, and DP (Heidbreder & Groenewegen, 2003).

Expósito and colleagues (2020), using c-Fos immunohistochemistry, found greater activity in the vmPFC, but not the dorsal part, during the second exposure to a novel vinegar solution in comparison with the first and sixth exposure in adult rats. This

suggests that the vmPFC, but not the dorsal, is selectively related to the first stages of the familiarization process leading to AN but not with the neophobic response or recognition of familiar tastes. Among the vmPFC regions, the results pointed to DP as the most vulnerable to the ageing impact. Aged rats exhibited an altered activity pattern during the familiarization process, with an increased DP activity during the first exposure to the novel vinegar solution but this activity significantly decreased after one exposure and continued decaying with additional exposures.

Given that AN to a vinegar solution requires longer exposure in adolescent rats and that the vmPFC exhibits activity changes during AN being some of them susceptible to age-induced AN delay, we aimed to assess mPFC activity in adolescent and adult rats following the same approach used by Expósito and colleagues (2020). Thus, we designed an experiment exposing adolescent and adult rats to a novel cider vinegar solution. These animals were subsequently sacrificed after the first, second or sixth exposure to perform the c-Fos immunohistochemistry as an index of neural activity. We assessed the number of c-Fos positive cells in the different mPFC regions (ACC, dPrL, vmPrL, IL, DP). Additionally, we defined larger areas using the previous ones: PrL (dPrL + vPrL), dmPFC (ACC + dPrL) and vmPFC (vPrL + IL + DP).

5.2. Method

Subjects. Thirty-six adolescent (± 28 days old, ± 117 g, n = 18) and adult (5-month-old; ± 671 g, n = 18) male Wistar rats were housed individually and maintained on a 12 h light/dark cycle. Food was available *ad libitum*, but the animals were water-deprived during the experiment. Rats had access for 15-min in the morning (Experimental session) to either water or a non-palatable 3% cider vinegar solution and had 20-min access in the afternoon to water (Rehydration session).

Behavioural procedure. Water was available for four days to habituate the animals to the procedure and establish a baseline. During the following six experimental sessions, water was switched to the cider vinegar solution. Depending on the number of vinegar experimental sessions adult and adolescent rats were assigned to one of the following groups (n = 6 each): Novel (one vinegar exposure), Familiar I (two vinegar exposures), Familiar II (six vinegar exposures). Intake (g) was recorded by weighing the drinking bottles before and after each drinking session. In order to allow comparisons between different age groups, an intake index was calculated by obtaining the percentage of ingested solution during the experimental drinking sessions in comparison with that of the last baseline day (Day x 100)/LB). Thus, a low index indicated high taste neophobia.

Animals were euthanized 90 min after their corresponding experimental session, in order to perform the c-Fos immunohistochemistry. The procedures were approved by the University of Granada Ethics Committee for Animal Research and Junta de Andalucía (1/06/2022/078).

Immunohistochemistry. The immunohistochemical procedure has been described elsewhere (Expósito et al., 2020). In brief, under deep anaesthesia, the rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde and their brains were removed and -80 ºC frozen. Afterwards, using a cryostat (Leica CM1900), coronal sections between +3.24 mm and + 2.76 mm relative to bregma were cut at 20 µm. Tissue sections were rinsed in phosphate-buffered saline (PBS 0.01 M, pH 7.4) incubated in 3% goat serum and 0.4% Triton X-100 in PBS for 30 min. The slices were transferred to a c-Fos primary antibody (Rabbit anti c-Fos antibody, 1:10.000); Sigma Aldrich) for 48 h at 4 ºC. They were incubated in a secondary antibody (Biotinylated goat anti-rabbit IgG, 1:500; Calbiochem) for 120 min 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 again and processed using the ABC kit (Vector Laboratories, Burlingame, CA). The reaction was visualized using the peroxidase substrate kit (DAB) (Vector Laboratories, Burlingame, CA). Finally, they were rinsed, mounted on gelatin-subbed slides, rehydrated with ethanol and xylenes and finally, they were covered-slipped.

To quantify c-Fos positive cells, a light microscope (Olympus BX41) was used to capture 14 photos per hemisphere from two different sections of each brain at 40x magnification, following a rostro-caudal vision. The brain areas assessed and representative photos can be seen in Fig. 1A and Fig. 1B. The photos were individually processed and c-Fos labelled cells were automatically counted using the Image J software (National Institute of Mental Health). For all images, the software automatically counted all objects with a specific size (350-3200) and circularity (0.20-1.00). For each image, brightness and contrast parameters and several filters ("Fill Holes", "Remove Outliers", "Gaussian Blur" and "Watershed") were applied individually for each brain's photos, in order to favour a more precise counting. Both behavioural and c-Fos positive cells were analyzed using the SPSS software (IBM, USA).

5.3. Results

Mean intake I ndices of the adult and adolescent Familiar II groups are displayed in Fig. 2. A two-factor mixed ANOVA analysis that includes one between-subject factor Age (Adolescent × Adult) and one within-subject factor Days with six levels (Vin1, Vin2, Vin3, Vin4, Vin5 and Vin6) was performed. Mauchly's sphericity test yielded no assumption of sphericity ($p = .038$), so Greeinhouse-Geisser corrections were used. There was a significant effect of Days [$F(3.005, 30.048) = 13.007$; $p < .001$; $\eta_p^2 = .565$], and Bonferroni post-hoc tests indicated significant differences between Vin1 ($p < .05$) vs. All, and Vin2 vs. Vin4 and Vin6 ($p < .05$). However, neither the interaction Days*Age

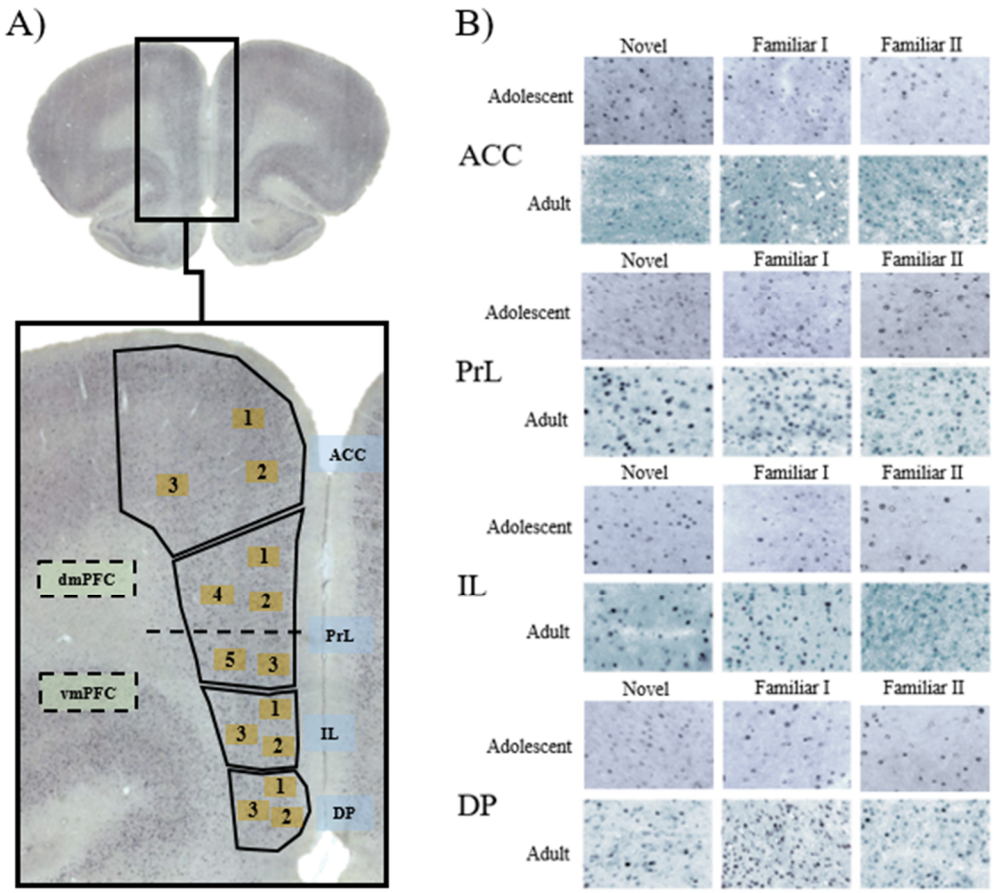

Fig 1. Representative images of **(A)** how c-Fos labelled samples photos of mPFC photos were taken and how mPFC is subdivided into ACC, PrL, IL and DP; and **(B)** representative 40x photos of each area (ACC, PrL, IL and DP) for each group (Novel, Familiar I and Familiar II) and for each age (Adolescent and Adult).

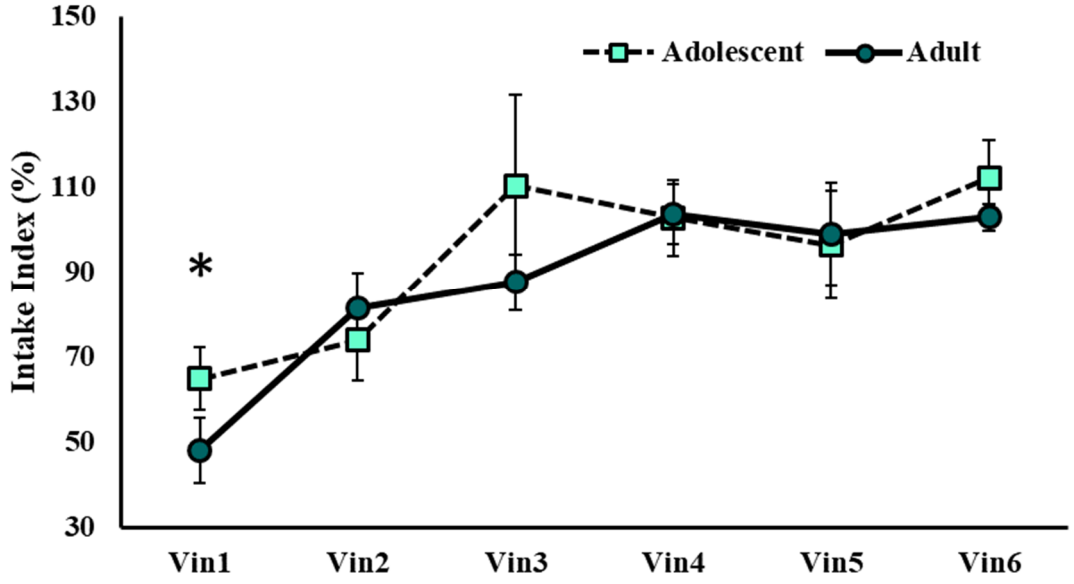

Fig. 2. Mean intake index (%; ± SEM) of animals exposed until Vin6 (Cider vinegar solution intake regarding last LB water intake). * = $p < .05$ vs. All; # = p .05 vs. Vin1, Vin4 and Vin6.

[$F(3.005, 30.048) = 1.224$; $p = .318$; $\eta_p^2 = .109$] or the main effect of of Age [$F(1, 10) = .337$; $p = .574$; $\eta_p^2 = .033$] were significant. In addition, although the interaction did not reach significance, independent repeated measures analyses were performed for each age group in order to assess the number of vinegar exposures required for AN. The results indicated that while in the Adult group, Vin2 intake index increased significantly during the second exposure in comparison with Vin1 [$F(5,25) = 7.496$; $p = .007$; $\eta_p^2 = .600$], the Adolescent group exhibited higher intake on Vin3 than Vin1 [$F(5,25) = 6.720$; $p = .001$; $\eta_p^2 = .573$] but no differences between Vin1 and Vin2. Since no other differences are significant, this indicates that adults required only one exposure to exhibit AN while adolescents required two exposures.

The mean number of c-Fos positive cells in the different mPFC regions assessed is shown in Fig. 3. A two-way ANOVA was carried out for each area, having two between-subject factors: Age (Adolescent × Adult) and Group (Novel × Familiar I × Familiar II). For dmPFC, the analysis did not yield significant effects of Age [$F(1,30) = .350$; $p = .558$; $\eta_p^2 = .012$], Group [$F(2,30) = 1.909$; $p = .166$; $\eta_p^2 = .113$] or Age × Group [$F(2,30) = .204$; $p = .817$; $\eta_p^2 = .013$]. However, for vmPFC there were significant effects of Age [$F(1,30) = 5.59$; $p = .025$; $\eta_p^2 = .157$], Group [$F(2,30) = 12.71$; $p < .001$; $\eta_p^2 = .459$] and Age × Group interaction [$F(2,30) = 6.58$; $p = .004$; $\eta_p^2 = .305$]. Bonferroni post-hoc tests indicated that the interaction effect was due to a lower number of c-Fos positive cells in the Adolescent than in the Adult Familiar I group ($p < .05$) but not in Novel or Familiar II. When mPFC was evaluated following a more traditional subdivision, similar two-way ANOVAs were performed with ACC, PrL, IL and DL. In ACC, there was not effect of Age [$F(1,30) = 1.4942$; $p = .231$; $\eta_p^2 = .047$], Group [$F(2,30) = .2494$; $p = .791$; $\eta_p^2 = .016$] or Age × Group [$F(2,30) = .0820$; $p = .921$; $\eta_p^2 = .005$]. Regarding PrL, there

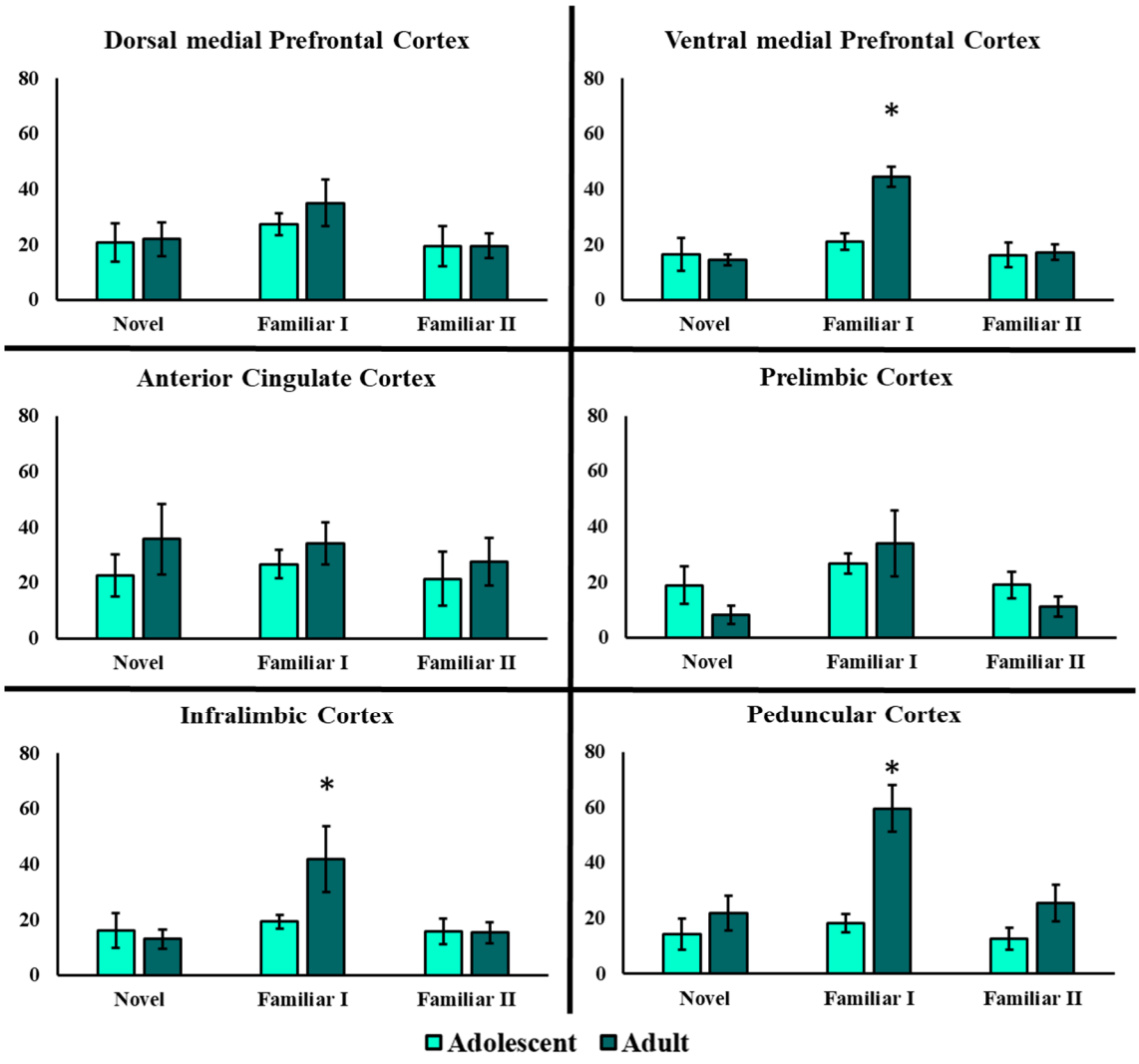

Fig. 3. Mean (± SEM) c-Fos positive cells in different mPFC areas. Anterior cingulate cortex (ACC), dorsal (dPrL) and ventral (vPrL) portion of prelimbic cortex, infralimbic cortex (IL), dorso-peduncular cortex (DP), prelimbic cortex (PrL; dPrL + vPrL), dorsal (dmPFC; ACC + dPrL) and ventral (vmPFC; vPrL + IL + DP) prefrontal cortex. * = $p < .05$.

was a significant effect of Group [$F(2,30) = 5.271$; $p = .011$; $\eta_p^2 = .260$] but not Age [$F(1,30) = .646$; $p = .428$; $\eta_p^2 = .021$], nor Age × Group [$F(2,30) = 1.379$; $p = .267$; $\eta_p^2 = .084$.

Bonferroni post-hoc tests indicated that those animals in the Familiar I groups exposed twice to the taste had a higher number of c-Fos positive cells than those in the groups Novel or Familiar II regardless of age. Concerning IL, while there was no effect of Age [$F(1,30) = 1.99$; $p = .169$; $\eta_p^2 = .062$], it was found a Group effect [$F(2,30) = 5.19$; $p =$

.012; η_p^2 = .257] that relayed, again, in a higher number of c-Fos positive cells found in Familiar I vs. Novel and Familiar II groups (p < .05). The interaction Age × Group was close to significance [F(2,30) = 3.20; p = .055; η_p^2 = .176], since the adolescent Familiar I group tended to exhibit a lower number of c-Fos positive cells than the corresponding adult Familiar I group. Accordingly, the Age × Group interaction was significant in DP [F(2,30) = 4.65; p = .017; η_p^2 = .237] in addition to the significant effects of Age [F(1,30) = 18.01; p < .001; η_p^2 = .375] and Group [F(2,30) = 7.92; p = .002; η_p^2 = .346]. In this case, the lower number of c-Fos positive cells exhibited by the adolescent than the adult Familiar I groups reached significance (p < .05).

5.4. Discussion

The behavioural results indicated that adolescent vinegar neophobia required two exposure trials to be attenuated in contrast to the adult group that attenuated neophobia to this taste after one exposure. In contrast to the widely accepted increased novelty seeking and reduced neophobia associated with physical environments, adolescent and adult rats exhibited similar taste neophobia. Moreover, adolescent taste neophobia was even higher since it required one more exposure to be attenuated. This is consistent with previous results in our lab that found a higher sensitivity to the acid but not sweet taste in comparison to adults (Expósito et al., 2023). It can be proposed that a longer evaluation of the potentially dangerous tastes consequences might be adaptive during adolescence to favour survival. However, this might not be adaptive in the case of tastes such as sweet or salty that predict sources of great value during the adolescent growth spurt. Therefore, the present results together with those of Expósito et al. (2023) show that the critical difference between adolescents and adults during the evaluation of novel vinegar taste lies in the protracted early stage of AN.

The main finding obtained in the present experiment is the selectively reduced activity of the vmPFC, but not dmPFC, during the vinegar taste evaluation process. This is mainly attributable to the reduced activity in DP, even though a similar tendency was found in IL. DP activity was reduced during the entire process from taste neophobia to consolidated taste familiarity after six exposures, however, only vmPFC activity during the second taste exposure differed from the increased activity seen in adults. Hence, it seems reasonable to think that the reduced activity of this area involvement during the early stage of the taste evaluation process is responsible for AN lengthening in adolescents.

The reduced activity could be attributed to DP immaturity in the younger animals. This does not seem likely because it is a selective effect which is not seen in the rest of the mPFC areas assessed, such as ACC, PrL or even IL. It is more feasible for a developmental change in DP function so its activity could not be yet related to taste AN during adolescence. It can be proposed that later DP involvement during adulthood would contribute to accelerating AN even though this activity is not required for completing the evaluation process. Indeed, the activity of several cortical and subcortical brain areas such as PRh (Gómez-Chacón et al., 2012, 2015), Pir (Grau-Perales, Gómez-Chacón, Morillas, et al., 2019), amygdala (Gómez-Chacón et al., 2012; Lin et al., 2018; Menchén-Márquez et al., 2023), NAcb (Grau-Perales et al., 2020; Grau-Perales, Gómez-Chacón, & Gallo, 2019) has been associated with taste neophobia and AN in rats. Moreover, lesion and pharmacological studies have supported the involvement in taste neophobia and familiarization of some of these areas such as PRh (Morillas et al., 2017; Ramos, 2020b, 2020a) and amygdala (Gómez-Chacón et al., 2016; Lin et al., 2018) as well as the hippocampus in context-dependent AN modulation (Grau-Perales, Levy, et al., 2019).

Previous reports with aged rats support that in comparison to adults, DP activity decreases at advanced ages in parallel to a delayed AN (Expósito et al., 2020). Moreover, these authors found that DP activity increased during the first exposure to a vinegar solution, thus supporting a functional change of the area over the lifetime period. It can be envisaged that increased DP activity at advanced ages could contribute to facilitating taste neophobia which has been demonstrated to be enhanced during aging (Gallo, 2018). Thus, during ageing increased DP activity during the first exposure together with decreased activity during the second exposure could promote the reported increased taste neophobia and delayed AN, which has a great adaptive value for survival since older organisms are highly vulnerable to even low doses of toxicity. Likewise, reduced DP activity during the second exposure has the great value of elongating the evaluation of potentially dangerous taste intake consequences.

To our knowledge, this is the first assessment of the mPFC activity related to taste neophobia and its attenuation. The results indicate a reduced dmPFC activity during the first stages of AN. Moreover, a further distinction between ACC, PrL, IL and DP areas unveiled a selective decline in DP. This prompts more research on the role of DP in taste memory and prompts research on its interaction with a proposed network mediating neophobia and its attenuation that includes PRh, amygdala and NAcb along the development.

Capítulo 6

Discusión General

Los resultados que conforman esta tesis doctoral ponen de manifiesto las posibilidades que ofrece la modalidad gustativa para estudiar los circuitos neurales responsables de la respuesta a la novedad y de la memoria de reconocimiento de sabores en ratas. Comprender los mecanismos neurales implicados en la respuesta a la novedad adquiere especial relevancia durante la adolescencia. Esta etapa del desarrollo se caracteriza por la transición del ambiente familiar conocido de la infancia hacia el mundo adulto. Se trata de enfrentarse a un ambiente que ofrece una variedad de estímulos y situaciones novedosas, incluyendo modificaciones de la dieta. Ello se corresponde con una organización peculiar de los circuitos cerebrales en sus fases tardías de formación. El empleo de un procedimiento comportamental sencillo, consistente en aplicar repetidamente un sabor desconocido para el animal, permite una aproximación multidisciplinar al estudio de la neofobia gustativa y su atenuación. La ingestión de una solución de sabor desconocido produce una respuesta cautelosa, reflejada en una reducción del consumo durante la primera presentación, consumo que se incrementa a medida que el sabor se convierte en familiar y no va asociado a consecuencias adversas, gracias a procesos de memoria de reconocimiento gustativa. Aunque la neofobia gustativa y la atenuación de la neofobia (AN) son fenómenos bien conocidos, se desconoce en buena medida la naturaleza de los procesos involucrados, los mecanismos cerebrales subyacentes y las posibles diferencias sexuales. Una comprensión completa requiere un abordaje psicobiológico. Para ello se ha combinado el análisis comportamental complejo mediante el registro de la cantidad consumida y la evaluación tanto del microanálisis de la estructura del lamido (MLS) como las reacciones orofaciales al sabor (TRT), con el análisis de la actividad cerebral empleando histoquímica de citocromo oxidasa e inmunohistoquímica de c-Fos como índices de actividad neural.

La primera serie experimental se dirige a investigar los procesos afectivos involucrados en la respuesta neofóbica y su atenuación. El registro únicamente del consumo no permite distinguir entre procesos disociables tales como la AN y la extinción de aversiones o preferencias gustativas aprendidas. Evaluaciones adicionales mediante el MLS y la TRT permiten explorar procesos afectivos y motivacionales que pueden diferir entre protocolos. En esta serie experimental se han empleado ambas técnicas para evaluar cambios potenciales en palatabilidad asociados al proceso de familiarización del sabor en ratas de ambos sexos empleando una solución acida de vinagre de sidra. Estudios previos empleando soluciones de sacarina y quinina en ratas macho habían arrojado resultados contradictorios en función de la técnica empleada. Mientras Lin y colaboradores (2012) aplicando el MLS describieron que la familiaridad del sabor incrementaba la palatabilidad, Neath y colaboradores (2010) no hallaron cambios en las respuestas orofaciales apetitivas mediante la TRT.

Los resultados de la primera serie experimental de esta tesis permiten extender estos resultados al sabor ácido y a ambos sexos. Efectivamente, si se acepta que ambas pruebas reflejan palatabilidad, el MLS se presenta como más sensible que la TRT para medir los cambios afectivos asociados a la degustación de un sabor desconocido y su posterior familiarización. Sin embargo, no pueden descartarse diferencias entre las pruebas que impliquen diversos procesos registrados ni el papel crítico del consumo voluntario para la AN que tiene menor impacto en la TRT. Por otra parte, el sexo se revela como una variable importante en el estudio de la neofobia gustativa y su atenuación, variable que no había sido incluida en los estudios previos sobre la AN. Las ratas hembra muestran mayor reactividad que las ratas macho en el MLS ante el sabor ácido, así como un mayor efecto inespecífico de reactividad al procedimiento de infusión intraoral en la TRT.

En conjunto, combinar en el mismo estudio el microanálisis de la estructura del lamido y de las respuestas orofaciales junto al consumo, pone de manifiesto la complejidad de los procesos afectivos y motivacionales implicados en la neofobia gustativa y la AN, así como la importancia de incluir el sexo como variable.

La segunda serie experimental se centra en la participación de la amígdala basolateral (BLA) en los procesos afectivos implicados en la AN. Estudios previos habían mostrado que lesiones permanentes y reversibles de esta zona interfieren con la AN a soluciones de sacarina sódica (Lin et al., 2009, 2018). Sin embargo, no interfirieron con la neofobia cuando se emplearon soluciones olfativas de acetato de amilo (Lin et al., 2009). Ello plantea la cuestión de si la participación de la BLA durante el proceso de la familiarización con un sabor depende de su componente olfativo. Estudios previos empleando una solución de vinagre de sidra, que posee tanto componente gustativo como olfativo, indicaron que la integridad de la BLA es necesaria para que se produzca el patrón de actividad asociado a la AN en la corteza perirrinal (Gómez-Chacón et al., 2012). Ello permite proponer la existencia de cambios en la actividad de la BLA durante la AN empleando dicho sabor. Para explorar esta cuestión se ha evaluado la actividad metabólica de la zona gracias a la cuantificación de los niveles de la enzima citocromo oxidasa. Se trata de una técnica ampliamente empleada para identificar circuitos neurales activados en diversas fases del aprendizaje y la memoria (Banqueri et al., 2018; Conejo et al., 2010, 2013; Méndez-Couz et al., 2016; Zorzo et al., 2019). Los resultados muestran un incremento selectivo de la actividad metabólica en la BLA durante la segunda presentación del sabor, pero no en otras áreas cerebrales tales como el estriado o la corteza piriforme. Ello puede interpretarse a favor de la participación de la zona en el proceso de familiarización de un sabor con un importante componente olfativo.

Dada la implicación de la amígdala en el procesamiento emocional, no se puede descartar su contribución a los cambios afectivos ligados a la palatabilidad que pueden acompañar a la familiarización con un sabor desconocido de acuerdo con los resultados obtenidos en la primera serie experimental. Por otro lado, estos resultados contribuyen a identificar una red neural implicada en el procesamiento de los componentes afectivos y motivacionales de la AN en la que la BLA interaccione con el sistema de recompensa meso-cortico-límbico. Efectivamente, estudios previos empleando inmunohistoquímica de c-Fos han demostrado que la AN está asociada a un aumento de la actividad en el NAcb (Grau-Perales et al., 2020; Grau-Perales, Gómez-Chacón, & Gallo, 2019) y en la mPFC (Expósito et al., 2020).

Por último, la tercera serie experimental se dedica a iniciar una aproximación de desarrollo al estudio de la neofobia gustativa y su atenuación con especial interés en la adolescencia dada la relevancia de estos fenómenos durante la transición infanto-juvenil. Se ha descrito previamente que las ratas adolescentes muestran un retraso en la AN del sabor ácido empleando una solución de vinagre de sidra. Dicho retraso parece corresponder a una evaluación más detallada del sabor antes de reconocerlo como seguro. La mPFC forma parte del sistema de recompensa crucial para regular los aspectos motivacionales del comportamiento y su actividad ha sido relacionada con la AN a una solución de vinagre en ratas adultas (Expósito et al., 2020). Teniendo en cuenta que la mPFC es una de las áreas cerebrales que madura tardíamente, ha resultado de especial interés investigar si su actividad se diferencia de la del adulto, ya que ello pudiera estar relacionado con las alteraciones comportamentales del adolescente. Para ello, se emplea inmunohistoquímica de c-Fos realizando un preciso análisis de la actividad en distintas regiones de la mPFC durante la exposición a la solución de vinagre por primera, segunda y sexta vez. Los resultados indican una reducida actividad en los adolescentes

en comparación con los adultos. Ello puede atribuirse a la ausencia del incremento selectivo de actividad que exhiben los adultos durante la segunda exposición al sabor en la región ventral de la mPFC correspondiente a la corteza peduncular dorsal (Expósito et al., 2020). Efectivamente, a diferencia de lo descrito en adultos, la actividad de ninguna región en la mPFC muestra cambios relacionados con la familiaridad del sabor en ratas adolescentes, lo que puede relacionarse con la inmadurez de la zona durante la adolescencia. Sin embargo, los resultados conductuales indican que tanto la neofobia gustativa como la AN se producen en estas edades, lo que implica que la participación de la mPFC no es necesaria para la familiarización del sabor. Aun así, el análisis independiente del consumo de adultos y adolescentes a lo largo de las presentaciones apoya resultados previos con la misma solución indicando un retraso en la AN durante la adolescencia (Expósito et al., 2023). Dicho retraso podría estar relacionado con la ausencia de la participación cortical durante esta etapa. Esta interpretación permite proponer un papel de la región ventral de la mPFC correspondiente a la corteza peduncular dorsal, que aún no es funcional durante la adolescencia, en la aceleración del proceso de familiarización.

En conjunto, los resultados que integran esta tesis doctoral aportan nueva evidencia sobre el papel de los procesos afectivos y motivacionales en la neofobia gustativa y su atenuación, poniendo de manifiesto la participación de una compleja red neural formada por circuitos emocionales y de recompensa cuya investigación se beneficia de una aproximación de desarrollo.

Capítulo 7

Conclusiones

1. Los resultados con respecto a los cambios en el valor hedónico durante la neofobia gustativa y su atenuación difieren según la prueba utilizada. Mientras el MLS encuentra un aumento del valor hedónico a medida que el sabor se atenúa, al utilizar la TRT no se encuentran cambios atribuibles a la AN.

2. Existen diferencias sexuales en la respuesta emocional durante la exposición a un sabor novedoso y su aplicación repetida, mostrando las hembras una mayor reactividad que los machos tanto en el MLS como en la TRT. Las ratas hembras muestran mayor LCS y mayor número de respuestas aversivas.

3. El patrón de cambios en palatabilidad observado durante la neofobia y la AN en el MLS es opuesto al que se encuentra durante la extinción de un CTA. Mientras la AN induce el aumento abrupto del consumo en la segunda exposición en comparación con la primera, la palatabilidad del sabor aumenta gradualmente. Sin embargo, durante la extinción la palatabilidad del sabor condicionado se recupera con relativa rapidez mientras que el consumo lo hace más lentamente o, incluso, no llega a hacerlo.

4. El uso de histoquímica de CCO revela una mayor activación en la BLA en los grupos expuestos dos veces a una solución de vinagre de sidra que en aquellos que han sido expuestos solo una vez. Ello apoya la participación de la BLA en la AN.

5. En ratas adolescentes no aparece el patrón adulto identificado mediante inmunohistoquímica de c-Fos que consiste en aumento de la actividad

de la parte ventral de la mPFC durante la segunda exposición a una solución de vinagre de sidra en comparación con la primera. Esto puede atribuirse a inmadurez cerebral y puede relacionarse con la AN más lenta descrita en esta etapa del desarrollo, lo que apoya un papel facilitador de la zona.

Chapter 7

Conclusions

1. The results regarding changes of the flavour hedonic value during taste neophobia and its attenuation differ depending on the test used. While licking microanalysis indicates that the hedonic value increases as the flavour becomes familiar, no evidence of palatability changes during the attenuation of taste neophobia was found in the taste reactivity.

2. There were sex differences in flavour neophobia and its attenuation. Both microanalysis of the licking structure and taste reactivity test evidenced higher reactivity in female rats than male rats. Females exhibited higher LCS and greater number of aversive responses.

3. The pattern of palatability changes observed during neophobia and AN in licking microanalysis is opposite to that previously reported during the extinction of a CTA. While AN induced an abrupt increase in consumption after the first exposure, palatability increased gradually. During extinction, however, the palatability of the conditioned taste recovers relatively quickly, while consumption increases gradually or not at all.

4. CCO histochemistry revealed increased BLA metabolic activity in the groups exposed twice to an apple cider vinegar solution compared to those exposed only once. This supports the BLA involvement in AN.

5. The adult pattern of c-Fos vmPFC immunoreactivity associated with AN using cider vinegar is absent in adolescent rats. This might be attributed to brain immaturity and can be related with the slower AN reported in adolescent, thus supporting a facilitating role of the area.

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Índice de abreviaturas

ACC: corteza cingulada anterior

AN: atenuación de la neofobia

ANOVA: análisis de la varianza

ATP: adenosín trifosfato

BAT: tarea de acceso breve

BL: línea base

BLA: amígdala basolateral

CCO: citocromo c oxidasa

CeA: amígdala centromedial

CFP: preferencia al sabor condicionada

CPu: caudado-Putamen

CS: estímulo condicionado

CTA: condicionamiento aversivo-gustativo

D1DR: receptor dopaminérgico D1

DAB: diaminobenzidina

dmPFC: porción dorsal de la corteza prefrontal medial

DP: corteza peduncular dorsal

dPrL: porción dorsal de la corteza prelímbica

g: gramo

GC: corteza gustativa

HC: hipocampo

HET: heterocigótico para CACNA1c

IC: corteza insular

ID: identificación

IL: corteza infralímbica

L: litro

LCS: tamaño del clúster de lametazos

LH: hipotálamo lateral

M: molar

mg: miligramo

min: minuto

MLM: modelo linear mixto

MLS: microanálisis de la estructura del lamido

mM: milimolar

mm^3: metro cúbico

mPFC: corteza prefrontal medial

mRNA: ácido ribonucleico mensajero

NAcb: núcleo accumbens

NAcb-C: zona central del núcleo accumbens

NAcb-Sh: corteza del núcleo accumbens

NaCl: cloruro de sodio

NTS: núcleo del tracto solitario

OD: densitometría óptica

OFC: corteza orbitofrontal

PBN: núcleo parabraquial

pERK: fosforilación de la quinasa regulada extacelular

PFC: corteza prefrontal

Pir: corteza piriforme

PND: día postnatal

PRh: corteza perirrinal

PrL: corteza prelímbica

rNTS: porción rostral del núcleo del tracto solitario

ROI: región de interés

s: segundo

SEM: error estándar de la media

T1R: receptor gustativo tipo 1

T2R: receptor gustativo tipo 2

T3R: receptor gustativo tipo 3

TRT: prueba de reactividad al sabor

US: estímulo incondicionado

v/v: volumen por volumen

v/w: volumen por peso

vmPFC: porción ventral de la corteza prefrontal medial

VP: pálido ventral

VPMpc: porción parvocelular del núcleo ventral posterolateral del tálamo

w/v: peso por volumen

WT: cepa sin mutaciones específicas

Index of abbreviations

ACC: anterior cingular cortex

AN: attenuation of neophobia

ANOVA: analysis of variance

ATP: adenosine triphosphate

BAT: brief access task

BL: baseline

BLA: basolateral amydgala

CCO: cytochrome c oxidase

CeA: centromedial amygdala

CFP: conditioned flavor preference

CPu: caudate-putamen

CS: conditioned stimulus

CTA: conditioned taste aversion

D1DR: D1 dopamine receptor

DAB: diaminobenzidine

dmPFC: dorsal part of the medial prefrontal cortex

DP: dorsal peduncular cortex

dPrL: dorsal prelimbic cortex

g: gram

GC: gustatory cortex

HC: hippocampus

HET: CACNA1c heterozygous

IC: insular cortex

ID: identification

IL: infralimbic cortex

L: litre

LCS: lick cluster size

LH: lateral hypothalamus

M: molar

mg: milligram

min: minute

MLM: mixed linear model

MLS: microanalysis of licking structure

mM: millimolar

mm^3: cubic millimetre

mPFC: medial prefrontal cortex

mRNA: messenger ribonucleic acid

NAcb: nucleus accumbens

NAcb-C: nucleus accumbens core

NAcb-Sh: nucleus accumbens shell

NaCl: sodium chloride

NTS: nucleus of the solitary tract

OD: optic densitometry

OFC: orbitofrontal cortex

PBN: parabrachial nucleus

pERK: phosphorylated extracellular signal-regulated kinase

PFC: prefrontal cortex

Pir: piriform cortex

PND: postnatal day

PRh: perirhinal cortex

PrL: prelimbic cortex

rNTS: rostral portion of the nucleus of the solitary tract

ROI: region of interest

s: second

SEM: standard error of the mean

T1R: taste 1 receptor

T2R: taste 2 receptor

T3R: taste 3 receptor

TRT: taste reactivity test

US: unconditioned stimulus

v/v: volume per volume

v/w: volume per weight

vmPFC: ventral part of the medial prefrontal cortex

VP: ventral pallidum

VPMpc: parvocellular portion of the ventral posteromedial nucleus of the thalamus

w/v: weight per volume

WT: wild type

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