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PAPEL DE LA ARGININA VASOPRESINA EN LA MODULACIÓN AMIGDALINA DE MIEDO INNATO EN LA RATA

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"Gracias por ser y por estar"
$(\partial + m)\,\psi = 0$

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“A todos nos mueve una pasión dominante: la pasión por el progreso y la verdad”

“La verdad es una gran coqueta: no hay que buscarla con demasiada pasión, pues con frecuencia se rinde más bien a la indiferencia. Se escapa cuando parece que la tenemos presa, pero se entrega si se le espera pacientemente; se revela ella misma después de habernos despedido de ella, pero es inexorable cuando se la ama con excesivo fervor”

Paul de Kruif

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1. RESUMEN

Fundamentos. La amígdala cerebral desempeña un papel fundamental en la modulación de la ansiedad. Numerosos estudios han demostrado que la arginina vasopresina (AVP) induce efectos ansiogénicos después de ser administrada tanto de manera sistémica como intra septal. El núcleo central de la amígdala (CeA) presenta una inervación vasopresinérgica significativa así como la expresión de los receptores V1a y V1b para la AVP.

Objetivo. El objetivo de este trabajo fue estudiar la importancia de la neurotransmisión vasopresinérgica en la modulación amigdalina de la ansiedad innata, y esclarecer si los subtipos de receptores de la AVP pueden tener un papel diferencial en la modulación de la ansiedad innata.

Método. Las conductas de ansiedad fueron evaluadas utilizando la prueba de enterramiento defensivo y la prueba de caja luz/oscuridad, después de la microinfusión bilateral de la AVP sola o en conjunto con antagonistas para el receptor V1a o V1b en el CeA.

Resultados. La microinfusión de AVP indujo en dosis bajas (1ng/lado), pero no en dosis altas (10 ng/lado) efectos ansiogénicos, únicamente en la prueba del enterramiento defensivo. El antagonista V1b (SSR149415), a diferencia del V1a (compuesto de Manning) canceló los efectos ansiogénicos de la AVP en la prueba del enterramiento defensivo. En ninguna de las dos pruebas se observaron efectos en las conductas de ansiedad tras administrar los antagonistas por sí solos.

Conclusiones. Nuestros resultados indican que el subtipo de receptor V1b contribuye en la modulación amigdalina de la ansiedad al utilizar un modelo para evaluar ansiedad en el cual los estímulos resultan altamente aversivos.

ABSTRACT

The amygdala plays a paramount role in the modulation of anxiety and numerous studies have shown that arginine vasopressin (AVP) elicits anxiogenic effects following either its systemic or septal administration. The aim of this paper was to study the involvement of vasopressinergic neurotransmission in the amygdaloid modulation of unconditioned anxiety and to ascertain whether or not AVP receptor subtypes may have a differential role in this modulation. Anxiety behavior was evaluated both in Shock-Probe Burying Test and Light-Dark Box following the bilateral microinfusion of AVP alone or AVP together with either AVP 1a or AVP V1b receptor antagonists into the central amygdala (CeA). AVP microinfusion elicited at low (1 ng/side) but not at high doses (10 ng/side) anxiogenic-like responses in the Shock-Probe Burying Test but not in the Light-Dark Box. SSR149415, an AVP V1b antagonist unlike Manning compound, an AVP V1a antagonist, fully prevented AVP effects in the Shock-Probe Burying Test when it was administered simultaneously with AVP. In addition, oxytocin receptor blockade also failed to affect AVP effects. No effects of any AVP antagonist by itself were observed in both anxiety paradigms. Our results indicate that AVP V1b receptor contribute to the amygdaloid modulation of anxiety at least in the context of the Shock-Probe Burying Test since no effects were noticed in the Light- Dark Box. It remains to the future to ascertain whether AVP receptor subtypes have indeed differential actions either in the modulation of global or specific features of unconditioned anxiety.

2. ANSIEDAD

La ansiedad es un estado del organismo causado por la anticipación de un peligro o daño, que se da ante una amenaza desconocida, vaga o indefinida, que incluso puede o no estar presente (LeDoux 1996). Desde el punto de vista biológico, la ansiedad puede ser definida como la respuesta adaptativa que prepara a un individuo para detectar y contender contra un peligro real o potencial (Pérez de la Mora et al. 2010). Aunque de acuerdo con esto la ansiedad es una respuesta normal en los individuos, cuando su presencia no guarda relación con el estímulo que la desencadena u ocurre aún en ausencia de este, se habla de ansiedad patológica.

La ansiedad representa un estado de alta excitación que posee una valencia negativa (Russell 1980) y que da como resultado una mayor vigilancia, en ausencia de una amenaza inmediata (Davis et al. 2010). Los trastornos de ansiedad son altamente prevalentes durante la edad adulta (Remes et al. 2016), tienen un comienzo temprano (Kessler et al. 2005) y afectan a una proporción significativa de los adultos jóvenes (Remes et al. 2016). Tales trastornos representan económicamente una carga enorme para los recursos de atención de la salud (Gustavsson et al. 2011) y son comórbidos con una amplia variedad de afecciones médicas, incluidas diversas enfermedades psiquiátricas, neurológicas y cardiovasculares, con una consiguiente importante reducción en la calidad de vida de los sujetos (Shah & Han 2015).

El Manual Diagnóstico y Estadístico de los Trastornos Mentales, en su quinta edición (DSM V), divide a la ansiedad patológica en varias categorías entre los que se incluyen: la ansiedad por separación, el mutismo selectivo, la ansiedad social, la fobia específica, la ansiedad generalizada y los ataques de pánico dentro de otras entidades.

Clínicamente la ansiedad se caracteriza por experiencias subjetivas de tensión, de pensamientos de preocupación y por numerosos cambios físicos que incluyen a la sudoración, mareos e incremento en la presión sanguínea así como del ritmo cardiaco.

Es necesario hacer mención, que los trastornos de ansiedad a nivel mundial tienen una prevalencia estimada, a lo largo de la vida de un individuo, de entre el 13,6% y el 28,8% (Alonso et al. 2004; Kessler 2007) y que debido a que en su mayoría no son atendidos, provocan un gran sufrimiento a los individuos que la padecen y frecuentemente los invalidan.

Para controlar y/o mitigar las consecuencias asociadas a los trastornos de ansiedad, se han implementado, de manera general, dos tipos de tratamientos: la terapia farmacológica y la psicoterapia.

Dentro de la terapia farmacológica de manera general se prescriben los inhibidores de la recaptación de serotonina (SRI) (v.g. fluoxetina), los inhibidores de la recaptación de serotonina y norepinefrina (SNRI) (v.g. venlafaxina), las benzodiacepinas (v.g. clonazepam), los medicamentos anticonvulsivos (v.g. gabapentina) y raramente los antidepresivos tricíclicos (v.g. clomipramina) (Bystritsky et al. 2013). En cuanto a la psicoterapia, la terapia cognitivo conductual es la más utilizada debido a que le enseña a los pacientes a reconocer y cambiar los patrones de pensamiento y las conductas que desencadenan a la ansiedad (Roy-Byrne et al. 2010).

Tanto la terapia farmacológica como la psicoterapia, han demostrado ser efectivas solas o en combinación; sin embargo cuando de ambos tratamientos son combinados se ha demostrado tener una mayor eficacia (Roshanaei-Moghaddam et al. 2011).

2.1 Estudio de la ansiedad

Debido a los altos costos que conlleva el tratamiento de la ansiedad, las pruebas clínicas requeridas para su diagnóstico, así como la dificultad en el desarrollo de terapias farmacológicas específicas que mitiguen sus efectos, resultan indispensables estudios tendientes a entender la etiopatogenia de estos trastornos. Por ello, dado que por razones tanto prácticas como éticas, la experimentación en los humanos resulta limitada, se han desarrollado diversos modelos animales para su estudio que han contribuido a su entendimiento (Harro 2017).

Es necesario, sin embargo aclarar, que a diferencias de los humanos en los cuales la ansiedad es identificada a través de un amplio repertorio de expresiones faciales o por medio de auto-informes, en los roedores su detección resulta más compleja (Adhikari 2014; Degroot & Treit 2004), por lo cual es necesario analizar cuidadosamente sus comportamientos innatos de defensa. Debido a lo anteriormente expuesto, es posible definir a la ansiedad en animales como un estado temporal de comportamiento inducido por estímulos amenazantes difusos (Sylvers et al. 2011).

Los modelos animales de ansiedad se basan en la capacidad de un individuo para responder ante un estímulo que reconocen como ajeno o potencialmente peligroso y que normalmente propicia en ellos una respuesta endócrina y autónoma, como un aumento en la frecuencia cardiaca, la temperatura corporal, el nivel de adrenalina, noradrenalina y de la corticosterona en la sangre (Benelli et al., 2000) (Fig. 1). Adicionalmente, en dichos modelos existen alteraciones de la conducta que tienden a ser extremas y pueden verse reflejadas en un incremento en la locomoción, en conductas de evitación o bien en “inmovilidad” conductual (Steimer 2002).

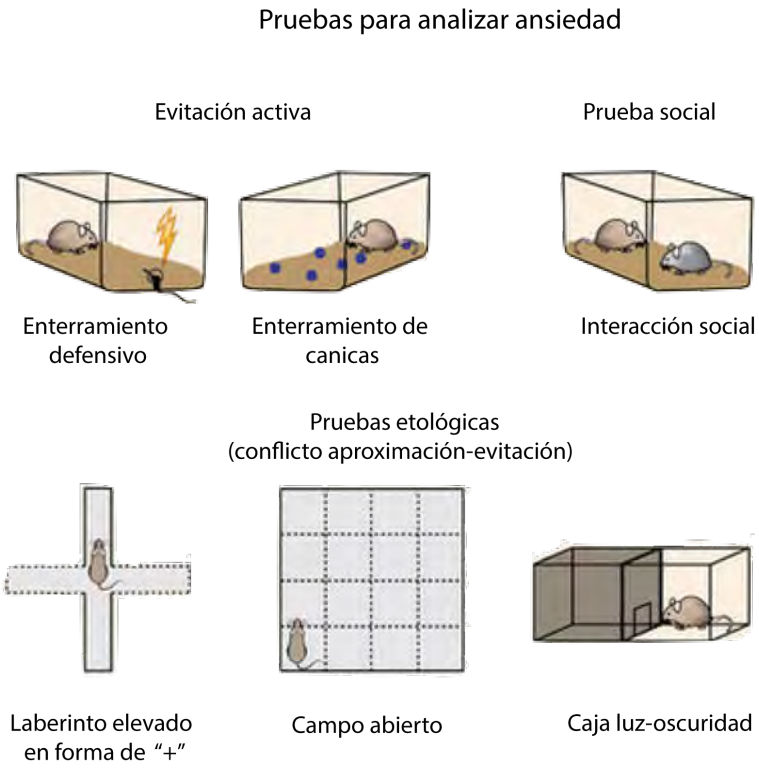

Figura 1. Diferentes tipos de pruebas conductuales empleadas en el estudio de la ansiedad innata en los roedores. En dichas pruebas, se explota dentro de otros rasgos, la tendencia natural de los roedores a evitar espacios abiertos e iluminados (laberinto elevado en forma de "+", campo abierto, caja luz-oscuridad), la presencia de objetos (enterramiento de canicas, enterramiento defensivo) o de conespecíficos (interacción social) potencialmente o abiertamente amenazantes. Imagen modificada de (Calhoon & Tye 2015).

Los modelos desarrollados para el estudio de la ansiedad pueden ser divididos en dos (Rodgers, 1997): los modelos condicionados y los no condicionados. Los modelos condicionados hacen referencia al condicionamiento clásico" o "condicionamiento Pavloviano" que se basan en el "aprendizaje asociativo". En el caso de los modelos no condicionados, explotan comportamientos filogenéticamente heredados o innatos que presentan los animales ante circunstancias que se asemejan a lo que podría ocurrirle a un individuo de su especie en su ambiente natural.

Dentro de los modelos innatos es posible diferenciarlos en aquellos que son empleados preferentemente para evaluar ansiogénesis (v.g. la prueba del enterramiento defensivo) o ansiolísis (v.g. caja luz-oscuridad).

Prueba del enterramiento defensivo

La prueba del enterramiento defensivo fue introducido en el estudio de las neurociencias por Pinel y Treit en 1978. Este paradigma conductual se basa en las respuestas defensivas instintivas de los roedores dirigidas hacia objetos amenazantes. La prueba tiene como objetivo reproducir en un ambiente controlado lo que sucede de forma cotidiana en la vida del roedor, cuando éste se enfrenta a un estímulo aversivo. Durante la evaluación conductual, el animal explora libremente el ambiente que incluye un electrodo que al tocarlo descarga una corriente eléctrica de aproximadamente 0.4 mA. La prueba del enterramiento defensivo representa un modelo adecuado para desentrañar neurobiología de las respuestas de afrontamiento activo y pasivo que se relacionan con ansiedad debido a que son fáciles de cuantificar, las respuestas son observables a partir de un solo choque, cualquier roedor lo presenta sin importar su experiencia previa y no requiere de entrenamientos complejos.

La caja luz-oscuridad

La prueba de exploración de la caja luz-oscuridad se desarrolló por Crawley y Goodwin a principios de la década de 1980. Este modelo animal se basa en aversión innata de los roedores a lugares con luz brillante en ambientes desconocidos, lo cual genera un conflicto inherente entre su impulso exploratorio y su evitación del compartimento iluminado. Durante una sesión de 5 minutos, los animales pueden libremente explorar un ambiente novedoso, a medida que la curiosidad lo impulsa y la ansiedad lo permite, compuesto por dos diferentes compartimentos: protegido (oscuro) y desprotegido (iluminado). El análisis de la conducta emplea medidas espacio temporales, mientras que las medidas dependientes en estos modelos se relaciona con la evitación de un peligro ambiental potencial. En esta prueba, como en otras pruebas para medir la actividad exploratoria, se debe prestar especial atención a la actividad locomotora basal y al comportamiento de búsqueda de novedad, ya que ambos parámetros pueden ser afectados por drogas o cambios genéticos que produzcan falsos resultados. Finalmente, es necesario tener en mente que, aun cuando la

cuantificación de los parámetros espacio-temporales son importantes, se ignoran otras conductas defensivas, tanto de tipo activo como pasivo (inmovilidad, huida, tigmotaxis y asimilación del riesgo).

Adicionalmente, en la actualidad existe una amplia variedad de modelos en los que los animales utilizados son obtenidos a través de líneas desarrolladas y seleccionadas genéticamente para obtener diferencias en su comportamiento en algunas de las pruebas utilizadas para medir la ansiedad experimental (Cryan & Sweeney 2011; Finn et al. 2003). Algunos de esos modelos utilizan cepas exogámicas como la rata Wistar-Kyoto (WKY) (Pare 1994) que presenta un comportamiento más reactivo en comparación con la cepa Sprague-Dawley (McAuley et al. 2009). En otros modelos se seleccionan cepas que difieren en su respuesta ante diversas situaciones ansiogénicas como las ratas de alta (HAB) y baja (LAB) ansiedad (Liebsch et al. 1998), las ratas Tsukuba de alta y baja emocionalidad (Fujita 1975) o las ratas de alta y baja vocalización ultrasónica (Brunelli 2005) por mencionar sólo a algunas. Sin embargo, después de años de experiencia y un análisis complejo de los resultados obtenidos en estos paradigmas fue posible determinar que los modelos animales si bien son útiles en el estudio de la ansiedad se debe tener en mente que cada uno de ellos muestra rasgos específicos de la ansiedad y que los resultados obtenidos en uno no puede ser generalizado a todos.

3. SUSTRATO CEREBRAL DE LA ANSIEDAD

La ansiedad es un fenómeno complejo que por su naturaleza involucra numerosas estructuras anatómicas que la modulan y expresan. Así, en un intento por definir éstas, Papez en (1937) agrupó algunas de ellas dentro de un circuito que a la postre se denominaría “circuito de las emociones ó circuito de Papez”. Dentro de dicho circuito quedaban incluidas estructuras tales como el tálamo, el hipotálamo, el hipocampo y la corteza cingulada, además de la amígdala, y la corteza prefrontal. Posteriormente Paul MacLean (1949) retomando dichas consideraciones y con el ánimo de darles un valor evolutivo propuso la teoría del “cerebro triuno”, que de acuerdo con él, se estructuraba en tres partes:

- *El cerebro instintivo o reptiliano*, que sería el más antiguo desde el punto de vista evolutivo y que incluía el complejo estriatal y los ganglios basales, atribuyéndoles la generación de emociones tan básicas como el miedo y la agresión.
- *El cerebro límbico o mamífero,* que procesaría respuestas más complejas y que incluía a la totalidad de los componentes propuestos en el circuito de Papez.
- *El cerebro cognitivo-ejecutivo,* que evolutivamente sería el más reciente y estaría constituido esencialmente por la neocorteza. Dicho cerebro, de acuerdo con MacLean, conectaría a la emoción con la cognición y ejercería el control sobre las respuestas producidas por los otros sistemas.

En la actualidad se acepta que la actividad de las estructuras que conforman el llamado sistema límbico, conducen a la expresión de conductas emocionales (pendientes de las circunstancias), así como la expresión del sistema nervioso autónomo y las respuestas hormonales en el cuerpo (LeDoux 2012).

A pesar de que como se dijo antes, numerosas estructuras nerviosas están involucradas en la modulación de la ansiedad, la amígdala es sin duda una de las más importantes, pues a ella llega toda la información sensorial relevante que recoge un sujeto de su entorno y en ella se implementa el tipo y grado de sus respuestas ansiosas (LeDoux 2007). Más aún, a pesar de que existen diferencias entre las especies, los aspectos básicos de esta estructura en términos de estructura y función han sido conservados a través de la evolución (Pabba 2013).

La primera referencia a la amígdala fue realizada en 1819 por el anatomista alemán Karl Friedrich Burdach, quien descubrió una masa de color gris localizada en la profundidad del lóbulo temporal con una apariencia similar a la de una almendra, a la que denominó amígdala (del latín almendra) (Burdach 1819).

A lo largo de los años, la amígdala ha sufrido adiciones estructurales y ha sido objeto de diferentes clasificaciones. La primera descripción anatómica de la amígdala fue realizada por Meynert en 1867 (Meynert 1867). Años más tarde, como consecuencia de las numerosas adiciones que esta estructura sufrió se introdujo el término de “complejo amigdalino” ya que algunas de ellas tenían incluso un origen embriológico distinto. Se estableció asimismo la primera clasificación de sus componentes (Johnston 1923), que ha servido de base para las nuevas clasificaciones. Así, con base en la localización anatómica de los núcleos amigdalinos (Price et al. 1987) éstos se agruparon en tres grupos principales: el grupo profundo o basolateral, el grupo superficial o cortical y el grupo centromedial, así como, algunos agrupamientos neuronales que no pudieron incluirse en ninguno de los grupos anteriores. Por otro lado, Swanson y Petrovich (1998) tomando en consideración el origen ontogénetico de los núcleos componentes de la amígdala considera que ellos pertenecen a dos categorías, aquellos que, de acuerdo con ellos, constituyen la extensión ventromedial del claustro y tienen por consiguiente un origen cortical v.g. los núcleos lateral y basolateral y aquellos que provienen del estriado como son los núcleos central y medial, así como las islas intercaladas paracapsulares.

La relación de la amígdala con las emociones fue inicialmente explorada por Brown y Schäfer (1888) así como por Cannon y Bard (1920) quienes la relacionaron con las respuestas emocionales. Sin embargo, fue MacLean quien incluyó a la amígdala en el sistema límbico (MacLean 1949). Cabe señalar, sin embargo, que la relación de la amígdala con las respuestas defensivas del organismo a estímulos amenazantes fue inicialmente estudiada por medio de ablaciones del lóbulo temporal realizadas en monos Rhesus (Macaca mulatta) por Klüver y Bucy (1937), quienes encontraron cambios en los niveles de agresión, miedo y conductas defensivas cuando las lesiones incluían a prácticamente la totalidad del lóbulo temporal. Dichos hallazgos fueron refinados posteriormente identificándose a la amígdala como la responsable de dichos cambios (Weiskrantz 1956).

3.1 Anatomía

Cómo se señaló antes, los núcleos de la amígdala de acuerdo a la clasificación de Price et al. (1987), que será la empleada en este trabajo, se pueden dividir en tres grupos:

1) El grupo profundo o basolateral, que incluye al núcleo lateral, el núcleo basal y el núcleo basal accesorio.

2) El grupo superficial o cortical, que Incluye los núcleos corticales y el núcleo lateral del tracto olfativo.

3) El grupo centromedial. Compuesto de los núcleos medial y central.

Finalmente, hay un conjunto de núcleos separados que no caen fácilmente en ninguno de estos tres grupos y se tratan por separado. Estos incluyen las islas intercaladas y el área amigdalo-hippocampal (Fig. 2).

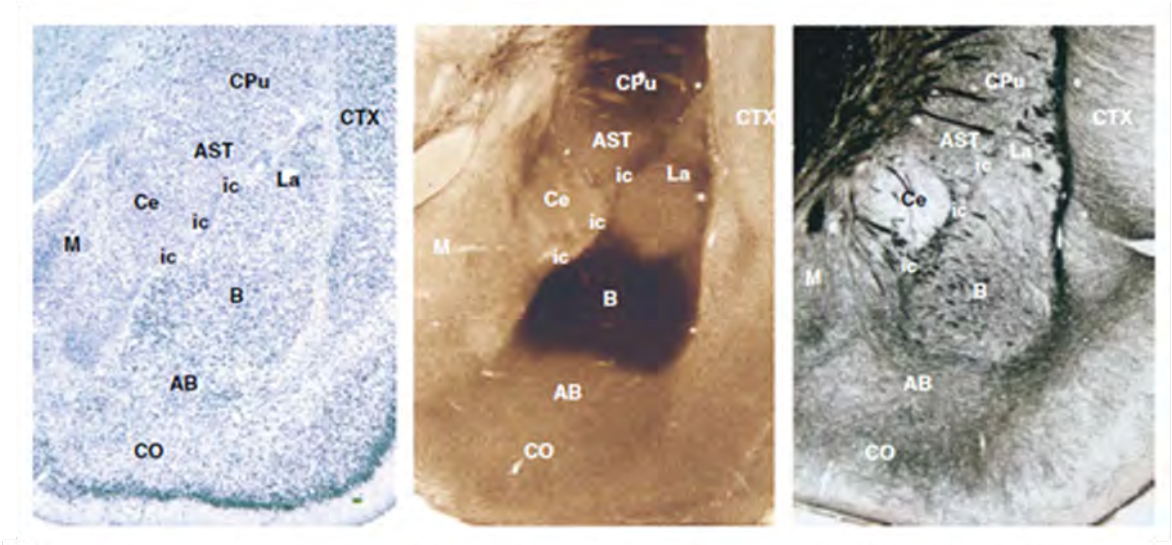

Figura 2. Núcleos amigdalinos en el cerebro de la rata teñidos con diferentes métodos. Panel izquierdo: tinción de Nissl. Panel medio: tinción para la acetilconlinesterasa. Panel derecho: tinción con plata. Abreviaturas: AB, núcleo accesorio basal; B, núcleo basal; Ce, núcleo central; itc, células intercaladas; La, núcleo lateral; M, núcleo medial; CO, núcleo cortical. Áreas que no son de la amígdala: AST, área estriato-amigdalina de transición; CPu, caudado putamen; CTX, corteza. Tomado de LeDoux 2007.

Núcleo Basolateral

El núcleo basolateral está comprendido por el núcleo lateral (LA), el núcleo basal (B), que en otras clasificaciones es llamado el núcleo basolateral (BLA), y el núcleo basal accesorio (AB), que en otras clasificaciones también es llamado el núcleo basomedial (Fig. 3). A menudo, estos tres núcleos son colectivamente referidos como el complejo basolateral (Johnston, 1923).

Núcleos corticales

A pesar que estas estructuras son superficiales se les denomina núcleos. Muchos tienen características corticales ya que están ubicados en la superficie del cerebro y tienen una estructura laminar (Price et al., 1987). Dentro de los núcleos corticales se encuentran el núcleo del tracto olfatorio lateral (NLOT), el núcleo de la cama del tracto olfatorio accesorio (BAOT), el núcleo anterior y posterior cortical (CoA y CoP, respectivamente), y la corteza periamígdalina (PAC).

Núcleos centromediales

El grupo nuclear centromedial se encuentra en la parte dorsomedial del complejo amigdalino y consta de la parte central (CeA), medial (MeA), y el núcleo de la cama de la estria terminal (BNST), que se encuentra fuera de los límites tradicionales de la amígdala, pero dentro de lo que se ha dado en llamar la amígdala extendida (de Olmos & Heimer, 1999).

Otros núcleos amigdalinos

Otros núcleos que no fueron incluidos en la clasificación anterior pero que se encuentran dentro de la amígdala son el área anterior de la amígdala (AAA), el área amígdala-hipocampal (AHA) y las islas intercaladas paracapsulares (IPC). El AHA es la más caudal de los núcleos de la amígdala y se compone de una subdivisión medial y otra lateral. Las islas intercaladas son pequeños grupos de neuronas que se encuentran dentro de haces de fibras que separan a diferentes núcleos amigdalinos (Millhouse 1986; Palomares-Castillo et al. 2012).

La clasificación anterior se ha adoptado por muchos autores, sin embargo algunos otros han sugerido una clasificación diferente que sería más adecuada basada en diferentes características, por ejemplo las conexiones conocidas de la amígdala (Alheid & Heimer, 1988; Alheid et al. 1995), o por las características estructurales y funcionales propias de cada núcleo como lo mencionado por Swanson y Petrovich (Swanson & Petrovich, 1998; Swanson 2000).

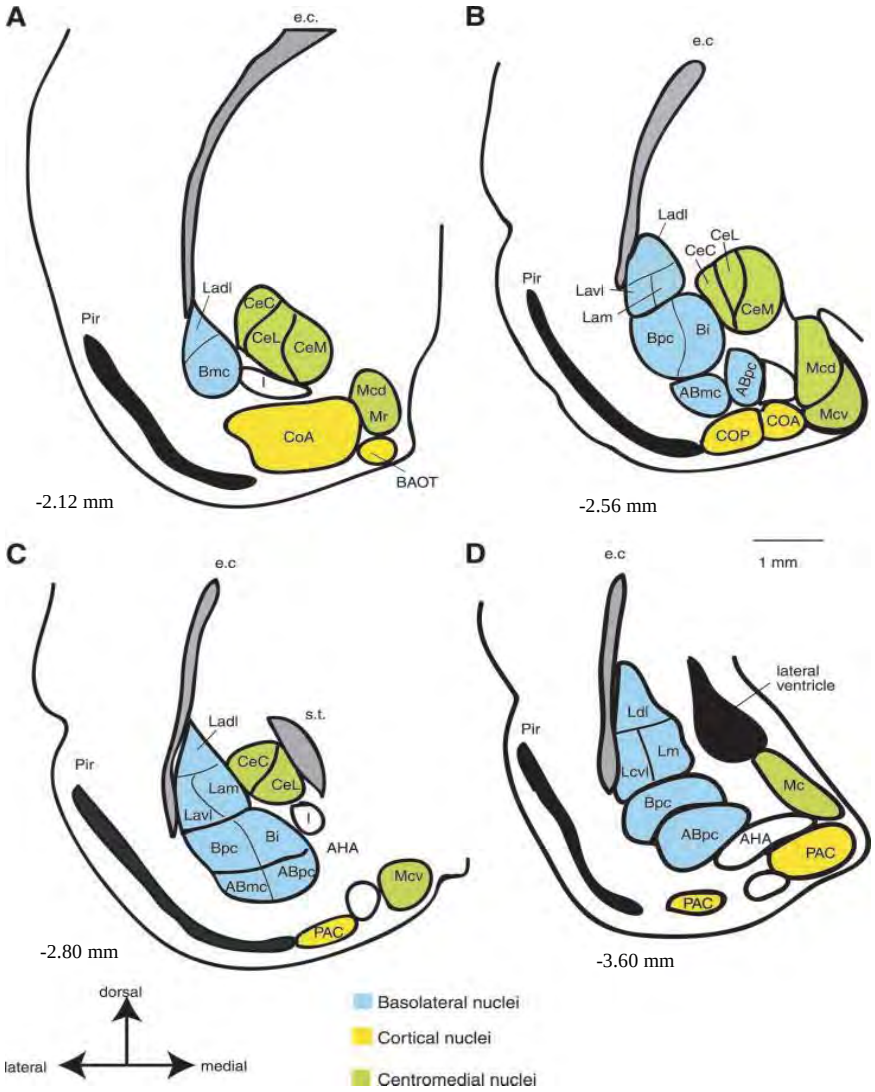

Figura 3. Los núcleos de la amígdala en la rata. Se muestran secciones coronales sucesivas en sentido rostro-caudal (A–D). Los diferentes núcleos se dividen en tres grupos como se describe en el texto. Las áreas en color azul forman el grupo basolateral, áreas en amarillo son el grupo cortical, y las zonas en color verde el grupo centromedial. Abreviaturas: ABmc, núcleo accesorio basal, subdivisión magnocelular; AB, núcleo accesorio basal, subdivisión parvicellular; Bpc, núcleo basal, subdivisión magnocelular; AHA, área amígdala-hipocampal; BAOT, núcleo del tracto olfatorio accesorio; CeC, núcleo central división capsular; CeL, núcleo central división lateral; CeM, núcleo central división medial; CoA, Núcleo anterior cortical; CoP, núcleo posterior cortial; E.C., cápsula externa; I, isla medial; Ladl, amígdala lateral, subdivisión medial; Lam, amígdala lateral, subdivisión medial; Lavl, amígdala lateral, subdivisión ventrolateral; Ldl, amígdala lateral, subdivisión dorsolateral; Lcvl, amígdala lateral, subdivisión ventrolateral; Lm, amígdala lateral, subdivisión medial; Mc, amígdala medial; Mcd, amígdala dorsal subdivisión medial; Mcv, amígdala medial subdivisión ventral; Mr, amígdala medial subdivisión rostral; PAC, corteza periamígdalina; Pir, corteza piriforme; ST, estría terminal. Las coordenadas con respecto a Bregma son mostradas debajo de cada corte coronal. Tomado de Sah, et al. 2003.

3.2 Participación de la amígdala en la integración de la ansiedad

Considerable trabajo realizado en diversos laboratorios ha permitido elaborar un modelo sobre la participación de la amígdala en la ansiedad. Dicho arquetipo (LeDoux 2000) ha sido desarrollado esencialmente utilizando resultados provenientes de paradigmas de ansiedad condicionada, que utilizan pruebas basadas en el aprendizaje asociativo, por lo que podría no necesariamente ajustarse a situaciones más complejas, como las que se presentan en la vida de un sujeto, ni ser válido para explicar la generación de la ansiedad no condicionada. Sin embargo, a pesar de sus limitaciones, dicho esquema ha demostrado ser hasta la fecha convincente ya que permite responder a muchas de las preguntas que se generan en torno a la ansiedad (LeDoux 2000; 2003; 2007).

Para que un organismo reaccione ante una amenaza, ya sea potencial o real es necesario que la información sea procesada por diversas estructuras del SNC para hacer que un sujeto dé una respuesta adecuada al estímulo que la produce (Dalgleish 2004).

A continuación se describen las fases y regiones cerebrales con mayor importancia en este procesamiento:

1. *Percepción del estímulo.*

El primer paso es la percepción del estímulo por las vías sensoriales primarias (visual, sensorial) que envían su información hacia el tálamo (de Olmos & Heimer, 1999).

2. *Procesamiento talámico de la información*

El tálamo es considerado una importante “estación retransmisora” debido a que toda la información sensorial que proviene del medio ambiente, con excepción de la olfativa, es retrasmitida directamente a la amígdala. La información recibida por esta estructura, dependiendo de la situación aversiva que la genera, puede seguir una vía larga o una corta (Debiec & LeDoux, 2004). La

vía corta se refiere a la transmisión de información directa del tálamo hacia la amígdala, lo que genera una representación rápida y poco precisa del estímulo, pero suficiente para conocer si esta es amenazante o no. Esta vía permite dar una respuesta rápida y exitosa al peligro que pone en entredicho la supervivencia del sujeto (Debiec & LeDoux, 2004). Sin embargo, no toda la información sensorial que llega a la amígdala procede del tálamo, ya que se ha reportado que información nociceptiva llega al BLA y CeA a través de la vía espino–parabraquial-amigdalina (Bernard et al. 1989; 1993; Jiménez-Velázquez et al. 2010).

La vía larga involucra a la par de la comunicación del tálamo con la amígdala, el envío de información hacia las cortezas sensoriales primarias y su posterior reenvió hacia la corteza prefrontal medial (mPFC), en donde se realiza un procesamiento más detallado que permite obtener una representación más precisa del estímulo amenazante. Una vez que ésta información ha sido procesada dentro de la corteza prefrontal medial es enviada a la amígdala (Debiec & LeDoux, 2004).

3. *Procesamiento de la información en la amígdala*

Complejo basolateral

La información sensorial proveniente del tálamo y de la corteza llega al complejo basolateral de la amígdala, y es contrastada con la información que le llega del tálamo (vía corta). Una vez que esta información ha sido procesada localmente es redireccionada hacia el CeA, en donde se implementan los componentes autonómicos y hormonales propios de la respuesta ansiosa. A la par de esto, información proveniente del BLA es enviada al núcleo accumbens, en donde la respuesta ansiosa adquiere un componente motivacional, y a la mPFC en donde posiblemente la ansiedad se haga consciente (Davis & Whalen, 2001; Pérez de la Mora et al. 2007b). Por otro lado, es comúnmente aceptado, que dentro del núcleo lateral es donde se produce la plasticidad que se asocia a la información contenida en los estímulos condicionado y no condicionados.

Núcleo central

Una vez que la información generada por la amenaza ha sido evaluada, es enviada al CeA, una estación vital de salida, que es el encargado de generar una respuesta somática y autonómica acorde con la naturaleza y magnitud de la amenaza. La respuesta ansiosa puede implementarse en dicho núcleo debido a que su porción medial es asiento de grupos neuronales con proyecciones hacia los centros responsables de regular funciones autonómicas tales como: la actividad cardiovascular, la respiración, el tono muscular y la intensidad de los reflejos (Pérez de la Mora et al. 2007b) así como de aquellos encargados de modular la actividad del eje hipotálamo-pituitaria-adrenal (HPA), localizados en el tallo cerebral y el hipotálamo respectivamente.

Islas intercaladas paracapsulares

Dado que no existen conexiones entre el núcleo lateral y la porción medial del núcleo central, que como se indicó antes, es la responsable de implementar la respuesta ansiosa se ha sugerido que la información integrada en el complejo basolateral tiene un relevo en las islas intercaladas paracapsulares (IPC) antes de llegar a la porción medial del CeA, pues dichas islas reciben numerosas aferencias del complejo basolateral y proyectan hacia la porción medial del CeA. Tales agrupamientos insulares son conglomerados muy densos de interneuronas GABAérgicas, que se encuentran localizados entre el BLA y CeA (Pare et al. 2004; Perez de la Mora et al. 2006; 2007a; 2008), y específicamente rodeando la mitad rostral del complejo basolateral.

En virtud de que hay evidencia experimental que sugiere que dichas interneuronas ejercen una influencia inhibitoria tónica entre ellas y sobre las neuronas de la porción medial del CeA (Royer et al. 1999), se ha sugerido que pudieran funcionar a manera de una interfase inhibitoria, con la capacidad para regular, tanto espacial como temporalmente el paso de impulsos nerviosos provenientes del complejo basolateral o de otras localidades extra-amigdalinas con dirección a la porción medial del CeA (Royer et al. 1999; Paré et al. 2004; De Celis 2007; Pérez de la Mora et al. 2008).

En resumen, de acuerdo al modelo más aceptado en la actualidad, la información sensorial relevante del medio ambiente alcanza directamente al complejo basolateral de la amígdala vía aferencias talámicas o corticales y despúes de ser procesada localmente la información es enviada a la porción medial del CeA en donde se controlan y median cambios fisiológicos y conductuales, asociados a respuestas defensivas, que ocurren en respuesta a una amenaza (Davis & Whallen 2001; Ledoux 2000). Dada la falta de conexiones entre el BLA y la porción medial del CeA, asiento de las neuronas que proyectan a los núcleos encargados de dar la respuesta ansiosa, se ha propuesto que sean las neuronas GABAérgicas de las islas IPC las encargadas de conectar anatómicamente a estas dos estructuras y modular funcionalmente el paso de impulsos nerviosos entre ellas (Fig. 4).

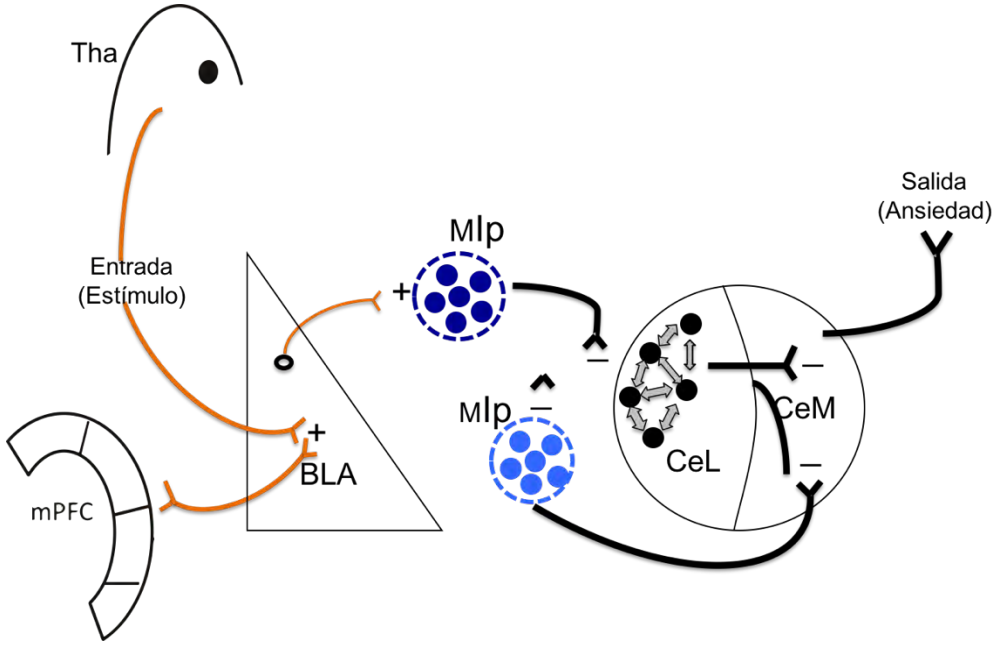

Figura 4. Procesamiento de la información en la amígdala. La información de peligro proveniente del tálamo (Tha) a través de proyecciones excitatorias glutamatérgicas llega al complejo basolateral (BLA), a la par existe comunicación bidireccional con la corteza medial prefrontal (mPFC). La información proveniente del BLA es retransmitida hacia las islas intercaladas paracapsulares (MIp) que forman una interface inhibitoria localizada entre el BLA y el núcleo central (CeA). Las MIp controlan el trafico del impulso nervioso proveniente de BLA modulando a las neuronas de salida del CeA inhibiendo tónicamente tanto en su parte lateral (CeL) en donde llegan proyecciones excitatorias provenientes de su parte medial (CeM) que envía proyecciones a núcleos autonómicos que dan respuesta al impulso ansioso. Tomado de la Mora et al. 2010.

4. EL SISTEMA VASOPRESINÉRGICO

La arginina vasopresina (AVP) es un péptido de nueve aminoácidos: Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2 (Stoop et al. 2015) con un puente disulfuro entre las posiciones 1 y 6 (Fig. 5). Dicho péptido fue conocido inicialmente como hormona antidiurética (ADH) por su participación en la colecta de agua en los túbulos renales (Starling & Verney 1925; Taylor et al. 1953). La arginina vasopresina fue caracterizada por Oliver y Schaefer en 1895 quienes realizaron extractos de la glándula hipofisaria y mostraron que dichos extractos poseían efectos sobre la presión arterial (Oliver & Schafer 1895). Sin embargo, su purificación fue lograda por Du Vigneuad y sus colaboradores hasta el año de 1951, pero no fue sino hasta tres décadas después que la vasopresina fue identificada como un neuropéptido producido en el cerebro.

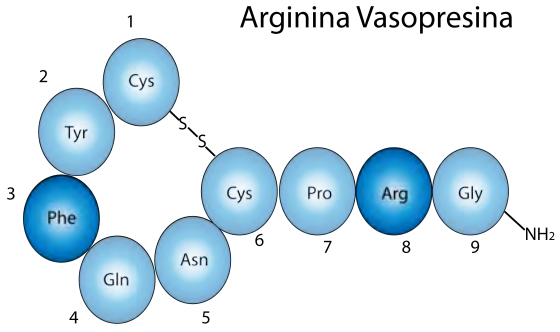

Figura 5. Representación de la estructura química de la arginina vasopresina. Se observan resaltados a la arginina en la octava y a la fenilalanina en la tercera posición.

El sistema vasopresinérgico participa en diversas funciones *e.g* regulación del equilibrio hidro-electrolítico, regulación de la presión sanguínea, regulación de la temperatura corporal, liberación de la corticotropina, participación en ritmos circadianos y conductualmente, en últimas fechas, se le ha relacionado con conductas sociales, aprendizaje, memoria, estrés, ansiedad y depresión (Caldwell et al. 2008; Landgraf & Neumann 2004a).

4.1 Síntesis y liberación

El gen que sintetiza para la vasopresina se localiza en la parte distal del brazo corto del cromosoma 20 (20p13) en humanos, 3 (3q41) en las ratas y 2 (2q73) en el ratón. El precursor de la arginina vasopresina es un pre-propéptido que contiene vasopresina, neurofisina II y la glicoproteína copeptina (Burbach et al. 2001; Young & Gainer 2003). La arginina vasopresina se sintetiza principalmente en el hipotálamo, en dos tipos diferentes de neuronas: las magnocelulares y parvocelulares. Las neuronas magnocelulares se encuentran principalmente en el núcleo paraventricular (PVN) y supraóptico (SON) del hipotálamo (Fig. 6). Extra hipotalámicamente se ha reportado en el núcleo de la cama de la estria terminalis (BNST) y en el núcleo medial amigdalino (MeA) como las dos mayores fuentes productoras de la AVP, ya que se ha demostrado tanto la presencia de RNAm para AVP (Szot & Dorsa 1993) así como cuerpos celulares y fibras (DiBenedictis et al. 2017) que muestran una densa inmunoreactividad para dicho péptido (Hallbeck et al. 1999).

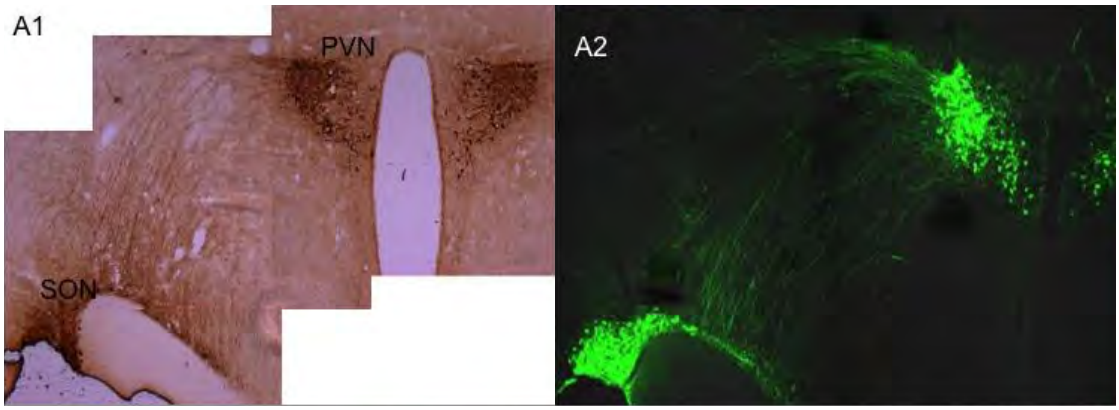

Figura 6. Visualización inmunohistoquímica para la arginina vasopresina dentro del hipotálamo. Note la densa distribución de dicho péptido tanto en el núcleo paraventricular (PVN) como el supraóptico (SON) del hipotálamo, sus principales sitios de producción de vasopresina (A1, A2).

Los axones de las neuronas magnocelulares en el PVN y SON proyectan hacia la pituitaria posterior (neurohipófisis) en donde la AVP se acumula en las terminales nerviosas y los cuerpos de Herring (Laycock 2010).

La AVP es liberada dentro de la glándula pituitaria fuera de la barrera hematoencefalica, lo que permite su rápida integración al torrente sanguíneo (Leng et al. 1999). En contraste, las neuronas parvocelulares del PVN proyectan sus axones a los capilares del plexo portal de la zona externa de la eminencia media del hipotálamo, de donde la AVP es transportada a la hipófisis anterior vía la circulación portal (Scott & Dinan 1998) (Fig. 7).

Cabe señalar, sin embargo, que las neuronas magnocelulares además de liberar a la AVP en la sangre circundante, a través de sus proyecciones axónicas hipotalámicas, liberan también AVP de sus somas y dendritas dentro del PVN y el SON (Ludwig et al. 2005). Dichas neuronas, envían proyecciones a diversas regiones del sistema nervioso central (SNC) tales como el hipocampo, el septo, el núcleo central de la amígdala (CeA) y diversas áreas del tronco cerebral en donde es probable que la AVP sirva como un neurotransmisor (Buijs et al. 1991; Hernández et al. 2016). Una vez liberada, la AVP puede llegar a otras áreas del cerebro a través del fluido extracelular (transmisión por volumen) para modular la actividad neuronal y por lo tanto el comportamiento de un organismo bajo condiciones de estrés o ansiedad (Albers 2015; Engelmann et al. 2004; Landgraf & Neumann 2004b; Ludwig & Stern 2015).

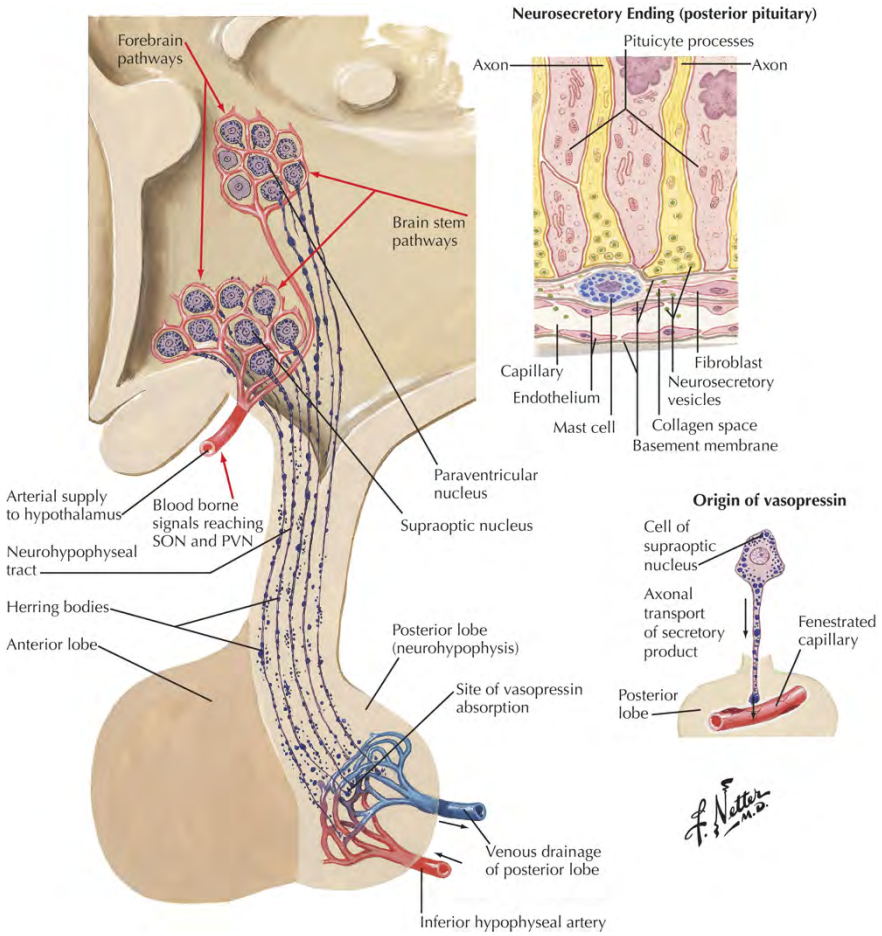

Figura 7. Síntesis y liberación de AVP. Note como la arginina vasopresina que es sintetizada en las neuronas magnocelulares de los núcleos PVN y SON es transportada por sus axones a la hipófisis posterior en donde es liberada al torrente circulatorio. PVN, núcleo paraventricular del hipotálamo; SON, núcleo supraóptico del hipotálamo. Tomado de Holt & Haspel, 2010.

4.2 Receptores vasopresinérgicos

La vasopresina ejerce sus efectos a través de tres receptores: V1a, V1b y V2 (Koshimizu et al. 2012; Zelena 2012). Dichos receptores son transmembranales y están acoplados a proteínas G (GPCR). Por consiguiente poseen siete dominios transmembranales, con estructura de α hélice, con un dominio N-terminal extracelular y un dominio citoplasmático C-terminal (Villabona 2010).

Los receptores V1 se localizan en el cerebro (V1a, V1b) y se acoplan a proteínas Gαq/11 que activan a la fosfolipasa C (PLC), la cual fosforila fosfoinosítidos membranales liberando inositol trifosfato (IP3) y dando lugar a la formación de diacilglicerol (DAG) que permanece en la membrana. El IP3, a su vez, facilita la liberación de calcio intracelular del retículo endoplásmico mientras que el DAG activa a la proteína quinasa C (PKC) en la superficie membranal. La PKC activada fosforila proteínas celulares que son responsables de las respuestas celulares características de estos receptores (Birnbaumer 2000; Treschan & Peters 2006) (Fig. 8).

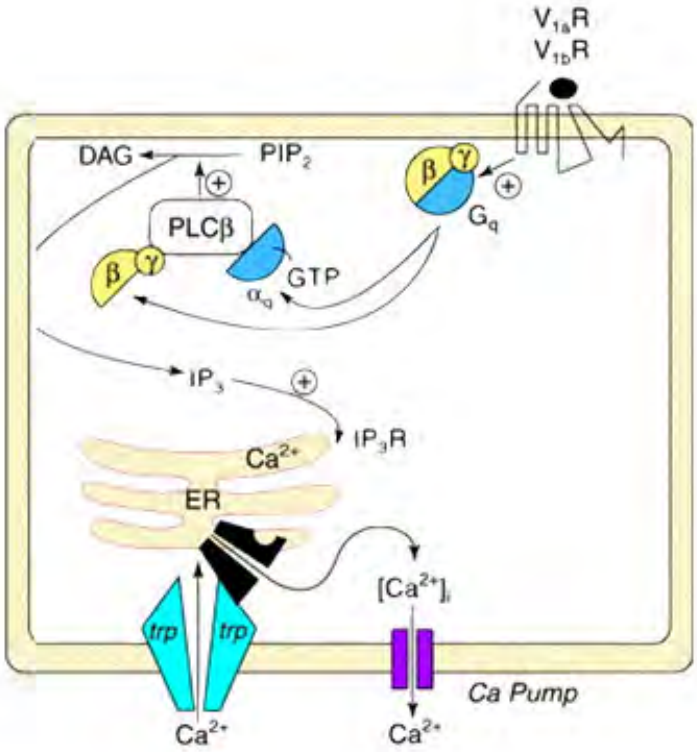

Figura 8. Transducción de señales a partir de los receptores V1. La señalización de los receptores V1 promueven la disociación de $G_{q/11}$ a GTP. La subunidad alfa (a_q) induce la estimulación de la actividad de PLC beta (b) que promueve la hidrólisis de PIP_2, aumentando los niveles intracelulares de DAG y IP_3. El IP_3, a su vez estimula los IP_3R localizados en la membrana del RE y promueve la liberación de Ca^{2+} de las reservas intracelulares. Además de activar la señalización cascada abajo, la liberación de Ca^{2+} activa canales catiónicos divalentes denominados *trp* que permiten el flujo de Ca^{2+} extracelular hacia el interior de la célula para reponer las reservas. Abs: DAG, diacilglicerol; ER, retículo endoplasmico; GTP, guanosín triifosfato; Gq α β γ , sub unidades de la proteína G; IP3R, receptor para inositol (1,4,5)-trifosfato; PIP2, fosfatidilinositol 4,5-bisfosfato; PLCb, fosfolipasa Cb. Imagen modificada de Birnbaumer 2000.

Los receptores V1a se encuentran en una gran variedad de tejidos que incluyen al músculo liso vascular, hígado, riñón, y bazo (Koshimizu et al. 2012), mediando diversos efectos biológicos, incluyendo la inducción de hipotensión, vasoconstricción, gluconeogénesis y agregación plaquetaria (Zingg 1996). Cabe señalar, que el receptor V1a es el receptor más prominente en diversos núcleos del cerebro como son el hipotálamo, el septum, el hipocampo, el núcleo de la cama de la estría terminal (BNST), la amígdala central (CeA), el cerebelo, el órgano subfornical y el plexo coroideo (Huber et al. 2005; Ostrowski et al. 1994; Szot et al. 1994). Por su lado, el receptor V1b que se describió inicialmente en la

adenohipófisis, como participante en la secreción de corticotropina (ACTH) a la circulación (Sugimoto et al. 1994) ha sido detectado en un buen número de regiones cerebrales tales como: el hipocampo, la zona exterior de la eminencia media y la amígdala (Corbani et al. 2017; Hernando et al. 2001; Lolait et al. 1995; Stemmelin et al. 2005; Vaccari et al. 1998; Young et al. 2006) entre otras (Herman et al. 1998). El receptor V2, se localiza principalmente en el riñón, en donde participa en las acciones antidiuréticas de la AVP. Resulta sin embargo interesante, que dicho receptor dentro del cerebro ha sido localizado únicamente en el cerebelo.

Llegados a este punto es necesario mencionar la existencia de la oxitocina (OT), un péptido muy similar a la AVP, de la que únicamente se diferencia por un cambio de dos aminoácidos en su secuencia. Debido a su similitud estructural se ha demostrado que los receptores Gq para OT son aparentemente no selectivos, ya que muestran una afinidad similar entre OT y AVP (Gimpl & Fahrenholz 2001). Los receptores para OT se expresan en el cerebro en el núcleo olfatorio, el BNST, el CeA, el hipocampo ventral y el núcleo ventromedial (Huber et al. 2005; de Kloet et al. 1986).

4.3 La arginina vasopresina y su relación con la ansiedad

Como se ha descrito antes, la AVP posee diversas actividades funcionales entre las que se incluyen la conducta, el aprendizaje, el proceso de memoria, la emocionalidad y el afrontamiento del estrés (Ebner et al. 1999; Engelmann et al. 1994; Wigger et al. 2004; Young 2001) así como el control de la actividad del eje hipotálamo-pituitaria-adrenal (HPA) (Keck et al. 2002; Wotjak et al. 2002).

Los primeros trabajos que relacionaron a la AVP con conductas relacionadas con la ansiedad fueron llevados a cabo por Gold y sus colaboradores en 1978, quienes postularon que la deficiencia de la AVP producía déficits conductuales que podían ser revertidos tras su administración (Gold et al.

1978) e intentaron relacionar a este neurotransmisor con padecimientos derivados del estrés, como son la ansiedad y la depresión mayor. Postularon asimismo, la existencia de vías vasopresinérgicas en SNC que participarían en el control de diversos procesos fisiológicos y en las enfermedades afectivas. Desde entonces se han desarrollado diferentes estudios para confirmar estas ideas.

Particularmente interesantes a este respecto resultan las ratas Brattleboro, que se descubrieron a principios de 1960 y que derivan de la cepa Long Evans. (Sawyer et al. 1964; Sokol and Valtin 1965; Sokol & Zimmerman 1982). Dichas ratas tienen una mutación en el gen de la neurofisina que está presente, como se indicó antes, en el prepropéptido que posee la secuencia requerida para la síntesis de la AVP y que como consecuencia de ello hace imposible su secreción (Bohus & de Wied 1998; Evans et al. 2000; Surget & Belzung 2008). Como resultado de dicha mutación, las ratas Brattleboro muestran una disminución en los niveles de ansiedad en comparación con las ratas Long Evans (Williams et al. 1983), una alta actividad exploratoria (Balazsfi et al. 2015) y un perfil de depresión baja (Mlynarik et al. 2007).

Así mismo, con la finalidad de estudiar a fondo los mecanismos por los cuales se origina la ansiedad, una de las primeras estrategias utilizadas para ello fue la de realizar una selección artificial de ratas, separando aquellas que mostraban mayores niveles de ansiedad (ratas HAB) en el laberinto elevado en forma de “+” (EPM, por sus siglas en ingles) (Hess et al. 1992) de aquellas con menores niveles de ansiedad (ratas LAB) en dicho modelo. El establecimiento de estas dos líneas de ratas coadyuvo a establecer una relación entre la AVP y la ansiedad, ya que al medir la expresión de la AVP y la de su RNAm, en el PVN fue posible encontrar que dichos marcadores estaban más expresados en las ratas HAB que en las LAB (Keck et al. 2003). Estos resultados son congruentes con los hallazgos de Bunck et al. (2009) quienes reportaron una disminución de la expresión del gen de AVP en el PVN y SON en ratones LAB con respecto con la expresión de dicho gen en ratones HAB.

Por otro lado, estudios sobre la existencia de diversos tipos de polimorfismos ligados al gen de la AVP demostraron que los individuos que poseían polimorfismos asociados a una sobreexpresión de dicho neuropéptido, mostraban un aumento de la ansiedad (Murgatroyd et al. 2004).

Con la intensión de explorar los efectos centrales y periféricos de la AVP, se han desarrollado diversos agonistas y antagonistas para los receptores vasopresinérgicos destacando por su efectividad y especificidad: el compuesto de Manning, un antagonista selectivo (Ki 0.7 nM; Manning et al. 2008) para los receptores V1a y el SSR149415, un antagonista selectivo (Ki 1.3 nM; Roper et al. 2011) para los receptores V1b. Debido a la similitud con la oxitocina también se ha empleado en diversos estudios el OTA, un antagonista selectivo (Ki 0.2 nM; Maning et al. 2008).

Los estudios farmacológicos realizados han demostrado en su mayoría efectos ansiogénicos inducidos por la AVP, sin embargo, en algunos casos pueden resultar confusos. Ejemplo de ello, es que en tanto que la administración intraseptal e intraperitoneal (i.p.) de la AVP produjo efectos ansiolíticos en el EPM (Appenrodt et al. 1998), el bloqueo de los receptores V1a con el antagonista $d(CH_2)_5$Tyr(Me)AVP disminuyó los niveles de ansiedad (Liebsch et al. 1996) en el mismo modelo y región sugiriendo un papel ansiogénico para la AVP. En línea con estos últimos resultados, la administración a ratas de un oligo-desoxiribonucleotido anti sentido para los receptores V1a en el septum redujo sus niveles de ansiedad cuando estas fueron expuestas al EPM (Landgraf et al. 1995). A pesar de la disparidad en los efectos, dichos resultados podrían ser explicados debido a las diferencias en cuanto a las vías de administración empleadas, así como los fármacos (v.g. agonista, antagonistas) y las dosis administradas durante los experimentos.

Por lo que toca al papel de los receptores V1b, la disponibilidad de un antagonista específico para ellos, el SSR149415 (Serradeil-Le Gal et al. 2005), ha permitido analizar su papel en la regulación de la ansiedad. Los resultados de experimentos en los que este compuesto ha sido administrado oralmente a ratas, han mostrado que este antagonista tiene un poderoso efecto ansiolítico en varios modelos de ansiedad, entre los que se incluye el EPM. Resulta interesante para este trabajo, que la administración de SSR149415 en el septum lateral no tuvo efectos observables en las ratas al ser evaluadas en el EPM, ni en la prueba de conflicto de Vogel (Stemmelin et al. 2005) pero sí cuando fue infundido dentro de la amígdala y las ratas fueron evaluadas en el EPM. Por otro lado, los efectos ansiolíticos del SSR149415 únicamente se registraron cuando este compuesto fue administrado en el BLA, y no en el CeA ni en el MeA (Salome et al. 2006). Los resultados obtenidos sugieren que los receptores V1b juegan un papel ansiogénico en la modulación amigdalina de la ansiedad.

Por otro lado, estudios realizados utilizando ratones transgénicos dieron apoyo adicional al papel ansiogénico de los receptores V1a en la modulación de la ansiedad. Así, en ratones macho knockout (KO) para el receptor V1a se observó una disminución en los comportamientos asociados a la ansiedad en comparación con ratones no manipulados (wild type) (Bielsky et al. 2004; Egashira et al. 2007). Congruentemente, la sobreexpresión del gen para V1a en el septum lateral resultó en un incremento de las conductas asociadas a la ansiedad (Bielsky et al. 2004).

En lo concerniente al receptor V1b, las manipulaciones genéticas para silenciar el gen que codifican para el receptor V1b, demostraron efectos conductuales inconsistentes al disminuir conductas asociadas a la ansiedad y en otros no se observó ningún efecto sobre la ansiedad (Egashira et al. 2005; Wersinger et al. 2002).

4.4 La arginina vasopresina en la amígdala

La presencia de la AVP ha sido reportada en diversas estructuras cerebrales, dentro de las que se encuentra la amígdala, una estructura que es reconocida como clave en el procesamiento de la ansiedad.

Dentro de la amígdala se ha indicado que la AVP se sintetiza, esto debido a que dentro del MeA y el BNST se ha demostrado la presencia de RNAm (Szot & Dorsa 1993) así como de células productoras y fibras vasopresinérgicas (Buis 1978, 1980; 1991; Caffé et al. 1987; DiBenedictis et al. 2017) que se comunican de manera indirecta con el hipotálamo (Jankord & Herman 2008). Así también se conoce que numerosas fibras vasopresinérgicas provenientes del PVN y el SON atraviesan tanto el AHA como el BLA (Zhang & Hernández 2013) y que terminales vasopresinérgicas inervan el BNST, el BLA y el CeA (Hernández et al. 2016; Zhang & Hernández 2013).

Como se mencionó antes en el SNC únicamente se han reportado los receptores V1 (a, b) para la AVP. En la amígdala, se ha descrito la presencia de los receptores V1a en la parte lateral del BNST y la parte medial del CeA (Huber et al. 2005; Stoop et al. 2015). En cuanto a los receptores V1b, su distribución ha sido ampliamente discutida, debido a que se creía que únicamente se hallaban en la pituitaria, en donde modulan el eje HPA. Sin embargo, se ha reportado tanto inmunoreactividad por el receptor (Hernando et al. 2001; Stemmelin et al. 2005) así como la presencia de RNA en la amígdala (Young et al. 2006). Estudios recientes han permitido identificar de manera especifica la existencia de los receptores V1b en el BLA y el CeA, utilizando manipulaciones genéticas y ligandos fluorescentes (Corbani et al 2017).

5. PLANTEAMIENTO DEL PROBLEMA

La ansiedad es el padecimiento psiquiátrico más frecuente en México y el mundo alcanzando una prevalencia cercana al 25 %. Su tratamiento, por tanto, representa una carga económica para los servicios de salud pública y un sufrimiento considerable para el caso de los pacientes, a los que en ocasiones puede llegar a incapacitar.

En la actualidad no se conoce en su totalidad, el sustrato neurobiológico de la ansiedad, ni los mecanismos que la subyacen. Es imperativo, por tanto, la realización de estudios que subsanen estas carencias y que permitan el desarrollo de estrategias tanto conductuales como farmacológicas para su control/tratamiento.

Diversos sistemas de neurotransmisión están involucrados en la modulación de la ansiedad, la arginina vasopresina ha sido implicada recientemente en dicha modulación, sin que se conozcan, los sitios anatómicos de su acción, ni menos aún, los mecanismos mediante los cuales ejerce sus efectos ansiogénicos.

Dado que la amígdala, tiene un papel fundamental en la modulación de la ansiedad y que a su núcleo central llegan numerosas terminales vasopresinérgicas provenientes del núcleo paraventricular del hipotálamo, creemos de gran interés, estudiar la participación de esta estructura en la generación de las acciones ansiogénicas de este neurotransmisor, concediendo particular énfasis a la participación de sus receptores V1b. Consideramos, que los resultados obtenidos en este trabajo serán de importancia para entender la participación de los receptores vasopresinérgicos en la modulación amigdalina de la ansiedad y la posible activación diferencial entre los receptores.

6. HIPÓTESIS

Los receptores vasopresinérgicos V1b presentes en el núcleo central de la amígdala (CeA) modulan los efectos ansiogénicos ejercidos por la AVP de manera independiente a los receptores V1a.

7. OBJETIVO GENERAL

Estudiar la neurotransmisión vasopresinérgica en la modulación amigdalina de la ansiedad innata, y esclarecer si los subtipos de receptores de la AVP pueden tener un papel diferencial.

7.1 Objetivos particulares

1. Establecer el papel de la arginina vasopresina en la modulación amigdalina de la ansiedad innata.
2. Establecer el posible papel diferencial que tiene los receptores V1a y V1b en la modulación amigdalina de la ansiedad innata.
3. Establecer si la actividad de los receptores V1b para la arginina vasopresina sobre la ansiedad innata es general o depende del modelo utilizado.

8. PROCEDIMIENTOS EXPERIMENTALES

8.1 Animales

Para las evaluaciones conductuales fueron empleadas ratas macho de la cepa Wistar con un peso de 250 a 260 g, criadas en el bioterio del Instituto de Fisiología Celular. Las ratas fueron hospedadas en un ambiente controlado (22°C; ciclo luz/oscuridad 12/12 h, luces encendidas de 7:00–19:00 h) con agua y alimento (dieta de nutrición constante para ratas de laboratorio, Rat Diet 500i) disponible *ad libitum*. Todos los experimentos fueron realizados en la fase de luz del día (9:00-18:00 h) de acuerdo con las especificaciones técnicas para el cuidado y uso de animales de laboratorio establecidas en la Norma Oficial Mexicana (NOM-062-Z00-1999), los lineamientos establecidos por el comité de ética local, y en acuerdo con el "International Guiding Principles for Biomedical Research Involving Animals", Council for International Organization of Medical Sciences, 2010. Todos los procedimientos experimentales fueron realizados reduciendo el sufrimiento de los animales al mínimo. En todos los experimentos las ratas fueron asignadas a los grupos experimentales de manera aleatoria.

8.2 Cirugía y microinyección

Para la implantación permanente de cánulas guía dentro de la amígdala, las ratas fueron anestesiadas por vía intraperitoneal con una mezcla de clorhidrato de ketamina (60 mg/kg) y xilazina (5 mg/kg), ambas adquiridas de Pisa Agropecuaria (Atalaquie, Hgo, México) y fijadas en un aparato estereotáxico (Kopf Instruments, Tujunga, CA, USA) con la barra de incisivos a **-**3.3 mm. La temperatura corporal de las ratas fue mantenida a 37°C usando un controlador de temperatura (CMA/150 (CMA/Microdialysis, Stockholm, Sweden). Cánulas de 46 mm de diámetro (C315G, Plastics One, Roanoke, VA, USA) fueron dirigidas bilateralmente hacia el CeA (coordenadas AP: -1.7 mm, ML: ± 4.2 mm a partir de bregma, DV: **-**7.7 mm de la superficie del cráneo, de acuerdo con el atlas de Paxinos y Watson (1986). Las cánulas guía fueron fijadas con ayuda de tornillos

de acero inoxidable y acrílico dental (Laboratorios Arias, México City, México) y se les colocaron dentro “mandriles” (C315DC, Plastics One) para mantenerlas sin ser bloqueadas. Para prevenir infecciones se administró Estreptobezetazil V-Fortificado (Fort Dodge Animal Health Laboratories, México City, México) por vía intra-muscular. Para evitar el desprendimiento de las cánulas, las ratas fueron hospedadas en cajas individuales hasta el día de la microinyección.

Después de 7 días de recuperación de la cirugía y de la anestesia, las ratas fueron manipuladas una vez al día durante 5 min por un lapso de cuatro días para la prueba del enterramiento defensivo y tres días para la caja luz-oscuridad antes de ser tratadas y evaluadas conductualmente.

8.3 Administración de fármacos

La arginina vasopresina ([Arg8]-Vasopressin acetate salt) fue adquirida de Sigma Chemical Co. (St. Louis, MO, USA), el antagonista V1a (d(CH_2)$_5^1$,Tyr(Me)^2,Arg^8)-Vasopressin trifluoroacetate salt (Compuesto de Manning) y el antagonista de la Oxitocina (OTA, d(CH_2)$_5^1$,Tyr(Me)^2,Thr^4,Orn^8,des-Gly-NH_2^9)-Vasotocin trifluoroacetate salt se adquirieron de BACHEM Pioneering partner for peptides (Torrance, CA, USA), el antagonista V1b (SSR149415) fue adquirido en Axon Medchem (Groningen, GRQ). El SSR149415 se preparó en una solución 0.9% (p/v) NaCl conteniendo el 5% DMSO (Sigma, Lyon, France) y 5% Cremophor EL (Sigma, Lyon, France). [FITC] [Ahx]CYIQNCPLG[Amida] fue adquirido de Biosynthesis Committed to Biomic Research, (Lewisville, TX, USA).

En el día de las evaluaciones conductuales, las ratas fueron administradas bilateralmente con: AVP (1 o 10 ng/lado); SSR149415 (1 o 10 ng/lado), compuesto de Manning (10 o 30 ng/lado), u OTA (15 ng/lado) como se muestra en la Tabla 1. Los tratamientos fueron administrados vía cánulas de microinyección (0.20 mm diámetro externo, C315I, Plastics One), que protruía 1 mm del final de la cánula guía durante un periodo de 5 min, usando dos bombas de microdialisis (CMA/ Microdialysis, Stockholm, Sweden). Los tratamientos

fueron microinfundidos en un volumen de 250 nl/lado. Las cánulas permanecieron en el sitio de la inyección durante 60 s después de la microinyección para prevenir el reflujo de los fármacos inyectados y para permitir su correcta difusión. Las observaciones conductuales en cada prueba de ansiedad comenzaron inmediatamente después de la microinyección de los fármacos. El rango de dosis de la AVP usados se basaron en los estudios de Appenrodt y Schwarzberg (2000). En el caso de los antagonistas, las dosis se basaron en lo reportado por Hernández et al. (2016) para los receptores V1a (compuesto de Manning), lo descrito en el trabajo de Salomé et al. (2006) para los receptores V1b (SSR149415) y el trabajo de Lazslo et al. (2016) para los receptores para la OT. Diferentes grupos de ratas fueron usadas para la prueba del enterramiento defensivo y la caja luz-oscuridad.

En los experimentos en los cuales fueron usados antagonistas, se infundió de manera simultánea tanto el agonista como el antagonista con intención de que la relación de sus concentración se mantuviera inalterada en su sitio de inyección y por consiguiente estar seguros de que la unión a sus receptores dependiera esencialmente de su afinidad por ellos. Además, al administrar bajo estas condiciones ambos fármacos se evitó una doble microinyección, lo que redujo el riesgo que el segundo fármaco siguiera un camino diferente debido al daño tisular o a cambios en la presión tisular producidos por el alto volumen inyectado.

Tabla 1. Tratamientos administrados durante los experimentos conductuales

Experimentos	Tratamientos por grupo (ng/lado)					
Exp. 1 Vasopresina	SSF	1 AVP	10 AVP			
Exp. 2 Receptor V1B	Vehículo	1 AVP	10 SSR	10 SSR	1 AVP 1 SSR	1 AVP 10 SSR
Exp. 3 Receptor V1A	SSF	1 AVP	10 MC	1 AVP 10 MC	1 AVP 30 MC	
Exp. 4 Receptor OT	SSF	1 AVP	15 OTA	1 AVP 15 OTA		

AVP, arginina-vasopresina; MC, compuesto de Manning; OT, oxitocina; OTA, antagonista para la oxitocina; SSF, solución salina fisiológica; SSR, SSR149415.

8.4 Extensión de la difusión de vasopresina después de su microinyección en la amígdala

Con la intención de conocer la magnitud de la difusión de la AVP dentro del CeA, en un grupo de ratas distintas a las empleadas para las evaluaciones conductuales, fue infundido en la amígdala [FITC] [Ahx]CYIQNCPLG[Amide], un análogo fluorescente de oxitocina con una composición de aminoácidos y un peso molecular similar a la AVP, bajo las mismas condiciones empleadas para la administración de la oxitocina. Diez minutos después de la infusión, las ratas fueron sacrificadas mediante una sobredosis de pentobarbital sódico. Los cerebros fueron removidos y congelados (30-60 s) en isopentano enfriado en hielo seco. Secciones de cerebro de 40 µm fueron obtenidos con ayuda de un criostato y montados con Krystalon en laminillas gelatinizadas. Las secciones de cerebros fueron aireadas por 5 días a temperatura ambiente y se observaron bajo un microscopio de fluorescencia (BX41 Olympus; filtro de emisión U-MNU2). El volumen de difusión del sitio de inyección se calculó usando el diámetro de la imagen fluorescente más grande y empleando la fórmula de una esfera ($3/4\pi R^3$) centrada en el sitio de inyección.

8.5 Evaluaciones conductuales

8.5.1 Prueba del enterramiento defensivo (SPBT)

El procedimiento se realizó de acuerdo con lo descrito por Treit et al. (1981) y por Fernandez-Guasti et al. (2001). La prueba se llevó a cabo en una caja elaborada con acrílico con medidas de 27x16x23 cm. El suelo de la caja utilizada se encontraba recubierto uniformemente por una cama de aserrín de pino de 5 cm de grosor. En una de las paredes del dispositivo, 2 cm por encima del nivel de la cama de aserrín, se colocó un electrodo (“probe”) electrificado a permanencia de 7 cm de largo y 0.5 cm de diámetro que generaba una descarga eléctrica de 0.4 mA (generador de choques constantes; LaFayete Instruments, Inc.) cada vez que la rata lo tocaba con el hocico o las extremidades. La rata fue colocada, al inicio

de la prueba, en el extremo contrario al electrodo y se le permitió explorar la caja por 10 min a partir de haber recibido el primer choque. Los experimentos se realizaron con una iluminación roja de 2,3 a 2.5 luxes. Durante la evaluación de la conducta se registraron como indicadores de ansiedad la latencia al primer episodio de enterramiento tras recibir el primer choque eléctrico, el número de choques recibidos y el tiempo total que pasó la rata enterrando al electrodo. El tiempo de enterramiento se tomó como una medida de ansiedad, la medida de latencia como índice de reactividad y el número de choques como una indicación del grado de nocicepción/aversión a la descarga eléctrica.

Además de las respuestas conductuales de tipo activo reflejadas durante la prueba, el efecto de los distintos tratamientos sobre el congelamiento conductual, una respuesta ansiosa de tipo pasivo, también fue monitoreada. El tiempo de congelamiento, definido como el porcentaje del tiempo de la prueba en que el animal realizaba únicamente aquellos movimientos necesarios para la respiración fue utilizado como parámetro. Los datos del congelamiento fueron obtenidos a partir de la siguiente manera operación:

$$\text{Porcentaje de congelamiento} = \frac{\text{Tiempo de inmovilidad (s)}}{\text{Tiempo total de la prueba del enterramiento defensivo (600 s)}} \times 100$$

8.5.2 Caja Luz-Oscuridad

La conducta se evaluó como fue descrito previamente en de la Mora et al. 2005. La prueba se realizó en una caja de acrílico dividida en dos compartimentos conectados por una pequeña abertura (7x7 cm). Las paredes y el suelo de uno de los compartimientos (27x27x27 cm) eran de color blanco y un compartimiento más pequeño (27x18x27 cm) era de color negro. Cuadrados de 9x9 cm fueron marcados en el suelo en ambos compartimientos usando líneas negras o blancas respectivamente. Durante los experimentos, el compartimiento blanco fue

iluminado intensamente con luz blanca fría (700 luxes), el compartimiento negro fue iluminados con luz de baja intensidad (70 luxes). Al inicio del experimento, cada rata fue colocada en el centro del compartimiento blanco en frente a la abertura de los dos compartimientos y la conducta fue videograbada durante 5 min. El tiempo de latencia a la primera entrada al compartimiento negro y el tiempo total de permanencia en el compartimiento blanco, fueron tomados como medidas de ansiedad (Costall et al. 1989; Henniger et al. 2000). La caja fue limpiada con Dextran y secada antes de cada prueba.

8.5.3 Campo abierto

Con la finalidad de analizar la influencia de la AVP sobre la locomoción de las ratas, se sometió a estas al paradigma de campo abierto inmediatamente después de haber sido probadas en cualquiera de los modelos de ansiedad utilizados. La prueba de campo abierto se llevó a cabo en una caja de acrílico de 50x50x30 cm equipada con celdas fotoeléctricas, que detectaban los movimientos de los animales dentro de la caja mediante el registro del número de rompimientos de los haces de luz que ocurrían entre ellas (OMNIALVA, Ciudad de México). Cada una de las paredes del dispositivo contenía 10 celdas fotoeléctricas separadas entre sí por un espacio de 5 cm. Las celdas fueron colocadas a 4 cm por encima del suelo. Para el registro de los movimientos de las ratas, la caja fue sincronizada con una PC que permitía estimar cuantitativamente el número de interrupciones de los haces de luz que ocurrían entre las fotoceldas. Dichas interrupciones fueron transformadas en “eventos arbitrarios” de locomoción con una frecuencia de muestreo de 10 Hz. El nivel de iluminación utilizado en el interior de la caja fue de 138 luxes, intensidad que se ha demostrado no afecta la locomoción de las ratas (Bouwknecht et al. 2007). Al comenzar la prueba, las ratas fueron colocadas en una de las esquinas de la caja y a continuación se les permitió explorarla libremente durante 5 min. Mediante el software OMNIALVA fue posible registrar los movimientos de la rata en la caja

(número de eventos) y determinar el tiempo de permanencia de las ratas tanto en su parte central como periférica (Prut & Belzung 2003).

Todos los experimentos conductuales fueron llevados a cabo en una habitación sono-amortiguada equipada con equipo para videograbar, los videos fueron posteriormente analizados.

8.6 Evaluación histológica

Una vez terminadas las pruebas de conducta, con objeto de verificar la correcta posición de las cánulas implantadas, los animales se anestesiaron profundamente con una sobredosis de pentobarbital sódico (65 mg por rata), y se les administró bilateralmente 250 nl/lado azul de Pontamina a través de las cánulas de inyección usadas para la administración de la AVP. Los cerebros fueron removidos y fijados en formaldehído al 10% durante una semana. Para protegerlos en contra de los efectos de la congelación los cerebros se sumergieron en soluciones de sacarosa con una concentración creciente (10%, 20% y 30%), permaneciendo en cada una de ellas, por un tiempo mínimo de 24 h. Los cerebros fueron posteriormente seccionados obteniéndose cortes coronales de 40 µm mediante un criostato (CM 1510-3, Leica Instruments, Nussloch, Germany) a una temperatura de -19°C. Los cortes obtenidos fueron teñidos con violeta de Cresilo y finalmente fueron montados en portaobjetos utilizando Permount. La posición de las cánulas fue determinada mediante la observación de las secciones teñidas con ayuda de un microscopio estereoscópico. Los datos de las ratas empleadas para el análisis estadístico fueron de aquellas en las cuales las cánulas guía se situaron dentro del CeA o en su proximidad inmediata.

8.7 Análisis estadístico

Los resultados son expresados como medias ± error estándar. Debido a que el análisis de Kolmogoroff–Smirnoff mostró que la población bajo estudio en los experimentos conductuales siguió una distribución normal, se llevó a cabo estadística paramétrica para la evaluación de los datos. Se llevó a cabo un análisis de varianza de una vía (ANOVA) seguida, cuando fue necesario ($P<0.05$), de un análisis *post hoc* de Dunnet para comparar los grupos tratados vs. el grupo control.

Se llevó a cabo la prueba de t de Student para evaluar el efecto de la AVP en la conducta de inmovilidad, así como para evaluar el efecto de DMSO (5%)+ Cremephor (5%) en el tiempo de enterramiento; la significancia en todas las pruebas fue de $P<0.05$. Todos los análisis estadísticos fueron realizados con ayuda del software GraphPad Prism V6.

9. RESULTADOS

9.1 Localización de las cánulas de inyección

Los sitios de implante de las cánulas guía, en los animales empleados en el análisis estadístico, se localizaron dentro del CeA. Las puntas de las cánulas estuvieron localizadas entre los niveles AP -1.8 a -3.1mm con respecto a Bregma, de acuerdo con el atlas de Paxinos y Watson (1986) (Fig. 9). Los datos de los animales con el posicionamiento de las cánulas fuera de la amígdala o con signos de alguna afección se excluyeron del análisis estadístico.

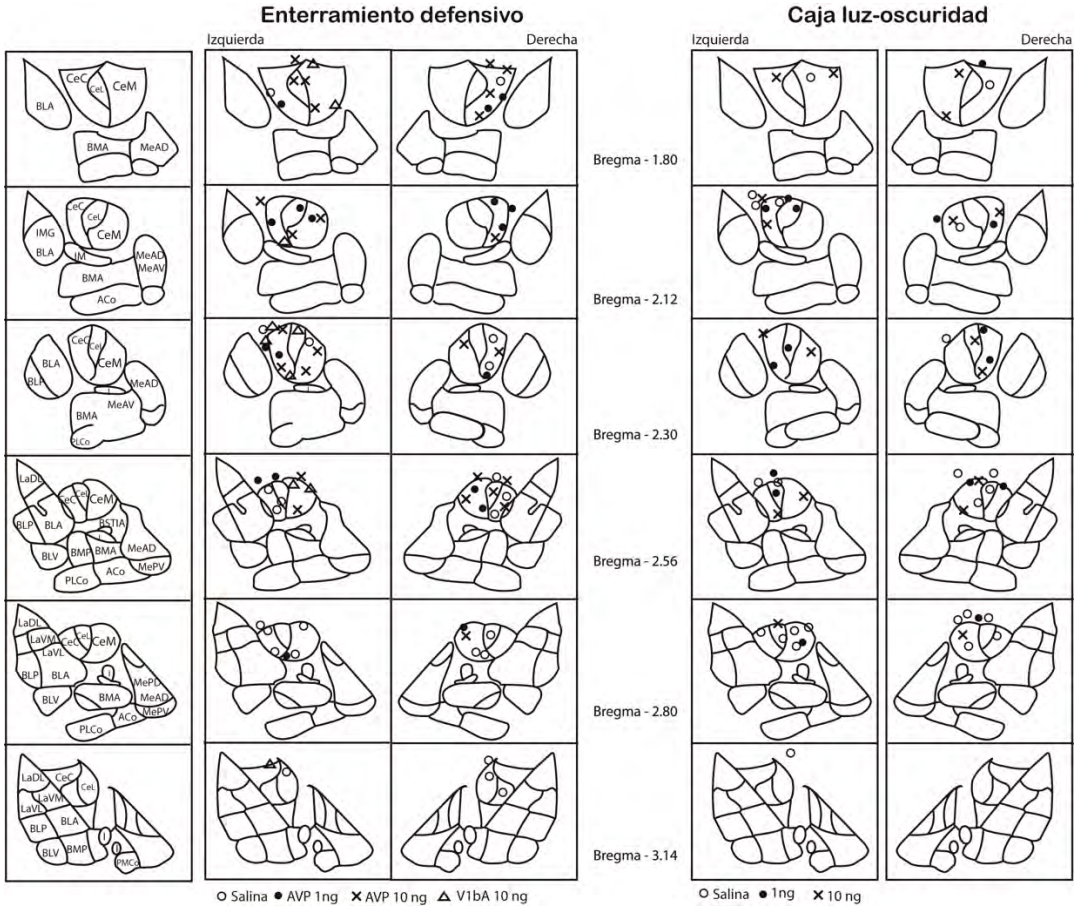

Figura 9. Representación esquemática de los sitios de implante de las cánulas en la amígdala en aquellos datos de animales usados tanto en la prueba de enterramiento defensivo como la caja luz-oscuridad. Las coordenadas estereotáxicas corresponden a lo establecido en el atlas de Paxinos y Watson (1986). Debido a la gran densidad de puntas de las cánulas, en algunas de las secciones, los símbolos se sobreponen. AVP, arginina vasopresina; V1bA, antagonista para receptores V1b; ACo, núcleo anterior cortical de la amígdala; BLA, núcleo basolateral amigdalino; BMA, núcleo amigdalino basomedial; CeL, núcleo central amigdalino, división lateral; CeM, núcleo central amigdalino, división medial; IM, isla intercalada principal; LA, amígdala lateral; MeA, núcleo medial amigdalino.

9.2 Magnitud de la difusión de la [FITC] [Ahx]CYIQNCPLG[Amide] tras su inyección en el CeA

Con el objeto de evaluar la magnitud de la difusión de la AVP administrada estereotaxicamente se infundió dentro del CeA, bajo las mismas condiciones experimentales que las usadas en este trabajo, [FITC] [Ahx]CYIQNCPLG[Amida], un análogo de oxitocina fluorescente que difiere de la AVP sólo en dos aminoácidos. Bajo estas condiciones se encontró que el análogo de la AVP permaneció dentro del CeA cerca del sitio de su inyección y que su difusión radial no llegó a ser mayor que 0.380 mm^3 del sitio de su inyección (Fig. 10). Más aun, como puede ser observado, el análogo de la AVP fue endocitado por los cuerpos de las células nerviosas circundantes al sitio de su inyección.

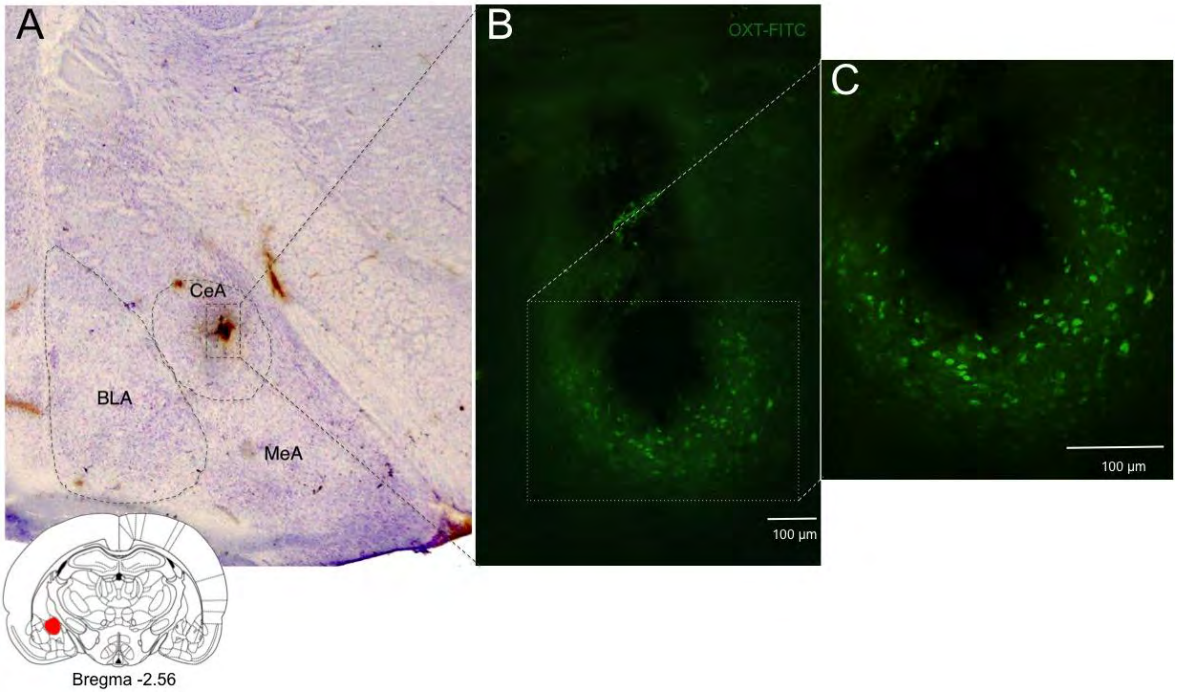

Figura 10. Magnitud de la difusión de la [FITC] [Ahx]CYIQNCPLG[Amida] microinyectada dentro de la amígdala. La [FITC] [Ahx]CYIQNCPLG[Amida], un análogo fluorescente de la oxitocina, que tiene una composición de aminoácidos y un peso molecular similar a la de la AVP fue microinyectado en la amígdala bajo las mismas condiciones experimentales que las empleadas para infundir la AVP. La extensión de la difusión de este compuesto fue evaluada después de 10 min de su infusión dentro del CeA. (A) La punta de la cánula es mostrada dentro del CeA en una sección coronal contra-teñida con violeta de cresilo en el nivel -2.56 mm de acuerdo con el atlas de Paxinos & Watson (1986). (B) El análisis de microscopia de fluorescencia (4X) indica que la [FITC][Ahx]CYIQNCPLG[Amida] permanece en el CeA cerca de la punta de la cánula, difundiendo radialmente para ocupar el volumen de una esfera de alrededor de 0.380 mm^3 centrada en el sitio de su inyección. Note que dicho análogo fue endocitado por los cuerpos de las células nerviosas que circundan al sitio de su inyección. (C) Amplificación (10X) de la misma zona. BLA, núcleo basolateral amigdalino; CeA, núcleo central amigadalino; MeA, núcleo medial amigdalino. OXT-FITC, [FITC] [Ahx]CYIQNCPLG[Amida].

9.3 Evaluación conductual en la prueba de enterramiento defensivo después de la administración de AVP en la amígdala central

La micro-infusión de la AVP (1ng/lado) dentro del CeA indujo en las ratas un aumento en el tiempo de enterramiento durante la prueba (Fig. 11A). Sin embargo, la AVP a dosis mayores (10 ng/lado) perdió su efecto ($F_{2,26}$= 4.743; P<0.05). Efectos similares fueron registrados en la latencia al enterramiento ($F_{2,26}$= 4.881; P<0.05) (Fig. 11B). El número de choques recibidos a lo largo de la prueba no registró un efecto estadísticamente significante (Fig. 11C) ($F_{2,26}$= 1.951; P>0.05). La actividad locomotora de las ratas no se encontró afectada por la administración de la AVP (1 y 10 ng/lado) al ser evaluada en el campo abierto inmediatamente después del enterramiento defensivo ($F_{2,26}$= 1.091; P>0.05) (Fig. 11D).

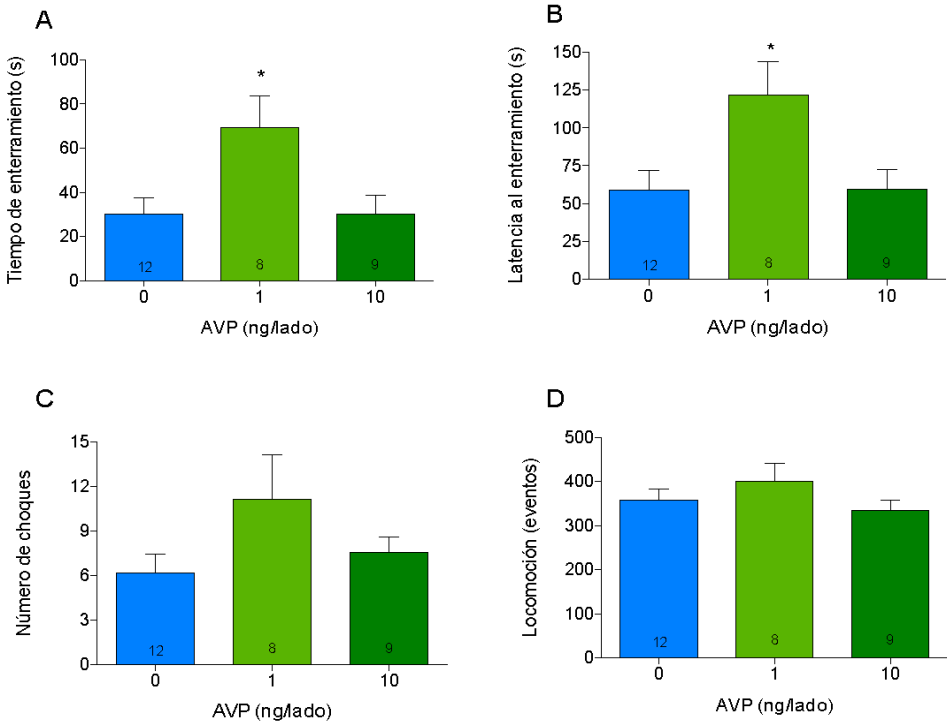

Figura 11. Efecto de la administración bilateral de la arginina vasopresina (AVP) en la prueba del enterramiento defensivo. La AVP a dosis bajas (1ng/lado) pero no altas (10ng/lado), incrementó tanto el tiempo de enterramiento (A) como su latencia (B). No se observaron a ambas dosis efectos estadísticamente significantes en el número de choques recibidos durante la prueba (C) ni en la actividad locomotora de los animales (D). Los resultados son expresados como medias ± SEM. El número de ratas (N) empleadas se indica dentro de cada barra. * P<0.05. ANOVA de una vía seguida de la prueba post hoc de Dunnett.

Por otro lado, aunque se observó una tendencia no significativa (*t*=1.613; P=0.1251) a que el grado de inmovilidad experimentado durante la prueba fuera menor entre el grupo tratado con la AVP vs su control esta no resultó significativa (Fig. 12). Prueba de t de Student; P>0.05.

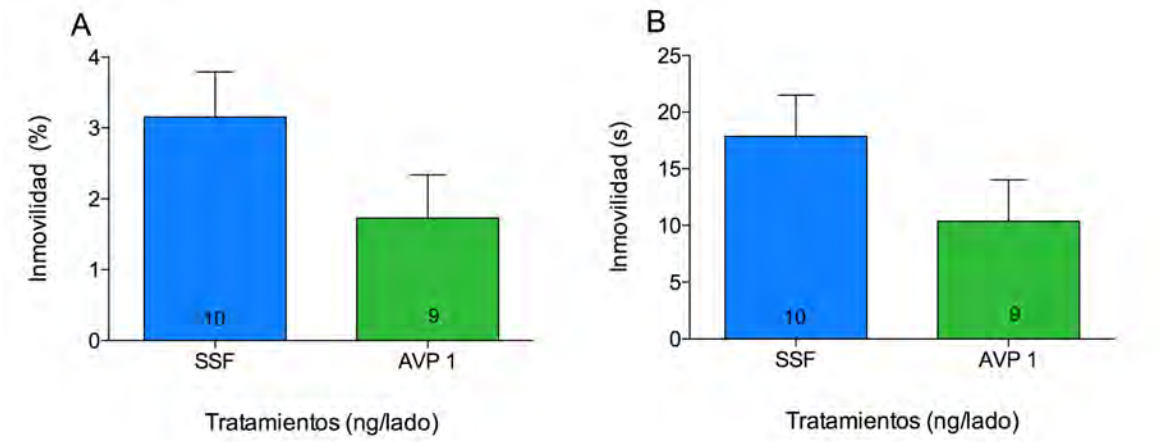

Figura 12. Efecto de la administración de la arginina vasopresina (AVP) en el grado de inmovilidad experimentada por las ratas durante la prueba del enterramiento defensivo. No se observaron efectos significativos con respecto al valor control ni en el porcentaje de inmovilidad con respecto al tiempo total de la prueba (A) ni en el tiempo de inmovilidad (B) tras la administración de la AVP (1ng/lado). Los resultados son expresados como medias ± SEM. El número de ratas empleadas se muestran en cada barra. Prueba de *t* de Student; P>0.05.

9.4 Bloqueo de los efectos ansiogénicos de la arginina vasopresina en la prueba del enterramiento defensivo por el antagonismo de los receptores V1b

Con la intención de conocer si los efectos inducidos por la AVP durante la prueba del enterramiento defensivo eran dependientes de un receptor específico para la AVP (V1a o V1b), fueron administrados junto con la AVP antagonistas selectivos tanto para el receptor V1a (Compuesto de Manning) o para el receptor V1b (SSR149415) en el CeA.

Como se observa en la Fig. 13A la administración del SSR149415, un antagonista específico para los receptores V1b, evitó ($F_{4,48}$=3.968; P<0.01) la aparición de los efectos de la AVP en la conducta de enterramiento. La administración del SSR149415, bloqueo por completo los efectos de la AVP en el tiempo de enterramiento (Fig. 13A). Sin embargo, no se observaron efectos de la AVP en la latencia al enterramiento tras la administración de la AVP (1ng/lado), SSR149415 (10 ng/lado), o la de ambos compuestos simultáneamente ($F_{4,48}$=2.562; P>0.05) (Fig. 13B). De igual manera, bajo ninguna condición experimental se observaron efectos en el número de choques recibidos por las ratas durante la prueba en relación con su control ($F_{4,48}$=0.16; P>0.05) (Fig. 13C). Por otro lado, la evaluación de la actividad locomotora en el campo abierto reveló que bajo ninguna de las condiciones experimentales usadas en este trabajo las ratas tratadas presentaron anomalías locomotoras cuando fueron comparadas con sus respectivos grupos control ($F_{4,48}$=0.753; P>0.05) (Fig. 14D).

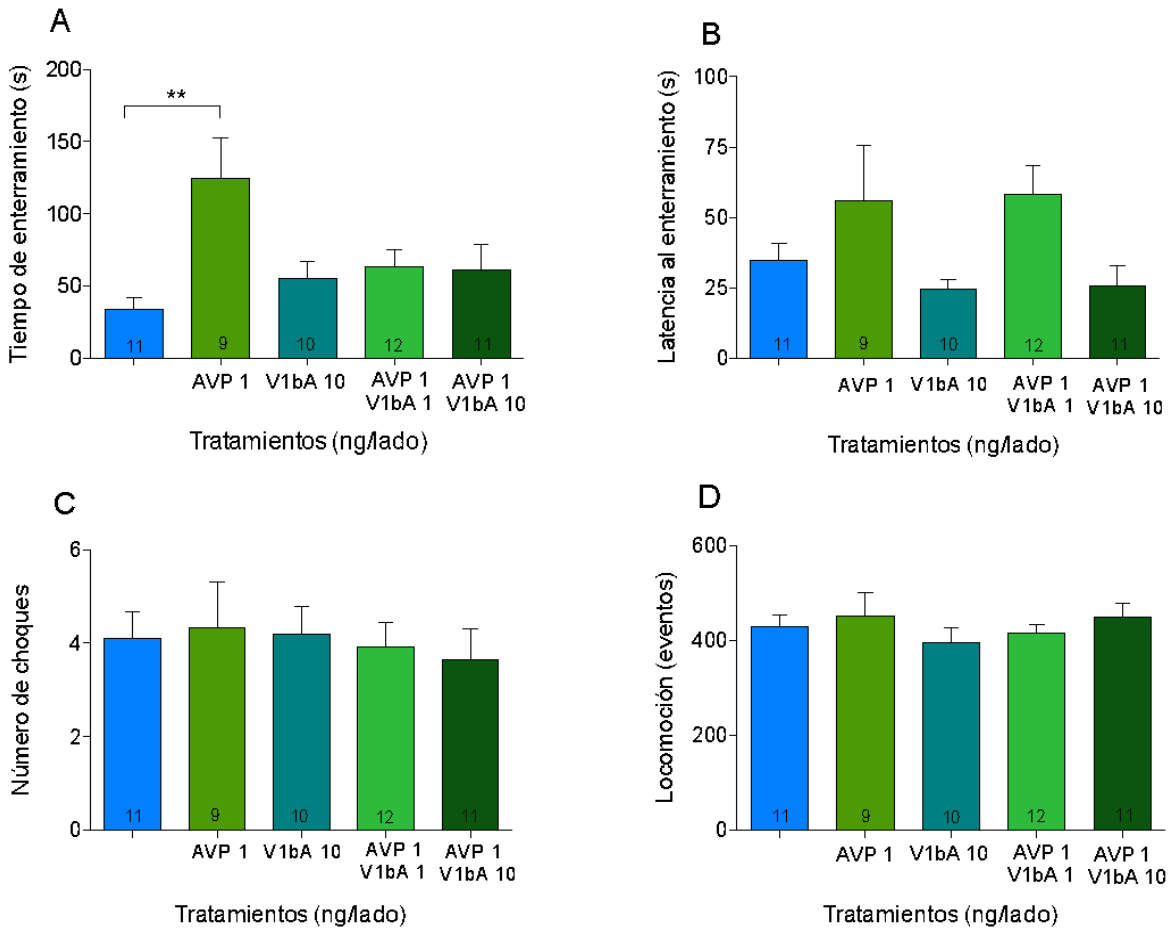

Figura 13. Efecto del bloqueo de los receptores V1b sobre la conducta de enterramiento inducida por la infusión bilateral intra-amigdalina de la AVP. (A) La infusión de SSR149415 bloqueo los efectos inducidos por la AVP en el tiempo de enterramiento sin presentar ningún efecto por si mismo sobre esta conducta. (B) Observe que ni la AVP, el SSR149415 ni su combinación tuvieron algún efecto en la latencia al enterramiento. Asimismo, note que tampoco se observaron efectos tanto en el número de choques recibidos durante la prueba (C) ni en la locomoción (D) bajo ninguna de las condiciones probadas. Los resultados son expresados como medias ± SEM. El número de ratas (N) empleado en cada tratamiento es mostrados en el interior de cada barra. * $P < 0.05$. ANOVA de una vía, seguido de la prueba post hoc de Dunnett.

9.5 Efecto del DMSO (5%) y Cremophor EL (5%) en la conducta de ratas evaluadas en la prueba del enterramiento defensivo.

Debido a que una mezcla de DMSO + Cremophor EL al 5% (c/u) fue usada como vehículo para la infusión de SSR149415, sus posibles efectos conductuales fueron estudiados en la prueba del enterramiento defensivo. Como se observa en la Fig. 14, no se encontraron efectos ni en el tiempo (A) (t=0.656; P =0.523) ni en la latencia al enterramiento (B) (t=0.301; P=0.768). No se encontraron asimismo efectos ni el número de choques recibidos durante la prueba (C) (t=0.847; P=0.411) ni en la locomoción de las ratas (D) (t=0.612; P=0.550) cuando esta fue evaluada en el campo abierto inmediatamente después de la prueba del enterramiento defensivo.

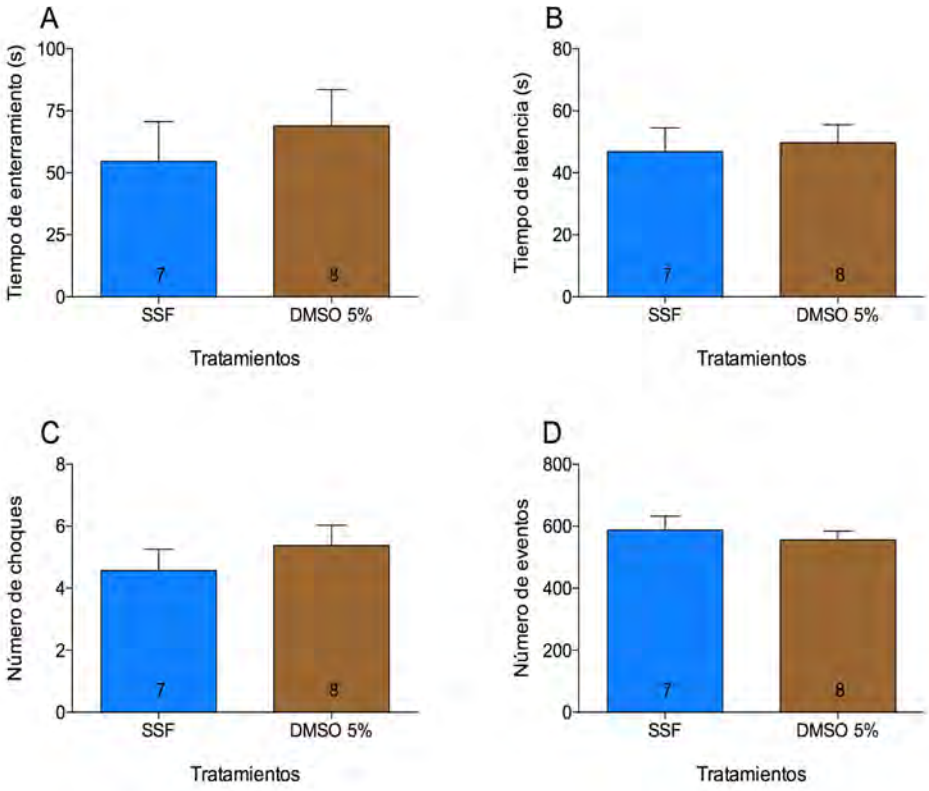

Figura 14. Efecto de la administración bilateral de DMSO+Cremophor EL en la prueba de enterramiento defensivo. La administración de una mezcla de DMSO + Cremophor EL al 5% (c/u) en el núcleo central de la amígdala no indujo efectos conductuales ni en (A) el tiempo de enterramiento, (B) la latencia al enterramiento ni (C) en el número de choques recibidos a lo largo de la prueba. No efectos fueron tampoco observados en (D) la actividad locomotora. Los resultados son expresados como medias ± SEM. El número de ratas (N) empleado en cada tratamiento es mostrados en el interior de cada barra. P> 0.05; Prueba de *t* de Student.

9.6 Efecto del bloqueo de los receptores V1a para vasopresina sobre la conducta de las ratas en la prueba del enterramiento defensivo

Con la intención de analizar la posibilidad de que los efectos de la AVP (1ng/lado) fueran mediados a través de los receptores V1a, y para corroborar la participación de los receptores V1b en la ansiedad, se administró simultáneamente con la AVP un antagonista selectivo (compuesto de Manning), a una dosis efectiva (10 ng), utilizada en experimentos anteriores (Hernández et al. 2016), para disminuir los niveles de ansiedad (Beiderbeck et al. 2007; Bredewold et al. 2014; Gray et al. 2012;), en el CeA y se evaluó la conducta de las ratas en la prueba del enterramiento defensivo.

La administración del compuesto de Manning fue incapaz de evitar el aumento del tiempo de enterramiento inducido por la AVP y no afectó ni por sí mismo ni en combinación con la AVP dicho parámetro ($F_{3,40}$ =3.137; P<0.05) (Fig.15A). Asimismo, no se observaron efectos ($F_{3,40}$=0.534; P>0.05) (Fig.15B) de este antagonista por separado (10ng/lado) ni en combinación con la AVP (1ng+10ng/lado respectivamente) en la latencia al enterramiento ni en el número de choques recibidos por las ratas durante la prueba ($F_{3,40}$=0.655; P>0.05) (Fig. 15C). La locomoción de estos animales no se vio afectada tampoco por ninguno de los tratamientos utilizados ($F_{3,40}$=0.230; P>0.05) (Fig. 15D).

Con el objeto de descartar la posibilidad de que la falta de antagonismo del compuesto de Manning sobre los efectos de la AVP en el enterramiento defensivo fuera debido al uso de una dosis baja de este compuesto los efectos de una dosis 3 veces mayor del antagonista fue probada en un nuevo experimento. De la misma manera que como ocurrió anteriormente, no se encontraron efectos (Fig. 16) en ninguno de los parámetros evaluados: (A) Tiempo de enterramiento ($F_{2,25}$=5.507; P<0.05). (B) Latencia al enterramiento ($F_{2,25}$=0.537; P>0.05). (C) Número de choques recibidos ($F_{2,25}$=3.011; P>0.05). (D) Locomoción en un campo abierto ($F_{2,25}$=0.611; P>0.05).

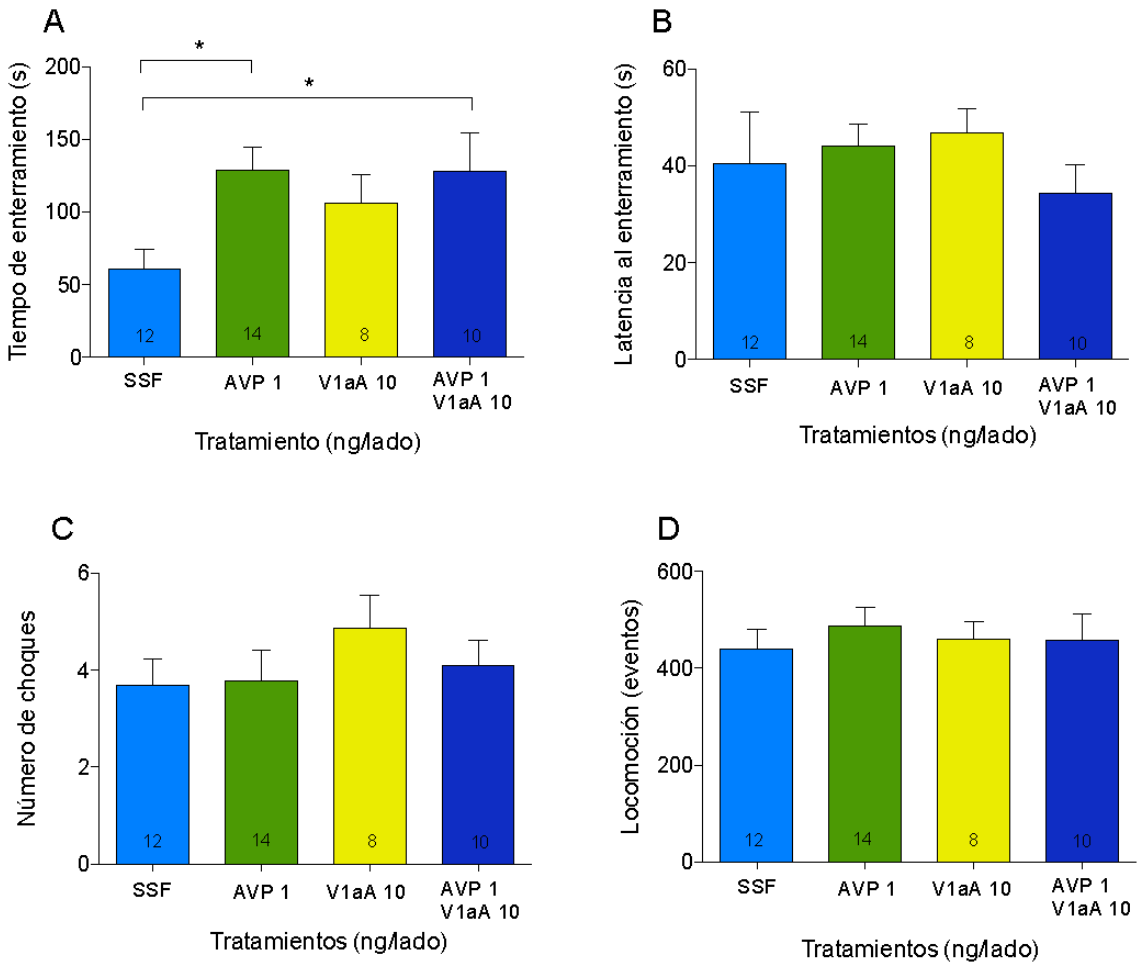

Figura 15. Efecto de la administración del compuesto de Manning en los efectos conductuales inducidos por la microinfusión de la arginina vasopresina (AVP) en el CeA. La administración del compuesto de Manning (10ng/lado) fue incapaz de bloquear los efectos de la AVP tanto en el tiempo de enterramiento (A), en su latencia (B) y en el número de choques recibidos durante la prueba (C). Ningún efecto fue tampoco observado sobre la locomoción cuando este antagonista fue infundido tanto por separado como en combinación con la AVP (D). Los datos son mostrados como medias ± SEM. El número de ratas empleadas se observan dentro de cada barra. * $P<0.05$, ANOVA de una vía seguido de la prueba post hoc de Dunnett.

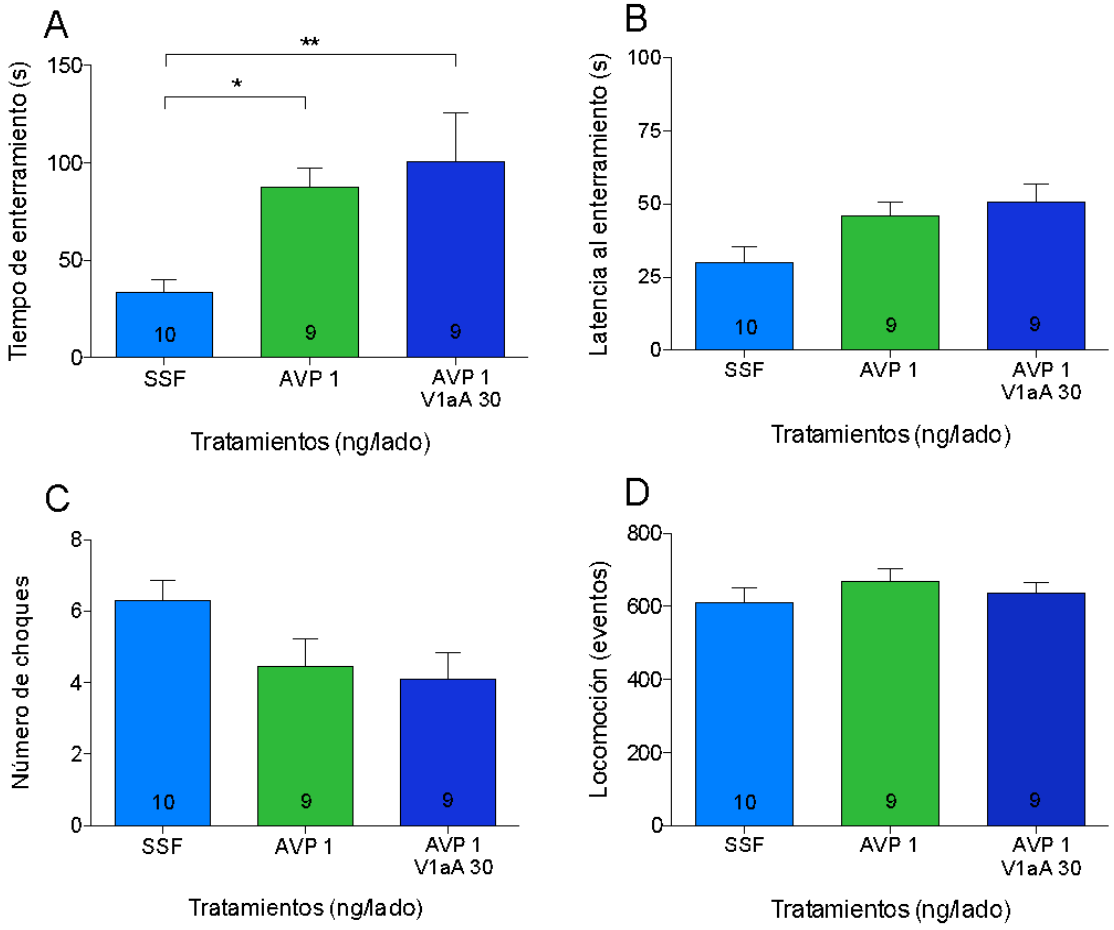

Figura 16. Efecto de la administración del compuesto de Manning en los efectos conductuales inducidos por la microinfusión de la arginina vasopresina (AVP) en el CeA. La administración del compuesto de Manning (30ng/lado) fue incapaz de bloquear los efectos de la AVP en el tiempo de enterramiento (A), no se observaron asimismo efectos en la latencia al enterramiento (B), el número de choques recibidos durante la prueba (C) ni en la actividad locomotora (D). Los datos se muestran como medias ± SEM. El número de ratas empleadas se observan dentro de cada barra. * P<0.05; ** P<0.01. ANOVA de una vía seguido de la prueba *post hoc* de Dunnett.

9.7 Efecto del bloqueo de los receptores oxitocinérgicos sobre los efectos de la AVP en la prueba del enterramiento defensivo

Debido a que los efectos de la AVP en la prueba del enterramiento defensivo podrían estar mediados por los receptores para la oxitocina, como ha sido reportado por Song et al. (2014; 2016), los efectos de la AVP en esta prueba fueron evaluados en presencia de un antagonista para los receptores a la oxitocina (OTA) utilizando una dosis que ha demostrado ser efectiva para bloquear los efectos de la OT (László et al. 2016). Como puede observarse en la Fig. 17, el OTA (15ng/lado) administrado simultáneamente con la AVP (1ng/lado) fue incapaz de modificar sus efectos. Así, ni el incremento del tiempo de enterramiento inducido por la AVP ($F_{3,38}$=3.775; P<0.05) ni su falta de efectos tanto en la latencia al enterramiento ($F_{3,38}$=1.090; P>0.05) como en el número de choques recibidos por las ratas ($F_{3,38}$=0.4012; P>0.05) fueron afectados. Adicionalmente no se observaron cambios en la falta de efectos de la AVP sobre la locomoción ($F_{3,38}$=1.216; P>0.05) (Fig.17).

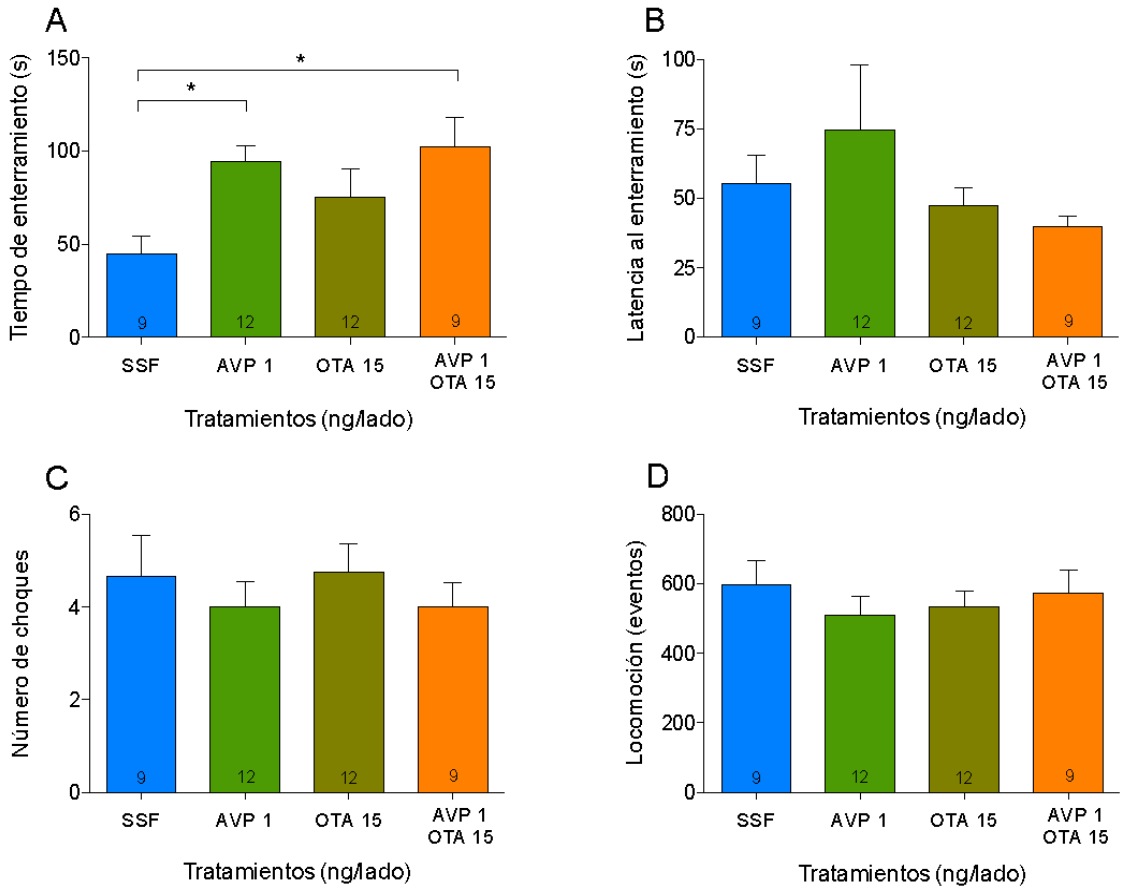

Figura 17. Efecto de la administración bilateral del OTA en el CeA sobre los efectos de la microinfusión simultanea de la arginina vasopresina (AVP) en la prueba del enterramiento defensivo. El OTA fue incapaz de modificar el incremento inducido por la AVP en el tiempo de enterramiento (A), ni la falta de efectos de este neurotransmisor sobre el tiempo de latencia al enterramiento (B), el número de choques recibidos durante la prueba (C) y la locomoción de los animales (D). Los resultados son expresados como medias ± SEM. El número de animales empleados se observan dentro de cada barra. * P<0.05. ANOVA de una vía, seguida de la prueba *post hoc* de Dunnett. CeA, Núcleo central de la amígdala; OTA, antagonista para los receptores a la oxitocina.

9.8 Efecto de la administración bilateral de la AVP en el CeA en la caja luz-oscuridad

No se observaron efectos tras la administración de la AVP (1ng/lado) y el SSR149415 (10ng/lado) tanto en forma individual como en combinación (1ng/lado + 1 o 10ng/lado respectivamente), en la latencia para pasar al compartimiento oscuro de la caja ($F_{4,80}$=0.173; P>0.05), en el tiempo de permanencia en su compartimiento iluminado ($F_{4,80}$=0.802; P>0.05) o en el número de transiciones entre los compartimientos oscuro e iluminado del dispositivo conductual ($F_{4,80}$=1.267; P>0.05). No se encontraron asimismo efectos de ninguno de los tratamientos en la locomoción de las ratas cuando sus valores fueron comparados con los valores registrados en el grupo control ($F_{4,80}$=0.089; P>0.05) (Fig. 18).

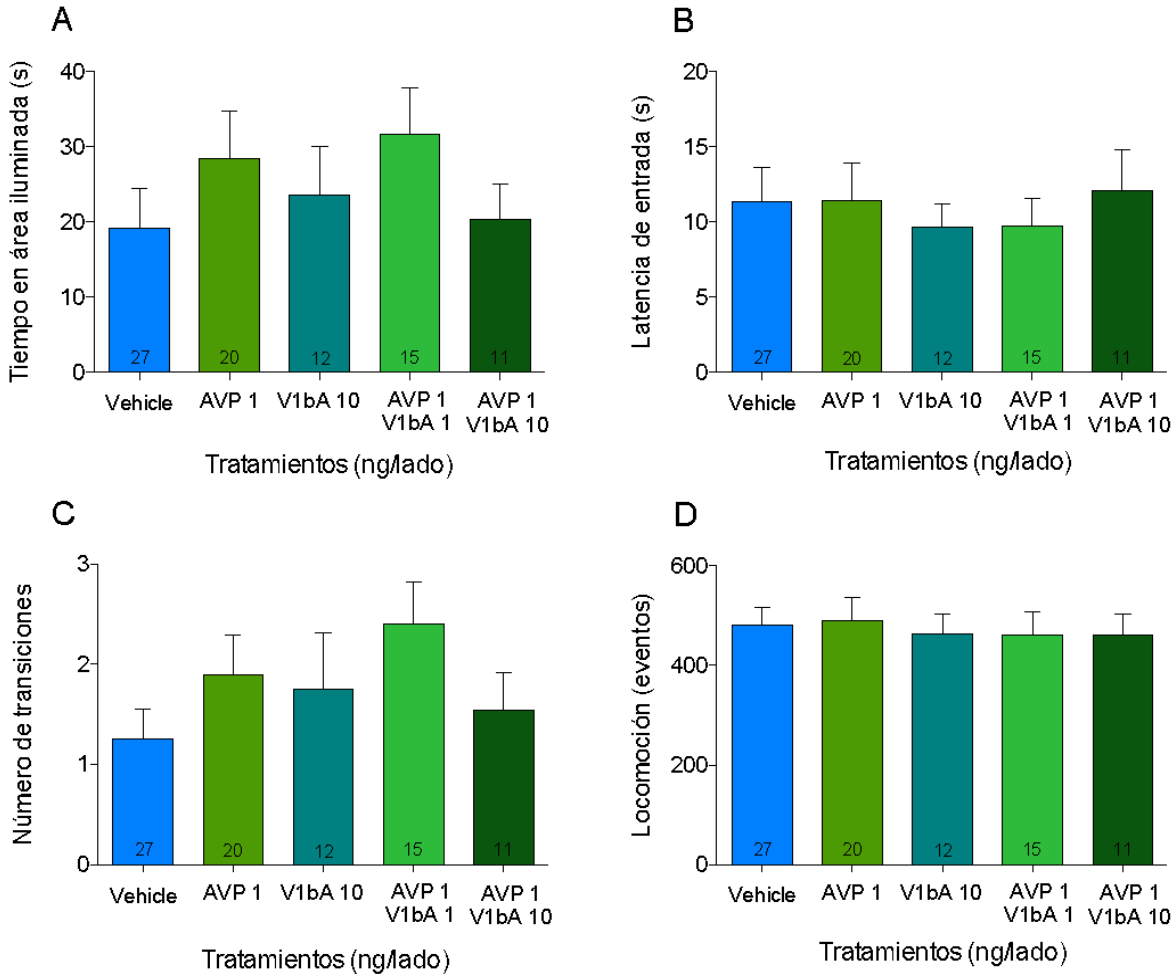

Figura 18. Efectos conductuales de la administración intra CeA de la arginina vasopresina (AVP) y del SSR149415 en la caja luz-oscuridad. Tanto la AVP como el SSR149415 (antagonista de los receptores V1b) administrados ya sea individualmente o en combinación fueron incapaces de afectar en la caja luz-oscuridad: el tiempo de permanencia en el compartimiento iluminado (A), la latencia para entrar al compartimiento iluminado (B) y el número de transiciones entre los compartimientos oscuro e iluminado (C). De manera similar, no se observaron efectos de ninguno de los tratamientos en la locomoción de los animales (D). Los resultados son expresados como medias ± SEM. El número de animales en cada grupo es mostrado dentro de sus barras respectivas. ANOVA de una vía. P>0.05.

10. DISCUSIÓN

El sistema vasopresinérgico se encuentra ampliamente representado en la amígdala, dado que han sido reportados tanto cuerpos celulares y fibras en su núcleo medial (MeA), así como terminales provenientes del PVN y receptores V1 (a,b) en su núcleo central (CeA). Cabe destacar que la amígdala es una estructura generalmente aceptada como clave en el procesamiento de la ansiedad. Sin embargo, a pesar de que su papel en dicha respuesta ha sido estudiado en el animal íntegro (Appenrodt et al. 1998; Mak et al. 2012) y en algunas regiones del cerebro tales como el septum (Appenrodt et al. 1998; 2000; Landgraft et al. 1995; Stemmelin et al. 2005) poco se sabe del papel que juega dicho sistema de neurotransmisión en la modulación amigdalina de la ansiedad. Más aún, dado que en la amígdala están presentes tanto receptores V1a y V1b que reconocen a la AVP como su neurotransmisor, se desconocía si ambos tipos de receptores participan en la supuesta modulación amigdalina de la ansiedad por parte de la AVP o si pudieran ejercer un papel diferencial en ella. El presente trabajo va dirigido a determinar el papel de la AVP en la modulación amigdalina de la ansiedad innata y a establecer el papel que en ella juegan sus distintos tipos de receptores y particularmente los V1b que han sido poco estudiados. Para ello determinamos los efectos conductuales de la AVP al ser infundida en el CeA y mediante la administración de antagonistas selectivos para sus dos tipos de receptores intentamos establecer la participación de cada uno de ellos utilizando dos paradigmas de ansiedad innata, el enterramiento defensivo (Treit et al. 1981) y la caja luz oscuridad (Costall et al. 1989).

Nuestros resultados sugieren que la AVP tiene una participación ansiogénica en la modulación amigdalina de la ansiedad innata y de que en estos efectos los receptores V1b, a diferencia de los V1a, juegan un papel fundamental. Así, en respuesta a la administración de la AVP, en la prueba del enterramiento defensivo nuestros resultados mostraron un aumento en el tiempo que las ratas estuvieron enterrando el electrodo (tiempo de enterramiento) que es el parámetro

de ansiedad más importante en este prueba (Treit et al. 1981; De Boer & Koolhaas 2003).

Cabe señalar, sin embargo, que los efectos ansiogénicos de la AVP se observaron únicamente cuando este neurotransmisor fue administrado a dosis bajas (1ng/lado) y que desaparecieron cuando la dosis fue incrementada a 10ng/lado. Es posible, que tales efectos se deban a una perdida en la especificidad de la AVP por su receptor y que como consecuencia de ello a dosis altas la AVP se hubiera unido también a receptores para la OT; que se encuentran también presentes en el CeA (Huber et al. 2005; Mouillac et al. 1995), poseen una afinidad similar por la AVP y tienen acciones ansiolíticas (Bale et al. 2001; De la Mora et al 2016; Lazlo et al. 2016). Adicionalmente, es posible que dosis altas de la AVP sensibilizaran a los receptores de OT compartiendo así su vía de señalización (Song et al. 2014, 2016) contribuyendo a amortiguar los efectos ansiogénicos de la AVP.

En este contexto resulta sorprendente, que la administración de la AVP a dosis que incrementan el enterramiento (1ng/lado) aumentaron la latencia para el inicio de dicha conducta únicamente en el primer experimento y no así en los subsecuentes. Dicho hallazgo resulta sorprendente debido a que este parámetro guarda una correlación negativa con el tiempo del enterramiento (De Boer & Koolhaas 2003) y en consecuencia debería haber disminuido en nuestros experimentos. La falta de constancia en los efectos por la administración de la AVP en los experimentos posteriores, podría ser explicado debido a diferencias individuales en las ratas que no permitieran observar de manera clara los efectos de la AVP en la reactividad de los roedores a comenzar la conducta del enterramiento. Dado que la latencia al enterramiento guarda una mayor relación con la reactividad de los roedores que con la ansiedad (De Boer & Koolhaas 2003), tales resultados pudieran ser explicados debido a una disminución en la sensibilidad de las ratas a los choques eléctricos recibidos. En apoyo de esta sugerencia, se ha reportado que la administración de la AVP en el CeA induce

efectos analgésicos en la región oro-facial de las ratas (Ahn et al. 2001). Cabe señalar que en línea con lo anterior, la tendencia observada en nuestras ratas a recibir un número mayor de choques como resultado de la infusión de la AVP, pudiera reflejar asimismo, dicha disminución en la sensibilidad de la región oro-facial de nuestras ratas.

Por otro lado, nuestros resultados muestran que los efectos de la AVP en esta prueba fueron observados únicamente en conductas de tipo activo (e.g. conducta de enterramiento) (De Boer & Koolhaas 2003) y no en aquellas de tipo pasivo (e.g. inmovilidad conductual).

Es importante hacer notar que los efectos de la administración bilateral de la AVP dentro del CeA en nuestros experimentos fueron específicos, ya que sus efectos se restringieron únicamente a la conducta de enterramiento y que su administración no afectó el número de choques recibidos a lo largo de la prueba ni la actividad locomotora de los animales.

Resulta sorprendente que los efectos ansiogénicos inducidos por la AVP en la prueba del enteramiento defensivo no se replicaran en la caja luz-oscuridad, otro paradigma empleado en el estudio de la ansiedad innata (Costall et al. 1989; Misslin et al. 1989). La razón de estas diferencias no resulta clara ya que este paradigma ha sido bien validado en su capacidad para detectar efectos tanto ansiogénicos como ansiolíticos (Hughes & Hancock 2016; Onaivi & Martin 1989). La razón de estas diferencias entre estos paradigmas no es fácilmente explicable pero es muy probable que obedezcan a disimilitudes significativas en los aspectos de la ansiedad innata que son modelados en cada prueba (Spooren et al. 2000). Es posible, que dentro de estas diferencias se encuentren el grado de ambigüedad y certeza de los estímulos ansiogénicos que ambas pruebas generan y la capacidad de los sistemas CCKérgicos amigdalinos para modificarlos en un sentido o en otro (ansiogénesis o ansiolísis).

Resulta tentador, con base en lo anterior, sugerir que mientras que los sistemas CCKérgicos no son capaces de identificar/afrontar estímulos difusos tales como cambios en la iluminación, característicos de la caja luz-oscuridad sí son capaces de hacerlo con estímulos más identificables y de mayor grado de aversión como son los choques eléctricos que se aplican en la prueba del enterramiento defensivo. Es claro, que se requiere de mas experimentación para poder entender a cabalidad la naturaleza de los efectos diferenciales encontrados en este trabajo.

Dado que en la amígdala el receptor más prominente es el receptor V1a (Ostrowsky et al. 1992;1994) resulta sumamente interesante que a diferencia del d(CH),Tyr(Me),Arg)-vasopresina (compuesto de Manning), un antagonista selectivo de receptores V1a (Manning et al. 1992), la administración bilateral intra-amigdalina del SSR149415, que antagoniza selectivamente a los receptores V1b (Serradeil-Le Gal et al. 2002), fue capaz de bloquear los efectos ansiogénicos de la AVP observados en el enterramiento defensivo. Dichos efectos, son sin embargo congruentes con la reducción de los niveles de ansiedad reportadas en modelos clásicos de esta respuesta (Engin & Treit 2008; Griebel et al. 2002; Hodgson et al. 2007; Salomé et al. 2006; Stemmelin et al. 2005) y sobre conductas sociales asociadas a la ansiedad (Amikishieva et al. 2011; Griebel et al. 2002; Litvin et al. 2011; Overstreet & Griebel 2005; Shimazaki et al. 2006). Por otro lado, la falta de efectos de este compuesto, por sí mismo, observados en nuestros experimentos sugiere que este antagonista se comportó como un antagonista clásico, carente de efectos propios, y descartan la posibilidad que dentro de la amígdala exista una liberación tónica de la AVP, que bajo condiciones basales afectara la actividad de los receptores V1b y por consiguiente su participación en la modulación basal que la amígdala ejerce sobre la ansiedad.

Por otro lado, cómo se señaló antes, el bloqueo de los receptores V1a no afectó los efectos ansiogénicos de la AVP cuando ambas sustancias fueron administradas de manera conjunta. Dado que diversos estudios han reportado

que los receptores V1a participan en la modulación de la ansiedad y que la administración del compuesto de Manning induce efectos ansiolíticos (Bayerl et al. 2016; Beiderbeck et al. 2007; Wigger et al. 2004), la falta de efectos atribuible al bloqueo de los receptores V1a cuando este compuesto fue infundido (10 y 30 ng/lado) resulta sorprendente ya que en experimentos anteriores, dicho compuesto bloqueo los efectos ansiogénicos de la AVP al ser evaluada en el EPM (Hernández et al. 2016). La posibilidad de que la falta de efectos del compuesto de Manning obedeciera a su administración simultánea con la AVP y no antes de ella resulta poco probable, ya que bajo estas mismas condiciones el SSR149415 fue capaz de inducir efectos ansiolíticos atribuibles al bloqueo de los receptores V1b. Más aún, bajo el esquema de inyección usado en este trabajo, los compuestos al ser infundidos simultáneamente alcanzan sus blancos bajo la misma relación de concentración que tenían al ser inyectados, al evitarse su difusión diferencial a consecuencia de los distintos tiempos de su inyección. Bajo estas condiciones, la mayor afinidad del compuesto de Manning por el receptor V1a que la mostrada por la AVP (Ki 0.7 nM vs 2.6 nM respectivamente) (Manning et al. 2008; Thibonnier et al. 1997) aunado al empleo de una dosis mayor del antagonista (10 y 30ng/lado) que la de la AVP (1ng/lado) asegurarían un bloqueo efectivo de los receptores V1a.

En línea con nuestros resultados en la caja luz-oscuridad, los ratones que no expresan el receptor V1b (knock out V1b) no exhibieron efectos del genotipo sobre las conductas de ansiedad al ser evaluados en la caja luz-oscuridad, el EPM ni en el campo abierto (Egashira et al. 2005; Wersinger et al. 2002). Es interesante, sin embargo, que ratones knock out para el receptor V1a mostraron, en los mismos modelos conductuales, una disminución de sus niveles de ansiedad (Bielsky er al. 2004; Egashira et al. 2007), lo que podría sugerir la existencia de una activación diferencial de los receptores para la AVP que dependería del tipo de paradigma conductual empleado para evaluar la ansiedad.

Queda por aclarar en el futuro si las distintas características de ansiedad innata modeladas en diferentes paradigmas no condicionados, son moduladas diferencialmente por diferentes subtipos de receptores para la AVP.

Aunque se podría argüir, que dadas las similitudes estructurales entre la AVP y OT los efectos mediados por la activación de los receptores V1b pudieran en realidad ser debidos a la activación de los receptores a la OT, esto parece poco probable, ya que la administración de OTA fue incapaz de bloquear los efectos ansiogénicos inducidos por la AVP cuando ambos compuestos fueron infundidos conjuntamente. Como argumento adicional, dado que la afinidad de la AVP es mayor por los receptores V1b (Ki 0.3 nM) que por los receptores para la OT (Ki 1.7 nM) es aún menos probable que la AVP haya ocupado un mayor número de receptores para la OT que para la AVP.

El sitio anatómico dentro de la amígdala en donde la AVP ejerció sus efectos ansiogénicos puede sugerirse que es el CeA. Esto, en vista de que la mayoría de las puntas de las cánulas estuvieron localizadas en el CeA y la infusión de los fármacos se realizó bajo condiciones que muy probablemente hicieron que dichos compuestos permanecieran cerca de su sitio de infusión es factible que el substrato anatómico de la acción ansiogénica de la AVP haya sido dicho núcleo.

Así, dado que en nuestros experimentos se utilizaron tanto un volumen de inyección muy pequeño (250 nl/lado) como una presión muy reducida (50 nl/min) es posible que la difusión de los fármacos haya estado limitada a una zona muy cercana a la punta de las cánulas inyectoras, como ha sido reportado por nosotros (de la Mora et al. 2006) y otros investigadores (James & Starr 1978; Peterson 1988). Como prueba de ello, nuestros resultados indican, que la difusión radial de [FITC][Ahx]CYIQNCPLG[amide], un análogo de la oxitocina (OT) fluorescente administrado dentro del CeA, bajo las mismas condiciones experimentales usadas en los experimentos farmacológicos, no haya ocupado

más de un volumen de 0.380 mm^3 del total de la amígdala (11.6 ± 0.2 mm^3) (Chen & Buckmaster, 2005).

Adicionalmente, han sido reportados receptores V1b dentro del CeA y una densa inervación vasopresinérgica proveniente del PVN del hipotálamo (Corbani et al. 2018; Hernández et al. 2016; Hernando et al. 2001; Huber et al. 2005; Stemmelin et al. 2011; Vaccari et al. 1998; Young et al. 2006). Sin embargo, se debe tener en cuenta la existencia de células vasopresinérgicas en el núcleo medial de la amígdala (MeA), que podrían liberar a la AVP de manera local, influyendo así los efectos conductuales observados.

Cabe señalar, que ha sido reportado (Salomé et al. 2006) que la micro-administración de dosis similares a las nuestras de SSR149415 dentro del BLA, pero no dentro del CeA, resultaron en la aparición de efectos ansiolíticos en el laberinto elevado en forma de signo de "+". Dichos efectos resultan sorprendentes pues la densidad de receptores V1b dentro del BLA es sumamente escasa (Hernando et al. 2001; Stemelin et al. 2005). Aunado a esto, la administración de SSR149415 en el CeA por si solo tuvo efectos ansiolíticos, mientras que en nuestros experimentos no se observaron dichos efectos. Es posible, sin embargo, que dado que las condiciones de inyección de estos investigadores fueron bastante más drásticas (300nl/lado a una velocidad de 200nl/min) que las nuestras, dicho antagonista haya alcanzado vía los ventrículos laterales, sitios fuera e incluso dentro de la amígdala en donde podría haber bloqueado receptores V1b con un papel importante en la modulación basal de la ansiedad.

El mecanismo a través del cual los receptores V1b ejercen los efectos ansiogénicos revelados en nuestros experimentos no es claro, pero dado que dichos receptores pudieran estar presentes en neuronas excitatorias localizadas en la porción medial del CeA, su activación por la AVP liberada de terminales vasopresinérgicas en dicho núcleo podría resultar en la aparición de efectos ansiogénicos. Alternativamente, dado que dichos receptores coexisten con

receptores que tienen un carácter ansiogénico y reconocen a la CRH/CRF es factible que la AVP liberada dentro de la porción medial del CeA pudiera promover la formación de dímeros facilitadores ansiogénicos entre los receptores V1b y los receptores para el factor liberador de corticotropina como ha sido descrito para otros receptores (Murat et al. 2012; Young et al. 2007).

11. CONCLUSIONES

La administración de 1 ng/lado de vasopresina en el núcleo central de la amígdala induce efectos ansiogénicos en la prueba de enterramiento defensivo.

La administración de un antagonista para receptores V1b (SSR149415), pero no para los receptores V1a bloquea los efectos ansiogénicos inducidos por la vasopresina durante la prueba de enterramiento defensivo.

La administración de vasopresina en el núcleo central de la amígdala no modifica los niveles de ansiedad en la caja luz-oscuridad.

En suma, los resultados de este trabajo subrayan el papel de la neurotransmisión vasopresinérgica en la modulación amigdalina de la ansiedad y muestran la participación de los receptores V1b localizadas en el CeA en dicha modulación. Adicionalmente, nuestros resultados sugieren que la AVP no tiene un efecto modulador global en la regulación amigdalina de la ansiedad y que sus efectos dependen de las características específicas modeladas por cada paradigma de ansiedad.

12. ABREVIATURAS

AAA.	Área anterior de la amígdala
AB.	Núcleo basal accesorio
AC.	Adenilato ciclasa
ACTH.	Corticotropina
ADH.	Hormona antidihuretica
AHA.	Área amigdalina hipocampal
AMPc.	Adenosin monofosfato 3´5´ciclico
AVP.	Arginina vasopresina
B.	Núcleo basal
BAOT.	Núcleo cama del tracto olfativo accesorio
BLA.	Núcleo basolateral
BNST.	Núcleo cama de la estria terminal
CeA.	Núcleo central de la amígdala
CoA.	Núcleo anterior cortical
CoP.	Núcleo posterior cortical
CRH.	Hormona liberadora de corticotropina
DAG.	Diacilglicerol
EPM.	Laberinto elevado en forma de signo “+”
GABA.	Ácido gamma-aminobutírico
GPCR.	Receptor acoplado a proteínas G
HAB.	Ratas Wistar de alta ansiedad
HPA.	Eje hipotálamo-pituitaria-adrenal
IP3.	Inositol trifosfato
IPC.	Islas intercaladas paracapsulares
LA.	Núcleo lateral
LAB.	Ratas Wistar de baja ansiedad
LS.	Septum lateral
MeA.	Núcleo medial de la amígdala
mPFC.	Corteza prefrontal medial
NLOT.	Núcleo del tracto olfativo lateral
OT.	Oxitocina
PAC.	Corteza periamígdalina
PKC.	Proteína quinasa c
PLC.	Fosfolipasa c
PVN.	Núcleo paraventricular del hipotálamo
SNC.	Sistema nervioso central
SON.	Núcleo supraóptico del hipotálamo
SPBT.	Prueba de enterramiento defensivo

13. REFERENCIAS

Adhikari A (2014) Distributed circuits underlying anxiety. Front Behav Neurosci 8: 112.

Ahn DK, Kim KH, Ju JS, Kwon S, Park JS (2001) Microinjection of arginine vasopressin into the central nucleus of amygdala suppressed nociceptive jaw opening reflex in freely moving rats. Brain Res Bull 55: 117-121.

Albers HE (2015) Species, sex and individual differences in the vasotocin/vasopressin system: relationship to neurochemical signaling in the social behavior neural network. Front Neuroendocrinol 36: 49-71.

Alheid GF, De Olmos J, & Beltramino CA (1995) Amygdala and extended amygdala. In: The Rat Nervous System. *Paxinos*, 495–578.

Alheid GF, & Heimer L (1988) New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. *Neuroscience, 27*, 1–39.

Alonso J, Angermeyer MC, Bernert S, Bruffaerts R, Brugha TS, Bryson H, de Girolamo G, Graaf R, Demyttenaere K, Gasquet I, Haro JM, Katz SJ, Kessler RC, Kovess V, Lepine JP, Ormel J, Polidori G, Russo LJ, Vilagut G, Almansa J, Arbabzadeh-Bouchez S, Autonell J, Bernal M, Buist-Bouwman MA, Codony M, Domingo-Salvany A, Ferrer M, Joo S, Martinez-Alonso M, Matschinger H, Mazzi F, Morgan Z, Morosini P, Palacin C, Romera B, Taub N, Vollebergh WA, EsemeD/Mhedea (2004) Prevalence of mental disorders in Europe: results from the European Study of the Epidemiology of Mental Disorders (ESEMeD) project. Acta Psychiatr Scand Suppl: 21-7.

Amikishieva AV, Ilnitskaya SI, Nikolin VP, Popova NA (2011) Effect of vasopressin V1b receptor antagonist, SSR149415, on anxiety-like behavior and Lewis lung carcinoma metastasis in mice. Exp Oncol 33: 126-9.

Appenrodt E, Schnabel R, Schwarzberg H (1998) Vasopressin administration modulates anxiety-related behavior in rats. Physiol Behav 64: 543-7.

Appenrodt E, Schwarzberg H (2000) Central vasopressin administration failed to influence anxiety behavior after pinealectomy in rats. Physiol Behav 103: 393-403.68: 735-9.

Balazsfi D, Pinter O, Klausz B, Kovacs KB, Fodor A, Torok B, Engelmann M, Zelena D (2015) Restoration of peripheral V2 receptor vasopressin signaling fails to correct behavioral changes in Brattleboro rats. Psychoneuroendocrinol 51: 11-23.

Bale TL, Davis AM, Auger AP, Dorsa DM, McCarthy MM (2001) CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior. J Neurosci 21: 2546-2552.

Bayerl DS, Honig JN, Bosch OJ (2016) Vasopressin V1a, but not V1b, receptors within the PVN of lactating rats mediate maternal care and anxiety-related behaviour. Behav Brain Res 305: 18-22.

Beiderbeck DI, Neumann ID, Veenema AH (2007) Differences in intermale aggression are accompanied by opposite vasopressin release patterns within the septum in rats bred for low and high anxiety. Eur J Neurosci 26: 3597-605.

Benelli A, De Pol A, Poggioli R, Cavazzuti E, Arletti R, Bertolini A, & Vergoni AV (2000) L-sulpiride, at antidepressant dosage, prevents conditioned-fear stress-induced gastric lesions in rats. Pharmacol Res, 42(2), 157-160.

Bernard JF, Alden M, and Besson JM (1993) The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: a Phaseolus vulgaris leucoagglutinin (Pha-l) study in the rat. J Comp Neurol 329: 201–229.

Bernard JF, Alden M, and Besson JM (1989) A possible spino (tri-gemino)-ponto-amygdaloid pathway for pain. Neurosci Lett 100: 83–88.

Bielsky IF, Hu SB, Szegda KL, Westphal H, Young LJ (2004) Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacol 29: 483-93.

Birnbaumer M (2000) Vasopressin Receptors. Trends in Endocrinol Metab 11: 406-410.

Bohus B, de Wied D (1998) The vasopressin deficient Brattleboro rats: A natural knockout model used in the search for CNS effects of vasopressin. Prog Brain Res 119: 555-573.

Bouwknecht JA, Spiga F, Staub DR, Hale MW, Shekhar A, and Lowry CA (2007) Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors; relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. Brain Res Bull /2 (1): 32-43.

Brown S & Schäfer E (1888) An investigation into the functions of the occipital and temporal lobes of the monkey's brain. Phil Trans R Soc B 179, 303–327.

Bredewold R, Smith CJW, Dumais KM, Veenema AH (2014) Sex-specific modulation of juvenile social play behavior by vasopressin and oxytocin depends on social context. Front Behav Neurosci 8 (216): 1-11.

Brunelli SA (2005) Selective breeding for an infant phenotype: rat pup ultrasonic vocalization (USV). Behav Genet 35: 53-65.

Buijs RM (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. Cell Tiss Res 192: 423-35.

Buijs RM (1980) Immunocytochemical demonstration of vasopressin and oxytocin in the rat brain by light and electron microscopy. J Histochem Cytochem 28: 357-360.

Buijs RM, Hermes MLHJ, Kalsbeek A, Vanderwoude T, Vanheerikhuize JJ (1991) Vasopressin Distribution, Origin and Functions in the Central-Nervous-System. Colloq Inse 208: 149-158.

Bunck M, Czibere L, Horvath C, Graf C, Frank E, Kessler MS, Murgatroyd C, Muller-Myhsok B, Gonik M, Weber P, Putz B, Muigg P, Panhuysen M, Singewald N, Bettecken T, Deussing JM, Holsboer F, Spengler D, Landgraf R (2009) A hypomorphic vasopressin allele prevents anxiety-related behavior. PloS one 4: e5129.

Burbach JP, Luckman SM, Murphy D, Gainer H (2001) Gene regulation in the magnocellular hypothalamo-neurohypophysial system. Physiol Rev 81: 1197-267.

Burdach K F (1819) Vom Baue und Leben des Gehims. 2 vols. Leipzig: Dyk'sche Buchhdl.

Bystritsky A, Khalsa SS, Cameron ME, Schiffman J (2013) Current diagnosis and treatment of anxiety disorders. P T. 38(1):30-57.

Caffé AR, van Leeuwen FW, Luiten PG (1987) Vasopressin cells in the medial amygdala of the rat project to the lateral septum and ventral hippocampus. J. Comp. Neurol. 261: 237-52.

Caldwell HK, Lee HJ, Macbeth AH, Young WS, 3rd (2008) Vasopressin: behavioral roles of an "original" neuropeptide. Prog Neurobiol 84: 1-24.

Calhoon GG, Tye KM (2015) Resolving the neural circuits of anxiety. Nat Neurosci 18: 1394-404.

Chen S, Buckmaster PS (2005) Stereological analysis of forebrain regions in kainate-treated epileptic rats. Brain Res.1057(1-2):141-52.

Corbani M, Marir R, Trueba M, Chafai M, Vincent A, Borie AM, Desarmenien MG, Ueta Y, Tomboly C, Olma A, Manning M, Guillon G (2017) Neuroanatomical distribution and function of the vasopressin V1B receptor in the rat brain deciphered using specific fluorescent ligands. Gen Comp Endocrinol.

Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM (1989) Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol Biochem Behav 32: 777-85.

Crawley J and Goodwin FK (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13(2):167-70.

Cryan JF, Sweeney FF (2011) The age of anxiety: role of animal models of anxiolytic action in drug discovery. Br J Pharmacol 164: 1129-61.

Dalgleish T (2004) The emotional brain. Nat Rev, 5, 582-589.

Davis M, Walker DL, Miles L, Grillon C (2010) Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacol 35: 105-35.

Davis M, Whalen PJ (2001) The amygdala: vigilance and emotion. Mol Psych 6: 13-34.

Debiec J & LeDoux J (2004) Fear and the Brain. Soc Res, 71(4), 807-818.

De Boer SF, Koolhaas JM (2003) Defensive burying in rodents: ethology, neurobiology and psychopharmacology. Eur J Pharmacol 463: 145-161.

De Celis R (2007) Investigación en neurociencias. Homenaje al Dr. Alfredo Feria Velasco. Editorial Bios-médica, México, D.F.

Degroot A, Treit D (2004) Anxiety is functionally segregated within the septo-hippocampal system. Brain Res 1001: 60-71.

de la Mora MP, Cardenas-Cachon L, Vazquez-Garcia M, Crespo-Ramirez M, Jacobsen K, Hoistad M, Agnati L, Fuxe K (2005) Anxiolytic effects of intra-amygdaloid injection of the D1 antagonist SCH23390 in the rat. Neurosci Letters 377:101-5.

de la Mora MP, Gallegos-Cari A, Arizmendi-García Y, Marcellino D, Fuxe K (2010) Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: Structural and functional analysis. Prog Neurobiol 90: 198-216.

de la Mora, MP Pérez-Carrera D, Crespo-Ramírez M, Tarakanov A, Fuxe K, Borroto-Escuela DO (2016) Signaling in dopamine D2 receptor-oxytocin receptor heterocomplexes and its relevance for the anxiolytic effects of dopamine and oxytocin interactions in the amygdala of the rat. Biochim Biophys Acta 1862: 2075-2085.

de Kloet ER, Voorhuis DA, Boschma Y, Elands J (1986) Estradiol modulates density of putative 'oxytocin receptors' in discrete rat brain regions. Neuroendocrinol 44: 415-21.

De Olmos JS, & Heimer L (1999) The concepts of the ventral striatopallidal system and extended amygdala. *Ann N Y Acad Sci, 877*, 1-32.

DiBenedictis BT, Nussbaum ER, Cheung HK, Veenema AH (2017) Quantitative mapping reveals age and sex differences in vasopressin, but not oxytocin, immunoreactivity in the rat social behavior neural network. J Comp Neurol 525(11):2549-2570.

DSM V (2013) Diagnostic and Statistical Manual of Mental Disorders, fifth edition Ed. American Psychiatric Association, Washington, DC.

Ebner K, Wotjak CT, Holsboer F, Landgraf R, Engelmann M (1999) Vasopressin released within the septal brain area during swim stress modulates the behavioural stress response in rats. Eur J Neurosci 11: 997-1002.

Egashira N, Tanoue A, Matsuda T, Koushi E, Harada S, Takano Y, Tsujimoto G, Mishima K, Iwasaki K, Fujiwara M (2007) Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice. Behav Brain Res 178: 123-7.

Egashira N, Tanoue A, Higashihara F, Fuchigami H, Sano K, Mishima K, Fukue Y, Nagai H, Takano Y, Tsujimoto G, Stemmelin J, Griebel G, Iwasaki K, Ikeda T, Nishimura R, Fujiwara M (2005) Disruption of the prepulse inhibition of the startle reflex in

vasopressin V1b receptor knockout mice: reversal by antipsychotic drugs. Neuropsychopharmacol 30: 1996-2005.

Engelmann M, Landgraf R, Wotjak CT (2004) The hypothalamic-neurohypophysial system regulates the hypothalamic-pituitary-adrenal axis under stress: an old concept revisited. Front Neuroendocrinol 25: 132-49.

Engelmann M, Ludwig M, Landgraft R (1994) Simultaneous monitoring of intracerebral release and behavior: endogenous vasopressin improves social recognition. J Neuroendocrinol 6: 391-5.

Engin E, Treit D (2008) Dissociation of the anxiolytic-like effects of Avpr1a and Avpr1b receptor antagonists in the dorsal and ventral hippocampus. Neuropeptides 42: 411-421.

Evans DA, De Bree FM, Nijenhuis M, Van Der Kleij AA, Zalm R, Korteweg N, Van Leeuwen FW, Burbach JP (2000) Processing of frameshifted vasopressin precursors. J Neuroendocrinol 12: 685-93.

Fernandez-Guasti A, Ferreira A, Picazo O (2001) Diazepam, but not buspirone, induces similar anxiolytic-like actions in lactating and ovariectomized Wistar rats. Pharmacol Biochem Behav 70: 85-93.

Finn DA, Rutledge-Gorman MT, Crabbe JC (2003) Genetic animal models of anxiety. Neurogenetics 4: 109-35.

Fujita O (1975) [Behavior-genetic analysis of responses in runway test as measures of emotional reactivity in rats: I.- phenotypic variations and heritability estimates based on offspring-parents regressions (author's transl)]. Shinrigaku Kenkyu 46: 281-92.

Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81: 629-83.

Gold PW, Goodwin FK, Reus VI (1978) Vasopressin in Affective-Illness. Lancet 1: 1233-1236.

Gray M, Innala L, Viau V (2012) Central Vasopressin V1A Receptor Blockade Impedes Hypothalamic–Pituitary–Adrenal Habituation to Repeated Restraint Stress Exposure in Adult Male Rats. Neuropsychopharmacol 37: 2712–2719

Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B, Maffrand JP, Soubrie P (2002) Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. Proc Natl Acad Sci USA 99: 6370-6375.

Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, Dodel R, Ekman M, Faravelli C, Fratiglioni L, Gannon B, Jones DH, Jennum P, Jordanova A, Jonsson L, Karampampa K, Knapp M, Kobelt G, Kurth T, Lieb R, Linde M, Ljungcrantz C, Maercker A, Melin B, Moscarelli M, Musayev A, Norwood F, Preisig M, Pugliatti M, Rehm J, Salvador-Carulla L, Schlehofer B, Simon R, Steinhausen HC, Stovner LJ, Vallat JM, Van

den Bergh P, van Os J, Vos P, Xu W, Wittchen HU, Jonsson B, Olesen J, Group CD (2011) Cost of disorders of the brain in Europe 2010. Eur Neuropsychopharmacol 21: 718-79.

Hallbeck M, Hermanson O, Blomqvist A (1999) Distribution of preprovasopressin mRNA in the rat central nervous system. J Comp Neurol 411: 181-200.

Harro J (2017) Animals, anxiety, and anxiety disorders: How to measure anxiety in rodents and why. Behav Brain Res

Henniger MS, Ohl F, Holter SM, Weissenbacher P, Toschi N, Lorscher P, Wigger A, Spanagel R, Landgraf R (2000) Unconditioned anxiety and social behaviour in two rat lines selectively bred for high and low anxiety-related behaviour. Behav Brain Res 111: 153-63.

Herman JP, Dolgas CM, Carlson SL (1998) Ventral subiculum regulates hypothalamo-pituitary-adrenocortical and behavioural responses to cognitive stressors. Neuroscience 86: 449-59.

Hernando F, Schoots O, Lolait SJ, Burbach JPH (2001) Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: Anatomical support for its involvement in the central effects of vasopressin. Endocrinology 142: 1659-1668.

Hernandez VS, Hernandez OR, Perez de la Mora M, Gomora MJ, Fuxe K, Eiden LE, Zhang L (2016) Hypothalamic vasopressinergic projections innervate central amygdala GABAergic neurons: implications for anxiety and stress coping. Front Neural Circuits 10: 92.

Hess J, Lesser D, Landgraf R (1992) Vasopressin and oxytocin in brain areas of rats selectively bred for differences in behavioral performance. Brain research 569: 106-11.

Hodgson RA, Higgins GA, Guthrie DH, Lu SX, Pond AJ, Mullins DE, Guzzi MF, Parker EM, Varty GB (2007) Comparison of the V1b antagonist, SSR149415, and the CRF1 antagonist, CP-154,526, in rodent models of anxiety and depression. Pharmacol Biochem Behav 86: 431-40.

Hughes RN, Hancock NJ (2016) Strain-dependent effects of acute caffeine on anxiety-related behavior in PVG/c, Long-Evans and Wistar rats. Pharmacol Biochem Behav 140: 51-61.

Huber D, Veinante P, Stoop R (2005) Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. Science (New York, NY) 308: 245-8.

James TA, Starr MS (1978) Effects and the rate and volume of injection of the pharmacological response elicited by intranigral microinjection of drugs. J Pharmacol Meth 1: 197-202.

Jankord R, Herman JP (2008) Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. Ann N Y Acad Sci 1148: 64-73.Johnston, J. B. (1923). Further contributions to the study of the evolution of the forebrain. *J Comp Neurol, 35*(5), 337-481. doi: Doi 10.1002/Cne.900350502

Jiménez-Velázquez G, López-Muñoz FJ, Fernández-Guasti A (2010) Parallel anxiolytic-like and antinociceptive actions of diazepam in the anterior basolateral amygdala and dorsal periaqueductal gray. Res Rep Brain Res 1349: 11-20.

Keck ME, Welt T, Muller MB, Uhr M, Ohl F, Wigger A, Toschi N, Holsboer F, Landgraf R (2003) Reduction of hypothalamic vasopressinergic hyperdrive contributes to clinically relevant behavioral and neuroendocrine effects of chronic paroxetine treatment in a psychopathological rat model. Neuropsychopharmacol 28: 235-43.

Keck ME, Wigger A, Welt T, Muller MB, Gesing A, Reul JM, Holsboer F, Landgraf R, Neumann ID (2002) Vasopressin mediates the response of the combined dexamethasone/CRH test in hyper-anxious rats: implications for pathogenesis of affective disorders. Neuropsychopharmacol 26: 94-105.

Kessler RC (2007) The global burden of anxiety and mood disorders: putting the European Study of the Epidemiology of Mental Disorders (ESEMeD) findings into perspective. J Clin Psychiatry 68 Suppl 2: 10-9.

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry 62: 593-602.

Klüver H, & Bucy PC (1937) “Psychic blindness” and other symptoms following bilateral temporal lobectomy in rhesus monkeys. *Am. J. Physiol., 119*, 352–353.

Koshimizu TA, Nakamura K, Egashira N, Hiroyama M, Nonoguchi H, Tanoue A (2012) Vasopressin V1a and V1b receptors: from molecules to physiological systems. Physiol Rev 92: 1813-64.

Landgraf R, Gerstberger R, Montkowski A, Probst JC, Wotjak CT, Holsboer F, Engelmann M (1995) V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. J Neurosci15: 4250-8.

Landgraf R, Neumann ID (2004a) Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. Front Neuroendocrinol 25: 150-176.

Landgraf R, Neumann ID (2004b) Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. Frontiers in neuroendocrinology 25: 150-76.

Laycock J (2010) Perspectives on Vasopressin. World Scientic

Laszlo K, Kovács A, Zagoracz O, Ollmann T, Péczely L, Kertes E, Lacy DG, Lénárd L (2016) Positive reinforcing effect of oxytocin microinjection in the rat central nucleus of amygdala.Behav Brain Res 296: 279-285.

LeDoux, J (1996). The Emotional Brain: The Mysterious Underpinnings of Emotional Life. New York: Simon & Schuster Paperback.

LeDoux JE (2000) Emotion circuits in the brain. *Annu Rev Neurosci, 23*, 155-184. doi: 10.1146/annurev.neuro.23.1.155

LeDoux J (2003) The emotional brain, fear, and the amygdala. Cell Mol Neurobiol, 23(4-5), 727-738.

LeDoux J (2007) The amygdala. Current Biol 17: R868-R874.

LeDoux J (2012) Rethinking the emotional brain. Neuron. 73(4):653-76.

Leng G, Brown CH, Russell JA (1999) Physiological pathways regulating the activity of magnocellular neurosecretory cells. Prog Neurobiol 57: 625-655.

Liebsch G, Montkowski A, Holsboer F, Landgraf R (1998) Behavioural profiles of two Wistar rat lines selectively bred for high or low anxiety-related behaviour. Behav Brain Res 94: 301-10.

Liebsch G, Wotjak CT, Landgraf R, Engelmann M (1996) Septal vasopressin modulates anxiety-related behaviour in rats. Neurosci Lett 217: 101-104.

Litvin Y, Murakami G, Pfaff DW (2011) Effects of chronic social defeat on behavioral and neural correlates of sociality: Vasopressin, oxytocin and the vasopressinergic V1b receptor. Physiol Behav 103: 393-403.

Lolait SJ, O'Carroll AM, Mahan LC, Felder CC, Button DC, Young WS, 3rd, Mezey E, Brownstein MJ (1995) Extrapituitary expression of the rat V1b vasopressin receptor gene. Proc Natl Acad Sci USA 92: 6783-6787.

Ludwig M, Bull PM, Tobin VA, Sabatier N, Landgraf R, Dayanithi G, Leng G (2005) Regulation of activity-dependent dendritic vasopressin release from rat supraoptic neurones. J Physiol 564: 515-22.

Ludwig M, Stern J (2015) Multiple signalling modalities mediated by dendritic exocytosis of oxytocin and vasopressin. Philos Trans R Soc Lond B Biol Sci 370.

MacLean Pd (1949) Psychosomatic disease and the visceral brain; recent developments bearing on the Papez theory of emotion. *Psychosom Med, 11*(6), 338-353.

Manning M, Stoev S, Chini B, Durroux T, Mouillac B, Guillon G (2008) Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents. Prog Brain Res 170: 473-512.

Manning M, Stoev S, Bankowski K, Misicka A, Lammek B, Wo NC, Sawyer WH (1992) Synthesis and some pharmacological properties of potent and selective antagonists of the vasopressor (V1-receptor) response to arginine-vasopressin. J Med Chem 35: 382-388.

Mak P, Broussard C, Vacy K, Broadbear JH (2012) Modulation of anxiety behavior in the elevated plus maze using peptidic oxytocin and vasopressin receptor ligands in the rat. J Psychopharmacol. 26(4):532-42. doi: 10.1177/0269881111416687.

McAuley JD, Stewart AL, Webber ES, Cromwell HC, Servatius RJ, Pang KC (2009) Wistar-Kyoto rats as an animal model of anxiety vulnerability: support for a hypervigilance hypothesis. Behav Brain Res 204: 162-8.

Meynert T (1867) Der Bau der Grosshirnrinde und seine ortlichen verschiedenheiten, nebst einem pathologisch-anatomischen Corollarium. Vjschr. Psychiat. 1, 77-93, 126-170, 198-217.

Millhouse O E (1986) The Intercalated Cells of the Amygdala. *Journal of Comparative Neurology, 247*(2), 246-271. doi: Doi 10.1002/Cne.902470209

Misslin R, Belzung C, Vogel E (1989) Behavioural validation of a light/dark choice procedure for testing anti-anxiety agents. Behav Processes 18: 119-132.

Mlynarik M, Zelena D, Bagdy G, Makara GB, Jezova D (2007) Signs of attenuated depression-like behavior in vasopressin deficient Brattleboro rats. Horm Behav 51: 395-405.

Mouillac B, Chini B, Balestre MN, Jard S, Barberis C, Manning M, Tribollet E, Trumpp-Kallmeyer S, Hoflack J, Elands J (1995) Identification of agonist binding sites of vasopressin and oxytocin receptors. Adv Exp Med Biol 395: 301-310.

Murat B, Devost D, Andrés M, Mion J, Boulay V, Corbani M, Zingg HH, Guillon G (2012) V1b and CRHR1 receptor heterodimerization mediates synergistic biological actions of vasopressin and CRH. Mol Endocrinol. 26(3):502-20. doi: 10.1210/me.2011-1202.

Murgatroyd C, Wigger A, Frank E, Singewald N, Bunck M, Holsboer F, Landgraf R, Spengler D (2004) Impaired repression at a vasopressin promoter polymorphism underlies overexpression of vasopressin in a rat model of trait anxiety. J Neuroscis 24: 7762-70.

Oliver G, Schafer EA (1895) On the Physiological Action of Extracts of Pituitary Body and certain other Glandular Organs: Preliminary Communication. J Physiol 18: 277-9.

Onaivi ES, Martin BR (1989) Neuropharmacological and physiological validation of a computer-controlled two-compartment black and white box for the assessment of anxiety. Prog Neuropsychopharmacol Biol Psychiatry 13: 963-76.

Ostrowski NL, Lolait SJ, Bradle DJ, O'Carroll AM, Brownstein MJ, Young WS 3rd (1992) Distribution of V1a and V2 vasopressin receptor messenger ribonucleic acids in rat liver, kidney, pituitary and brain. *Endocrinology*, 131(1): 533-535.

Ostrowski NL, Lolait SJ, Young WS, 3rd (1994) Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135: 1511-28.

Overstreet DH, Griebel G (2005) Antidepressant-like effects of the vasopressin V1b receptor antagonist SSR149415 in the Flinders Sensitive Line rat. Pharmacol Biochem Behav 82: 223-7.

Pabba M (2013) Evolutionary development of the amygdaloid complex. Front Neuroanat 7: 27.

Palomares-Castillo E, Hernandez-Perez OR, Perez-Carrera D, Crespo-Ramirez M, Fuxe K, & Perez de la Mora M (2012) The intercalated paracapsular islands as a module for integration of signals regulating anxiety in the amygdala. *Brain Res, 1476*, 211-234. doi: 10.1016/j.brainres.2012.03.047

Papez JW (1937) A poposed mechanism of emotions. Ach. Neurol. Pschiatr. 38: 725-743.

Pare WP (1994) Open field, learned helplessness, conditioned defensive burying, and forced-swim tests in WKY rats. Physiol Behav 55: 433-9.

Pare D, Quirk GJ, & Ledoux JE (2004). New vistas on amygdala networks in conditioned fear. J Neurophysiol, 92(1), 1-9. doi: 10.1152/jn.00153.2004

Paxinos G, Watson C (1986) The rat brain in stereotaxical coordinates, Fourth edn, Academic Press. San Diego.

Perez de la Mora, Lara-García D, Jacobsen KX, Vázquez-García M, Crespo-Ramírez M, Flores-Gracia C, Escamilla-Marvan E, Fuxe K (2006) Anxiolytic-like effects of the selective metabotropic glutamate receptor 5 antagonist MPEP after its intra-amygdaloid microinjection in three different non-conditioned rat models of anxiety. Eur J Neurosci 23: 2749-2759.

Perez de la Mora M, Hernandez-Gomez AM, Arizmendi-Garcia Y, Jacobsen KX, Lara-Garcia D, Flores-Gracia C, Fuxe K (2007a) Role of the amygdaloid cholecystokinin (CCK)/gastrin-2 receptors and terminal networks in the modulation of anxiety in the rat. Effects of CCK-4 and CCK-8S on anxiety-like behaviour and [3H]GABA release. Eur J Neurosci, 26(12), 3614-3630. doi: 10.1111/j.1460-9568.2007.05963.x

Pérez de la Mora M, Flores-García C, Crespo-Ramírez M, Hernández-Goméz, AM y Lara-García DA (2007b) La amígdala una estructura anatómica con relevancia para la ansiedad. In R. DeCelis (Ed.), Investigación en neurociencias. Homenaje al Dr. Alfredo Feria Velasco (pp. 169-175).

Perez de la Mora M, Jacobsen K X, Crespo-Ramirez M, Flores-Gracia C, & Fuxe, K (2008) Wiring and volume transmission in rat amygdala. Implications for fear and anxiety. Neurochem Res, 33(8), 1618-1633. doi: 10.1007/s11064-008-9722-9

Peterson SL (1988) Drug Microinjection Discrete Brain Regions. Kopf Carrier No. 50. David Kopf Instruments, Tujunga, California.

Price JL, Russchen FT, & Amaral DG (1987) The Limbic Region. II: The Amygdaloid Complex. *Elsevier Science.*

Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463: 3-33.

Remes O, Brayne C, van der Linde R, Lafortune L (2016) A systematic review of reviews on the prevalence of anxiety disorders in adult populations. Brain Behav 6: e00497.

Roper JA, O'Carroll AM, Young WS III, Lolait SJ (2011) The vasopressin Avprib receptor: molecular and pharmacological studies. Stress 14: 98–115.

Roshanaei-Moghaddam B, Pauly MC, Atkins DC, Baldwin SA, Stein MB, Roy-Byrne P (2011) Relative effects of CBT and pharmacotherapy in depression versus anxiety: is medication somewhat better for depression, and CBT somewhat better for anxiety?. Depress Anxiety. 28(7):560-7.

Roy-Byrne P, Craske MG, Sullivan G, Rose RD, Edlund MJ, Lang AJ, Bystritsky A, Welch SS, Chavira DA, Golinelli D, Campbell-Sills L, Sherbourne CD, Stein MB (2010) Delivery of evidence-based treatment for multiple anxiety disorders in primary care: a randomized controlled trial. JAMA 303(19):1921-8.

Royer S, Martina M, & Pare D (1999) An inhibitory interface gates impulse traffic between the input and output stations of the amygdala. J Neurosci, 19(23), 10575-10583.

Russell JA (1980) A circumplex model of affect. J Pers Soc Psychol 39: 1161-1178.

Sah P, Faber ESL, De Armentia ML, & Power J (2003) The amygdaloid complex: Anatomy and physiology. *Phys Rev, 83*(3), 803-834. doi: Doi 10.1152/Physrev.00002.2003

Salome N, Stemmelin J, Cohen C, Griebel G (2006) Differential roles of amygdaloid nuclei in the anxiolytic- and antidepressant-like effects of the V1b receptor antagonist, SSR149415, in rats. Psychopharmacology 187: 237-44.

Sawyer WH, Valtin H, Sokol HW (1964) Neurohypophysial Principles in Rats with Familial Hypothalamic Diabetes Insipidus (Brattleboro Strain). Endocrinology 74: 153-5.

Scott LV, Dinan TG (1998) Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: Implications for the pathophysiology of depression. Life Sci 62: 1985-1998.

Selye H (1936) A syndrome produced by diverse nocuous agents. Nature 138: 32-32.

Serradeil-Le Gal C, Wagnon J, Simiand J, Griebel G, Lacour C, Guillon G, Barberis C, Brossard G, Soubrie P, Nisato D, Pascal M, Pruss R, Scatton B, Maffrand JP, Le Fur G (2002) Characterization of (2S,4R)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3 dihydro-1H-indol-3-yl]-4-hydroxy-N,N-dimethyl-2-pyrrolidine carboxamide (SSR149415), a selective and orally active vasopressin V1b receptor antagonist. J Pharmacol Exp Ther. 300: 1122-1130.

Serradeil-Le Gal C, Wagnon J, 3rd, Tonnerre B, Roux R, Garcia G, Griebel G, Aulombard A (2005) An overview of SSR149415, a selective nonpeptide vasopressin V(1b) receptor antagonist for the treatment of stress-related disorders. CNS drug reviews 11: 53-68.

Shah AA, Han JY (2015) Anxiety. Continuum (Minneap Minn) 21: 772-82.

Shimazaki T, Iijima M, Chaki S (2006) The pituitary mediates the anxiolytic-like effects of the vasopressin V1B receptor antagonist, SSR149415, in a social interaction test in rats. Eur J Pharmacol 543: 63-67.

Sokol HW, Valtin H (1965) Morphology of the neurosecretory system in rats homozygous and heterozygous for hypothalamic diabetes insipidus (Brattleboro strain). Endocrinology 77: 692-700.

Sokol HW, Zimmerman EA (1982) The hormonal status of the Brattleboro rat. Ann N Y Acad Sci 394: 535-48.

Song Z, McCann KE, McNeill JK, Larkin TE2nd, Huhman KL, Albers HE (2014) Oxcytocin induces social communication by activating arginine-vassopressin V1a receptors and not oxytocin receptors. Psychoneuroendocrinol doi: 10.1016/jpsyneuen.20014.08.005.

Song Z, Larkin TE,Malley MO, Albers HE (2016) Oxytocin (OT) and arinine vasopressin (AVP) act on OT receptors an not AVP V1a receptors to enhance social recognition in adult Syrian hamsters (Mesocricetus aureatus). Horm Behav.doi:10.1016/j.yhbeh.2016.02.004.

Spooren WP, Vassout A, Neijt HC, Kuhn R, Gasparini F, Roux S, Porsolt RD, Gentsch C (2000) Anxiolytic-like effects of the prototypical metabotropic glutamate receptor 5 antagonist 2-methyl-6-(phenylethynyl)pyridine in rodents. J Pharmacol Exp Ther 295: 1267-1275.

Steimer T (2002) The biology of fear- and anxiety-related behaviors. Dialogues Clin Neurosci, 4(3), 231-249.

Starling EH, Verney EB (1925) The secretion of urine as studied on the isolated kidney. P R Soc Lond B-Conta 97: 321-363.

Stemmelin J, Lukovic L, Salome N, Griebel G (2005) Evidence that the lateral septum is involved in the antidepressant-like effects of the vasopressin V1b receptor antagonist, SSR149415. Neuropsychopharmacol 30: 35-42.

Stoop R, Hegoburu C, van den Burg E (2015) New opportunities in vasopressin and oxytocin research: a perspective from the amygdala. Annu Rev Neurosci. 38:369-88.

Sugimoto T, Saito M, Mochizuki S, Watanabe Y, Hashimoto S, Kawashima H (1994) Molecular cloning and functional expression of a cDNA encoding the human V1b vasopressin receptor. J Biol Chem 269: 27088-92.

Surget A, Belzung C (2008) Involvement of vasopressin in affective disorders. Eur J Pharmacol 583: 340-9.

Sylvers P, Lilienfeld SO, LaPrairie JL (2011) Differences between trait fear and trait anxiety: implications for psychopathology. Clin Psychol Rev 31: 122-37.

Swanson L W (2000) Cerebral hemisphere regulation of motivated behavior. *Brain Res, 886*(1-2), 113-164. doi: Doi 10.1016/S0006-8993(00)02905-X

Swanson L W, & Petrovich GD (1998) What is the amygdala? *Trends Neurosci, 21*(8), 323-331.

Szot P, Bale TL, Dorsa DM (1994) Distribution of messenger RNA for the vasopressin V1a receptor in the CNS of male and female rats. Brain Res Mol Brain Res 24: 1-10.

Szot P, Dorsa DM (1994) Expression of vasopressin mRNA in extrahypothalamic nuclei of the homozygous Brattleboro rat is not modulated by testosterone. Neuroendocrinol 58(4):381-7.

Taylor SP, Duvigneaud V, Kunkel HG (1953) Electrophoretic Studies of Oxytocin and Vasopressin. J Biol Chem 205: 45-53.

Thibonnier M, Preston JA, Dulin N, Wilkins PL, Berti-Mattera LN, Mattera R (1997) The human V3 pituitary vasopressin receptor: ligand binding profile and density-dependent signaling pathways. Endocrinology 138: 4109-22.

Treit D, Pinel JP, Fibiger HC (1981) Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. Pharmacol Biochem Behav 15: 619-626.

Treschan TA, Peters J (2006) The vasopressin system: physiology and clinical strategies. Anesthesiology 105: 599-612; quiz 639-40.

Vaccari C, Lolait SJ, Ostrowski NL (1998) Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. Endocrinology 139, 5015–5033.

Villabona C (2010) Antagonistas del receptor de vasopresina: los vaptanes. Endocrinología y Nutrición 57, Supplement 2: 41-52.

Weiskrantz L (1956) Behavioral changes associated with ablation of the amygdaloid complex in monkeys. *J Comp Physiol Psychol, 49*(4), 381-391.

Wersinger SR, Ginns EI, O´Carroll A-M, Lolait SJ, Young WS (2002) Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Mol Psychiatry 7:975-984.

Wigger A, Sanchez MM, Mathys KC, Ebner K, Frank E, Liu D, Kresse A, Neumann ID, Holsboer F, Plotsky PM, Landgrafl R (2004) Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: Critical role of vasopressin. Neuropsychopharmacol 29: 1-14.

Williams AR, Carey RJ, Miller M (1983) Behavioral differences between vasopressin-deficient (Brattleboro) and normal Long-Evans rats. Peptides 4: 711-6.

Wotjak CT, Ludwig M, Ebner K, Russell JA, Singewald N, Landgraf R, Engelmann M (2002) Vasopressin from hypothalamic magnocellular neurons has opposite actions at the adenohypophysis and in the supraoptic nucleus on ACTH secretion. Eur J Neurosci 16: 477-485.

Young LJ (2001) Oxytocin and vasopressin as candidate genes for psychiatric disorders: Lessons from animal models. Am. J. Med. Genet 105: 53-54.

Young WS, 3rd, Gainer H (2003) Transgenesis and the study of expression, cellular targeting and function of oxytocin, vasopressin and their receptors. Neuroendocrinology 78: 185-203.

Young WS, Li J, Wersinger SR, Palkovits M (2006) The vasopressin 1b receptor is prominent in the hippocampal area CA2 where it is unaffected by restraint stress or adrenalectomy. Neuroscience 143: 1031-9.

Young SF, Griffante C, Aguilera G (2007) Dimerization between vasopressin V1b and corticotropin releasing hormone type 1 receptors. Cell Mol Neurobiol. 27(4):439-61.

Zelena D (2012) Vasopressin in health and disease with a focus on affective disorders. Central nervous system agents in medicinal chemistry 12: 286-303.

Zhang L, Medina MP, Hernandez VS, Estrada FS, Vega-Gonzalez A (2010) Vasopressinergic network abnormalities potentiate conditioned anxious state of rats subjected to maternal hyperthyroidism. Neuroscience 168: 416-28.

Zhang L, Hernández VS (2013) Synaptic innervation to rat hippocampus by vasopressin-immuno-positive fibres from the hypothalamic supraoptic and paraventricular nuclei. Neurosci 228: 139-162

Zingg HH (1996) Vasopressin and oxytocin receptors. Baillieres Clin Endocrinol Metab 10: 75-96.

14. APÉNDICE: PRODUCCIÓN CIENTÍFICA DURANTE MIS ESTUDIOS DE DOCTORADO

14.1 Publicaciones científicas

- **Hernández-Pérez OR**, , Crespo-Ramírez M, Cuza-Ferrer Y, Anias-Calderón J, Zhang L, Roldan-Roldan G, Aguilar-Roblero R, Borroto-Escuela DO, Fuxe K, Perez de la Mora M. 2018 Differential activation of arginine-vasopressin receptor subtypes in the amygdaloid modulation of anxiety in the rat by arginine-vasopressin. Psychopharmacology doi: 10.1007/s00213-017-4817-0.

- Hernández VS, **Hernández OR**, Pérez de Mora M, Gomora MJ, Kjell F, Eiden L, Zhang L. 2016. Hypothalamic Vasopressinergic Projections Innervate Central Amygdala GABAergic Neurons: Implications for Anxiety and Stress Coping. Front Neural Circuits. 18;10:92

- Palomares-Castillo E, **Hernández-Pérez OR**, Pérez-Carrera D, Crespo-Rmírez M, Fuxe K, Pérez de la Mora M. 2012. The intercalated parcapsular islands as a module for integration of signals regulating anxiety in the amygdala. Brain Res. 1476:211-34.

14.2 Presentaciones en congresos nacionales e internacionales

Hernández-Pérez OR., Crespo-Ramírez M., Fuxe K., Pérez de la Mora M. 2017 Differential activation of vasopressin receptors on the amygdaloid modulation of anxiety in the rat by arginine-vasopressin. 47nd Annual Meeting Neuroscience of the Society for Neuroscience. Washington DC. USA

Hernández-Pérez OR., Crespo-Ramírez M., Fuxe K., Pérez de la Mora M. 2017 Differential activation of arginine-vasopressin receptor subtypes in the amygdaloid modulation of anxiety in the rat by arginine-vasopressin. 12th World Congress on Neurohypophysial Hormones. Mangaratiba, RJ, Brasil.

Hernández-Pérez OR., Crespo-Ramírez M., Fuxe K., Pérez de la Mora M. 2017 Papel de la arginina vasopresina en la modulación amigdalina del miedo innato en la rata. Encuentro de estudiantes de neurociencia y conducta. Ciudad de México, México.

Hernández VS., **Hernández-Pérez OR**., Pérez de la Mora M., Zhang L. 2016 Osmotic stress modifies rat exploratory behavior in the EPM: Is there an anxiogenic role of the hypothalamic magnocellular. Neurobiology of Stress Workshop. Newport Beach CA. USA.

Hernández-Pérez OR., Crespo-Ramírez M., Fuxe K., Pérez de la Mora, M. 2016 Differential activation of vasopressin receptors on the amygdaloid modulation of fear and anxiety in the rat. 46nd Annual Meeting Neuroscience of the Society for Neuroscience. Sandiego CA. USA

Hernández-Pérez OR., Favila S., Crespo-Ramírez M., Aguilar, R., Fuxe K., Pérez de la Mora M. 2016 1º Congreso de neurobiología. Role of arginine vasopressin on the amygdaloid modulation of fear and anxiety in the rat. Puebla, México.

Hernández-Pérez OR., Crespo-Ramírez M., Fuxe K., Pérez de la Mora M. 2015 Role of arginine vasopressin on the amygdaloid modulation of fear and anxiety. 45nd Annual Meeting Neuroscience of the Society for Neuroscience. Chicago, IL, USA.

Palomares, CE., **Hernández-Pérez OR**., Crespo-Ramírez M., Fuxe K., Pérez de la Mora M. 2015 Selective ablation of the intercalated neurons of the amygdala increased anxiety-like behavior in the Elevated Plus Maze. 45 Annual Meeting of the Society for Neuroscience. Chicago, IL, USA

Hernández-Pérez OR., Palomares CE., Crespo-Ramírez M., Fuxe K., Pérez de la Mora, M. 2013 Role of the vasopressin receptors within the amígdala in the modulation of unconditioned anxiety in the rata. 43nd Annual Meeting Neuroscience of the Society for Neuroscience. Sandiego CA.USA.

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ORIGINAL INVESTIGATION

Differential activation of arginine-vasopressin receptor subtypes in the amygdaloid modulation of anxiety in the rat by arginine-vasopressin

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Abstract

Rationale The amygdala plays a paramount role in the modulation of anxiety and numerous studies have shown that arginine vasopressin (AVP) elicits anxiogenic effects following either its systemic or septal administration.

Objectives The aim of this paper was to study the involvement of vasopressinergic neurotransmission in the amygdaloid modulation of unconditioned anxiety and to ascertain whether or not AVP receptor subtypes may have a differential role in this modulation.

Methods Anxiety behavior was evaluated both in Shock-Probe Burying Test and Light-Dark Box following the bilateral microinfusion of AVP alone or AVP together with either AVP 1a or AVP 1b receptor antagonists into the central amygdala (CeA).

Results AVP microinfusion elicited at low (1 ng/side) but not at high doses (10 ng/side) anxiogenic-like responses in the Shock-Probe Burying Test but not in the Light-Dark Box. SSR149415, an AVP 1b antagonist unlike Manning compound, an AVP 1a antagonist, fully prevented AVP effects in the Shock-Probe Burying Test when it was administered simultaneously with AVP. In addition, oxytocin receptor blockade also failed to affect AVP effects. No effects of any AVP antagonist by itself were observed in both anxiety paradigms.

Conclusions Our results indicate that AVP 1b receptor contribute to the amygdaloid modulation of anxiety at least in the context of the Shock-Probe Burying Test since no effects were noticed in the Light-Dark Box. It remains to the future to ascertain whether AVP receptor subtypes have indeed differential actions either in the modulation of global or specific features of unconditioned anxiety.

Keywords Amygdala · Vasopressin · AVP · SSR149415 · Manning compound · OTA · Anxiety Shock-Probe Burying Test · Light-Dark Box

José Anias-Calderón is deceased. This paper is dedicated to his memory.

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Introduction

Arginine-vasopressin (AVP), formerly known as antidiuretic hormone, is a neuropeptide mainly synthesized in magnocelullar neurosecretory neurons from both the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei (Buijs 1978). Copious evidence has indicated that AVP, in addition to contribute to plasma osmolality and blood pressure regulation, also has an important role in the modulation of both the hypothalamic-pituitary-adrenal (HPA) axis (Keck et al. 2002; Wotjak et al. 1996, 2002) and emotional responses such as anxiety and depression (Ebner et al. 1999; Scott and Dinan 1998; Wigger et al. 2004; Zelena 2012; Zhang et al. 2010).

According to this more generalized liability, extensive networks of AVP fibers have been disclosed in several extrahypothalamic nuclei including hippocampus, septum, and amygdala (Buijs 1978; Buijs et al. 1991; Caffe et al. 1987; De Vries et al. 1985), where AVP may be released as a neurotransmitter (Ebner et al. 2002; Landgraf et al. 1995a). In line with this, AVP V1a (Huber et al. 2005; Lolait et al. 1995; Ostrowski et al. 1994; Szot et al. 1994) and AVP V1b receptors (Corbani et al. 2017; Hernando et al. 2001; Stemmelin et al. 2005; Young et al. 2006) have been reported in several brain regions, including the amygdala.

On the other hand, septal microadministration of d(CH2)5Tyr(Me) AVP, an AVP V1a antagonist (Manning et al. 1992), has been shown to produce anxiolytic-like effects (Liebsch et al. 1996) and retro-dialysis septal infusion of an antisense oligo-desoxyribonuleotide for AVP V1a receptor was found to reduce anxiety-like behavior in the Elevated-Plus Maze (EPM) (Landgraf et al. 1995b). In addition, mice having a deletion of AVP V1a receptor exhibited a marked reduction in anxiety-like behavior in several unconditioned anxiety models (Bielsky et al. 2004).

Although the role of AVP V1b receptors in anxiety has been considerably less studied, the available evidence suggests that this receptor subtype may also participate in its modulation. Accordingly, anxiolytic-like effects were found in several models of conditioned and unconditioned anxiety following both the oral (Griebel et al. 2002; Shimazaki et al. 2006) and i.p. (Griebel et al. 2002; Hodgson et al. 2007; Litvin et al. 2011; Overstreet and Griebel 2005) administration of SSR149415, an AVP V1b receptor antagonist (Serradeil-Le Gal et al. 2002). In agreement with the above, microinfusion of SSR149415 into the lateral septum (Stemmelin et al. 2005) elicited anxiolytic-like effects in the EPM. Interestingly, the effects of SSR149415 on anxiety seem to involve specific pools of neurons, since anxiolytic effects were observed when this antagonist was infused within the amygdaloid basolateral nucleus (BLA) but not into the central (CeA) and medial (MeA) amygdaloid nuclei (Salomé et al. 2006). Likewise, anxiolytic-like effects were elicited after its infusion into the dorsal but not into the ventral hippocampus (Engin and Treit 2008). It is however noteworthy that whereas AVP V1a receptor KO mice show reduced anxiety-like behavior in the Light-Dark Box, Elevated Plus-Maze, and Open-Field Test, AVP V1b receptor KO mice do not exhibited any differences in anxiety-like behavior in the same paradigms as compared with wild type mice (Egashira et al. 2005).

It is commonly accepted that amygdala is a complex structure involved in anxiety and fear processing (Davis and Whalen 2001; de la Mora et al. 2010; Ehrlich et al. 2009; Le Doux 2007; Pare et al. 2004; Roozendaal et al. 1992). However, in spite of the fact that this region is innervated by numerous vasopressinergic terminals from PVN (Buijs 1978, 1980; Hernández et al. 2016) and both AVP V1a and V1b receptors populate its CeA and other nuclei (Corbani et al. 2017; Hernando et al. 2001; Huber et al. 2005; Stemmelin et al. 2005; Young et al. 2006), the role of vasopressinergic neurotransmission in the modulation of anxiety by this structure needs to be clarified. The AVP neurotransmission mediated by AVP V1b receptors within the amygdala has been, to the best of our knowledge, only considered by Salomé et al. 2006. The aim of this paper was to study the role of vasopressinergic neurotransmission in the amygdaloid modulation of anxiety and to ascertain whether or not AVP 1b receptors may have a differential role in this modulation.

In addition, we will also investigate whether AVP neurotransmission has either a global influence on the amygdaloid regulation of anxiety or exerts its effects by modulating specific defensive responses as revealed by different anxiety models. Therefore, in this work, we used two different anxiety paradigms, the Light-Dark Box and the Shock-Probe Burying Test, which differ in terms of both the aversive stimuli that induce anxiety and the kind of defensive strategy used by the animals to avoid damage. In the Light-Dark Box (Costall et al. 1989), which exploits the innate tendency of rodents to escape from highly illuminated spaces, a passive coping behavior is used to avoid light. In contrast, in the Shock-Probe Burying Test (De Boer and Koolhaas 2003; Treit et al. 1981), which takes advantage of the natural propensity of rats to bury any harmful object in their environment, an active burying behavior protects animals from receiving electrical shocks during the test. Additionally, these two animal models have not been used to evaluate the effect of AVP on the amygdaloid modulation of anxiety.

The effects of AVP might be mediated through its activity at oxytocin receptors, as reported by Song et al. (2016). Therefore, in order to control for the validity of the AVP effects reported in this work, the effects of AVP were also evaluated in the presence of d(CH_2)$_5^1$,Tyr(Me)^2,Thr^4,Orn^8,des-Gly-NH_2^9)-vasotocin, an oxytocin antagonist (OTA). It has been widely used (Barberis et al. 1999; Bosch et al. 2007; Laszlo et al. 2016) to block AVP V1a receptors.

Substantial sex differences have been reported in the rat brain for both vasopressin and oxytocin systems in the amygdala and other brain regions (Di Benedictis et al. 2017; Dumais and Veenema 2016). This includes changes in social behavior (Dumais and Veenema 2016) as well as in AVP V1a receptor binding (Dumais et al. 2013). Therefore, only male rats were used in this work.

Experimental procedures

Animals

Male Wistar rats (250–270 g), bred in the Instituto de Fisiología Celular, Universidad Nacional Autónoma de

México, México City, Mexico, were used in behavioral experiments. Rats were housed in a controlled environment (temperature 22 °C; lights on 7:00–19:00 h) with water and food ad libitum. The experiments were conducted during the light phase of the day (9:00–18:00 h) according to the guidelines established by the local Mexican Ethics Committee, according to the "International Guiding Principles for Biomedical Research Involving Animals," Council for International Organizations of Medical Sciences, 2010. Efforts were taken to minimize animals' suffering throughout all experimental procedures. In all behavioral experiments, rats were randomly assigned to groups and also tested randomly.

Behavioral experiments

Surgery and microinjection

For implantation of permanent guide cannulae into the amygdala, rats were anesthetized with a mixture of ketamine hydrochloride (60 mg/kg) and xylazine (5 mg/kg; Pisa Agropecuaria) (Atalaquie, Hgo, Mexico) i.p. and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) with the incisor bar set at − 3.3 mm. Body temperature was maintained at 37 °C using a CMA/150 temperature controller (CMA/Microdialysis, Stockholm, Sweden). Bilateral stainless steel cannulae of 46-mm outer diameter (C315G, Plastics One, Roanoke, VA, USA) were aimed to CeA (coordinates AP − 1.7 mm, ML ± 4.2 mm from bregma, DV − 7.7 mm from the skull surface, according to the atlas of Paxinos and Watson 1986. Guide cannulae were affixed with stainless steel screws and dental acrylic cement (Laboratorios Arias, México City, Mexico) and sealed with dummy cannulae (C315DC, Plastics One). Estreptobezetazil V-Fortificado (Fort Dodge Animal Health Laboratories, Mexico City, Mexico) was given to prevent infection. To avoid biting of the cannulae by rats in the same cage, the animals were housed in individual cages where they remained until they were injected.

After 7 days of recovery from surgery and anesthesia, rats were handled once a day (5 min) during either 4 or 3 days for the Shock-Probe Burying Test and Light-Dark Box, respectively. Open-Field was used following testing in both paradigms.

Drug administration

Vasopressin ([Arg8]-vasopressin acetate) was purchased from Sigma Chemical Co. (St. Louis, MO, USA); V1a antagonist ($d(CH_2)_5{}^1$,$Tyr(Me)^2$,Arg^8)-vasopressin trifluoroacetate salt (Manning compound) and OTA ($d(CH_2)_5{}^1$,$Tyr(Me)^2$,Thr^4,Orn^8,des-Gly-$NH_2{}^9$)-vasotocin trifluoroacetate salt were obtained from BACHEM Pioneering partner for peptides (Torrance, CA, USA); V1b antagonist SSR149415 was purchased from Axon Medchem (Groningen, GRQ). SSR149415 was prepared as a solution in 0.9% (*w*/*v*) NaCl containing 5% DMSO (Sigma, Lyon, France) and 5% Cremophor EL (Sigma, Lyon, France). [FITC] [Ahx]CYIQNCPLG[amide] was purchased from Biosynthesis Committed to Biomic Research (Lewisville, TX, USA).

On the day of the experiment, rats were treated bilaterally with AVP (either 1 or 10 ng/side), SSR149415 (either 1 or 10 ng/side), Manning compound (either 10 or 30 ng/side), or OTA (15 ng/side) as shown in "Results" via injection cannulae (0.20-mm outer diameter, C315I, Plastics One), which protruded 1 mm beyond the end of the guide cannulae over a period of 5 min, using two CMA/Microdialysis pumps (CMA/ Microdialysis, Stockholm, Sweden). Drugs were microinfused in a volume of 250 nl/side. The cannulae were kept in place for 60 s after the injection to prevent backflow and to allow for diffusion. Behavioral observations in every anxiety test were started immediately after AVP microinjection. The dose range of AVP used in this study was based in the study of Appenrodt and Schwarzberg (2000). V1b antagonist (SSR149415) was infused according to the work of Salomé et al. (2006) dealing with the effects of AVP on unconditioned fear in rats. Different cohorts of rats were used for either the Shock-Probe Burying Test or the Dark-Light Box.

In the experiments in which receptor antagonists were used, in order to facilitate that agonists and antagonists will reach their targets at nearly the same concentration ratio in which they were injected, and to be certain that their binding was mostly dependent on their receptor affinity, we infused both compounds simultaneously (for information see "Discussion"). Moreover, under these conditions, a double microinjection was avoided reducing the risk of tissue damage and preventing that the second compound, due to changes in tissue pressure produced by the high volume injected, will follow a different diffusion path.

Extent of vasopressin diffusion following its microinjection into the amygdala

In order to get an insight of the extent of AVP diffusion following its injection into the CeA, [FITC] [Ahx]CYIQNCPLG[Amide], a fluorescent oxytocin analog having a similar molecular weight and amino acid composition as AVP, was infused into the amygdala under the same conditions as used in this work. The animals were deeply anesthetized with sodium pentobarbital (65 mg per rat; Laboratorios Ttokkyo, Mexico City, Mexico). Ten minutes after infusion, the rats were killed under anesthesia and the brains removed from the animals and frozen (30–60 s) in dry ice-cooled isopentane. Cryostate sections (40 μm) were obtained and mounted on gelatinized slides with Krystalon. Sections were air-dried (5 days) at room temperature and observed in a fluorescence microscope (BX41 Olympus fitted

with a U-MNU2 emission filter). Volume of diffusion from the injection site was obtained by computing the volume of a sphere ($3/4\pi R^3$) centered at the site of injection using the larger diameter observed on the fluorescent image.

Shock-Probe Burying Test

The test was carried out essentially as described by Fernandez-Guasti et al. (2001) and Pesold and Treit (1995). The test was conducted in an acrylic cage (27 × 16 × 23 cm), which has its floor covered with a uniform layer (5 cm) of fine sawdust. The cage was equipped with an electrified probe (7-cm long, 0.5-cm thick) that protruded from one of its walls, 2 cm above the bedding and through which the rats receive an electric shock (0.4 mA) any time they come in contact with the probe. The current was generated by a constant-current shock generator (LaFayete Instruments, Inc.). During the test, the rat is introduced into the cage, and after the first electric shock, its behavior was recorded for 10 min. During the test, three parameters were recorded: the total amount of time that the rat spends burying the probe with the forepaws (burying behavior), the burying behavior latency, represented as the time from the first shock to the start of the burying behavior, and the number of shocks received by the rat during the test. The burying behavior was taken as the main measure of anxiety, the burying behavior latency as an index of reactivity, and the number of shocks as an indication of the harmful effects of the electric shocks. In addition, since the above responses reflect active coping behaviors, the effects of AVP on freezing (a passive coping behavior) was also evaluated. Freezing was taken as the % of the time of the test that the animal spends only executing the movements needed for respiration.

Light-Dark Box

Behavior was evaluated as previously described (de la Mora et al. 2005). Briefly, the box was of acrylic and divided into two compartments, connected by a small opening (7 × 7 cm). The walls and floor of one compartment (27 × 27 × 27 cm) were white, whereas those of a smaller compartment (27 × 18 × 27 cm) were black. Squares of 9 × 9 cm were marked on the floor of both compartments using either black or white lines. During the experiments, the white compartment was strongly illuminated with a cold light source (700 lx), whereas the black compartment was illuminated with a red light lamp (70 lx). At the beginning of the experiment, each rat was placed in the center of the white compartment facing the opening between the two compartments and behavior was video-recorded for 5 min. The latency of the first entry into the black compartment and the total time spent in the white compartment were taken as measures of anxiety-like behavior (Costall et al. 1989; Henniger et al. 2000). The box was cleaned with Dextran and dried before each trial.

Open-Field Test

To have an indication of the effects of either AVP or their antagonists (V1a, V1b) on locomotion, an Open-Field Test was used immediately after both the White and Black Box Test and the Shock-Probe Burying Test. Locomotion in this test was carried out in an acrylic box (50 × 50 × 30 cm) equipped with photoelectric cells to record the horizontal movements of the animals through the arena (OMNIALVA, Mexico City, Mexico). Each wall contained 10 photoelectric cells separated by 5 cm from each other that were located 4.0 cm above the arena. The box was interphased with a PC that allowed estimating quantitatively the number of beam interruptions through photocells during the locomotion of the animal in the arena and transforming them into arbitrary locomotion events (beam interruptions) with a sample frequency of 10 Hz. The illumination level inside the box was 138 lx. At the beginning of the test, the rats were placed in one of the corners of the box and were allowed to explore the arena for 5 min.

All behavioral experiments were carried out in a sound-attenuated room equipped with video-recording facilities. For each type of experiment, the apparatus used to evaluate the behavior was placed beneath the video camera, and the animal behavior was recorded in the absence of any observer and tested in a randomized way.

Histological evaluation

To verify the correct positioning of the implanted cannulae, at the end of the behavioral tests, the animals were deeply anesthetized with sodium pentobarbital (65 mg per rat; Laboratorios Ttokkyo, Mexico City, Mexico), and 250 nl of a diluted solution of pontamine sky blue was microinjected bilaterally via the injection cannulae.

Brains were removed and postfixed in 10% formaldehyde for 1 day and then cryoprotected by incubating the brains in PBS containing 10, 20, and 30% sucrose for 1 day in each solution. Serial coronal sections (40-μm thick) were made using a cryostat (CM 1510-3 Leica Instruments, Nussloch, Germany), counterstained with Cresyl Violet, and the exact positions of the cannulae were determined.

Statistical analysis

Results are expressed as means ± SEM. Since, KS distance, using the Kolmogoroff–Smirnoff test, suggested that the population under study in the behavioral experiments that followed a normal distribution parametric statistics was used to evaluate the results. Accordingly, one-way ANOVA was practiced for each parameter followed when necessary ($P < 0.05$) by Dunnett's test as a post hoc test for comparing treated groups against their respective vehicle-treated control group. Effects

of AVP on freezing behavior and effects of 5% DMSO 5% Cremephor were evaluated using the unpaired Student's *t* test. Significance in all tests was set at $P < 0.05$. Statistical parameters were computed using GraphPad Prism 6 statistical software.

Results

Microinjection location and diffusion

Cannulae tips in those animals, which were treated with either saline, AVP, or AVP V1b antagonist and evaluated in the Shock-Probe Burying Test or the Light-Dark Box, were found within the amygdala boundaries either inside or surrounding CeA. Cannulae tips were found spanning from AP level −1.8 to −3.1 mm according to the Paxinos and Watson (1986) stereotaxic atlas. Similar cannulae locations were found in the rest of the animals used in this work (see Fig. 1 in the Supplementary material). Animals with their cannulae tips in other locations were excluded from the statistical analysis.

[FITC] [Ahx]CYIQNCPLG[Amide], a fluorescent oxytocin analog, was administered into the amygdala under the same experimental conditions as used in current work. As shown in Supplementary Fig. 2, it was found that this fluorescent marker remains near its site of injection and radially diffused at a distance of no more than 0.380 mm^3 from this site.

Effects of the bilateral intra-amygdaloid administration of AVP on the behavior of rats in the Shock-Probe Burying Test

As shown in Fig. 1A, the microinfusion of low doses of AVP within CeA elicited an enhancement of burying behavior in the Shock-Probe Burying Test. These effects were, however, lost when AVP was infused at higher doses (one-way ANOVA: $F_{2,26} = 4.743$; $P < 0.05$). Similar effects were observed on the latency to bury (Fig. 1B) (one-way ANOVA: $F_{2,26} = 4.881$; $P < 0.05$). A trend for an increase in the number of shocks received during the test (control 6.16 ± 1.26, $N = 12$; AVP 1 ng; 11.1 ± 3.0, $N = 8$; 10 ng 7.5 ± 1.0, $N = 9$) was also noticed at low AVP doses but disappeared when higher doses were infused (one-way ANOVA: $F_{2,26} = 1.951$; $P > 0.05$). No effects of AVP (1 and 10 ng/side) were also observed on locomotion (control 357.8 ± 24.5 events, $N = 12$; AVP 1 ng, 399.8 ± 41.6 events, $N = 8$; 10 ng, 335.2 ± 23.1 events, $N = 9$) in the Open-Field (one-way ANOVA: $F_{2,26} = 1.091$; $P > 0.05$).

In addition, only a non-significant trend: control group (3.155 ± 0.635, $N = 10$) vs AVP-treated group (1.738 ± 0.608, $N = 9$) for a decrease in the percentage of freezing behavior was observed when an effective dose of AVP (1 ng/side) was infused. Two-way Student's *t* test ($P > 0.05$).

Effects of the blockade of either AVP V1a or V1b receptors on the behavior elicited by the bilateral intra-amygdaloid administration of AVP in the Shock-Probe Burying Test

In order to know whether the effects of AVP on the behavior of rats in the Shock-Probe Burying Test were mediated by either AVP V1a or AVP V1b receptors, either AVP + Manning compound (AVP V1a antagonist) or AVP + SSR149415 (AVP V1b antagonist) was microinfused bilaterally into the CeA and the rats were tested in this paradigm.

As indicated in Fig. 1C, SSR149415 ($F_{4,48} = 3.968$; $P < 0.01$) fully prevented the effects of AVP on burying behavior. No effects of this antagonist were observed when it was infused alone (Fig. 1C). In contrast, no effects of either AVP alone (1 ng/side), SSR149415 alone (10 ng/side), and AVP + SSR149415 (either 1 ng + 1 ng/side or 1 ng + 10 ng/side) were observed on latency to bury ($F_{4,48} = 2.562$; $P > 0.05$) (Fig. 1D). Likewise, no effects were also noticed on the number of shocks received during the test (vehicle 4.0 ± 1.9, $N = 11$; AVP 1 ng 4.3 ± 2.9, $N = 9$; SSR149415 10 ng 4.2 ± 0.5, $N = 10$; AVP 1 ng + SSR149415 1 ng 3.9 ± 0.5, $N = 12$; AVP 1 ng + SSR149415 10 ng 3.6 ± 0.6, $N = 11$) ($F_{4,48} = 0.16$; $P > 0.05$) as well as on locomotion in the Open-Field in rats infused with any of the above treatments (vehicle 428.8 ± 25.4 events, $N = 11$; AVP 1 ng 451.1 ± 50.2 events, $N = 9$; SSR149415 10 ng 395.7 ± 30.9 events, $N = 10$; AVP 1 ng + SSR149415 1 ng = 415.7 ± 17.3 events, $N = 12$; AVP 1 ng + SSR149415 10 ng 449.5 ± 29.5 events, $N = 11$) ($F_{4,48} = 0.753$; $P > 0.05$).

Manning compound unlike SSR149415 failed to prevent the effects of AVP on burying behavior ($F_{3,40} = 3.137$; $P < 0.05$) (Fig. 2A). No effects of either Manning compound alone (10 ng/side) or AVP + MC (1 ng + 10 ng/side) were observed on latency to bury ($F_{3,40} = 0.534$; $P > 0.05$) (Fig. 2B) and number of shocks received during the test (saline 3.7 ± 0.5, $N = 12$; AVP 1 ng 3.7 ± 0.6, $N = 14$; Manning compound 10 ng 4.8 ± 0.6, $N = 8$; AVP 1 ng + Manning compound 10 ng 4.1 ± 0.5, $N = 10$) ($F_{3,40} = 0.655$; $P > 0.05$). No effects on locomotion in the Open-Field were recorded with any of the above treatments (saline 440.5 ± 40.5 events, $N = 12$; AVP 1 ng 487.5 ± 37.4 events, $N = 14$; Manning compound 10 ng 459.0 ± 37.9 events, $N = 8$; AVP 1 ng + Manning compound 10 ng 457.9 ± 55.4 events, $N = 10$) ($F_{3,40} = 0.230$; $P > 0.05$). In line with these results, no effects were found in a new experiment using a higher dose of Manning compound (30 ng/side). Thus, no

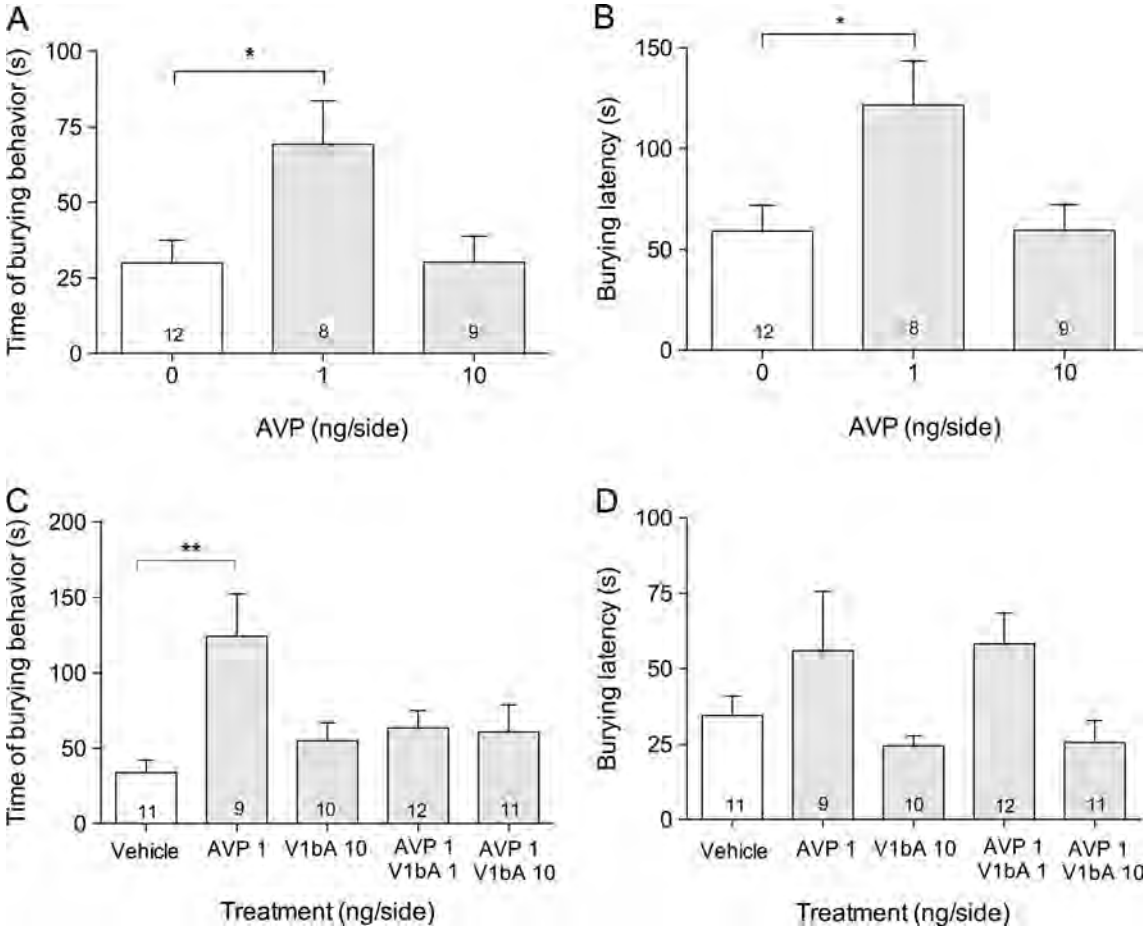

Fig. 1 Effects of the bilateral administration of arginine-vasopressin (AVP) on the behavior of rats in the Shock-Probe Burying Test and of its simultaneous administration with an AVP V1b receptor antagonist (V1bA). AVP at the lowest dose used increased both time of burying (**a**) and latency to bury (**b**). SSR149415, an AVP V1b antagonist, fully prevented the effects of AVP on burying behavior without having any effects by its own (**c**). Results are expressed as means ± SEM. *N* values are depicted inside each bar. * $P < 0.05$; ** $P < 0.01$. One-way ANOVA was followed by Dunnett's test

effects on AVP-induced increase on burying behavior ($F_{2,25} = 5.507$; $P < 0.05$) were noticed under these conditions (Fig. 2C). As earlier observed, latency to bury ($F_{2,25} = 0.537$; $P > 0.05$) (Fig. 2D) and number of shocks received during the test (saline 6.3 ± 0.5, $N = 10$; AVP 1 ng 4.4 ± 0.8, $N = 9$; AVP 1 ng + Manning compound 30 ng 4.1 ± 0.7, $N = 9$) were not affected ($F_{2,25} = 3.011$; $P > 0.05$). Effects of AVP on locomotion were also not affected (saline 611.5 ± 40.4 events, $N = 10$; AVP 1 ng 666.9 ± 36.3 events, $N = 9$; AVP 1 ng + Manning compound 30 ng 636.4 ± 27.6 events, $N = 9$) ($F_{2,25} = 0.611$; $P > 0.05$).

Effects of 5% DMSO 5% Cremophor EL on the behavior of rats in the Shock-Probe Burying Test

Since DMSO and Cremophor EL were used as a vehicle for infused SSR149415, the effects of this vehicle were studied in the Shock-Probe Burying Test. No effects were found in burying behavior (sec) (saline 54.6 ± 15.9 vs DMSO 68.8 ± 14.7) ($P = 0.523$; df = 13), latency to bury (s) (saline 46.8 ± 7.70 vs DMSO 49.6 ± 5.8) ($P = 0.768$; df = 13), number of shocks (saline 4.5 ± 0.68 vs DMSO 5.3 ± 0.65) ($P = 0.411$; df = 13), and locomotion (events) (saline 587.6 ± 44.9 vs DMSO 555.8 ± 26.68) ($P = 0.550$, df = 13). *N* in all cases was 7 for the saline group and 8 for the 5% DMSO group.

Effects of blockade of oxytocin receptors on the behavior elicited by AVP in the Shock-Probe Burying Test.

As shown in Fig. 2, OTA (15 ng/side) simultaneously infused with AVP (1 ng/side) failed to affect the effects of AVP on both burying behavior ($F_{3,38} = 3.775$; $P < 0.05$) (Fig. 2E), latency to bury ($F_{3,38} = 1.090$; $P > 0.05$) (Fig. 2F) and number of shocks received during the test (saline 4.6 ± 0.8, $N = 9$; AVP 1 ng 4.0 ± 0.5, $N = 12$; OTA 15 ng 4.7 ± 0.6, $N = 12$; AVP 1 ng + OTA

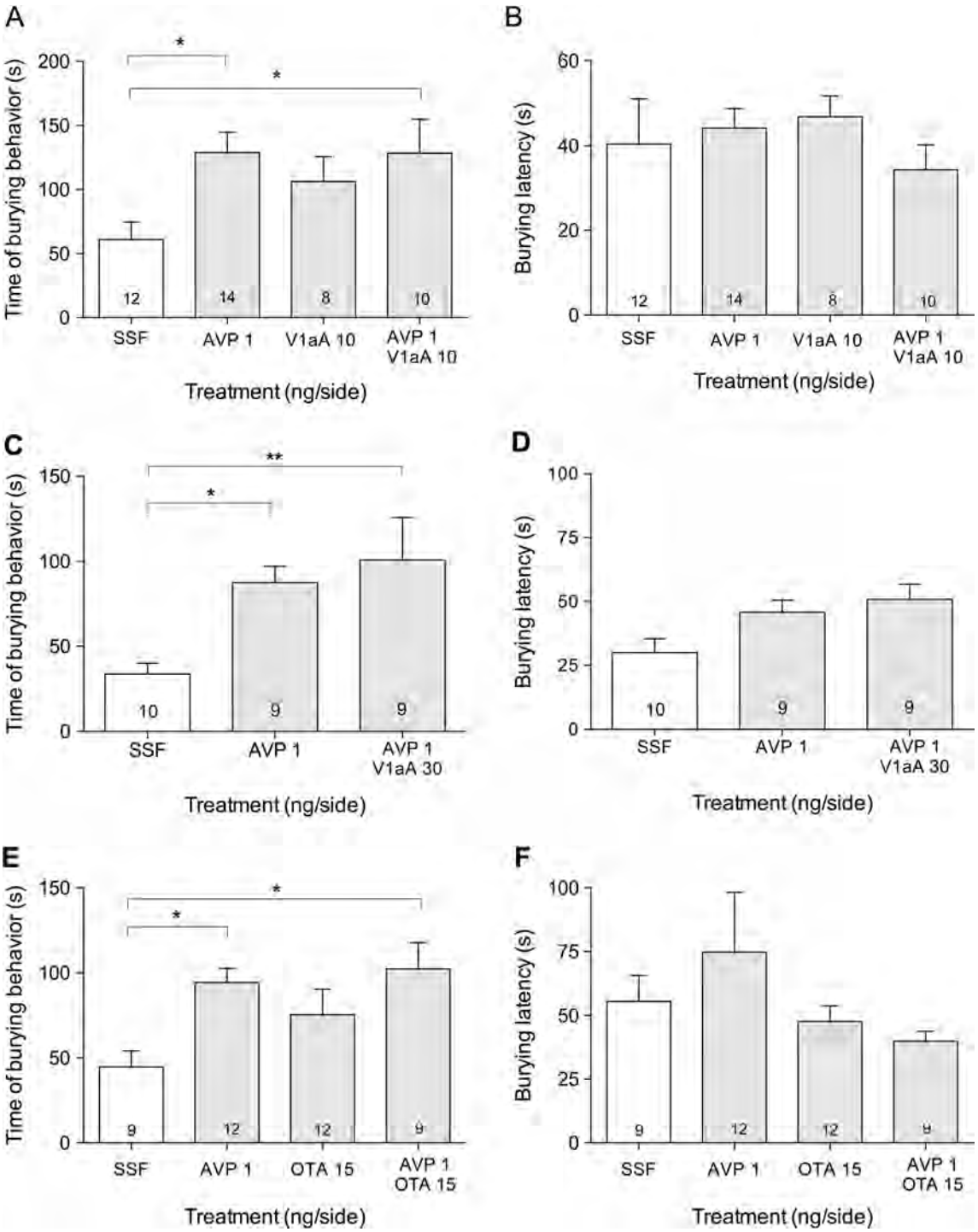

Fig. 2 Effects of the bilateral intra-amygdaloid administration of Manning compound (AVP V1a antagonist) and OTA (oxytocin antagonist) on the behavioral effects elicited by the microinfusion of arginine-vasopressin (AVP) into the CeA. Both Manning compound (**a**, **c**) and OTA (**e**) failed to prevent the effects of AVP on burying behavior. No effects on any of these antagonists were observed on burying latency when they were infused either alone or in combination with AVP (**b**, **d**, **f**). Results are expressed as means ± SEM. *N* values are depicted inside each bar. * $P < 0.05$; ** $P < 0.01$. One-way ANOVA was followed by Dunnett's test

15 ng 4.0 ± 0.5, $N = 9$) ($F_{3,38} = 0.4012$; $P > 0.05$). Effects of AVP on locomotion were also not affected (saline 647.4 ± 51.7 events, $N = 9$; AVP 1 ng 578.1 ± 30.5 events, $N = 12$; OTA 15 ng 571.8 ± 25.7 events, $N = 12$; AVP 1 ng + OTA 15 ng 634.9 ± 23.4 events, $N = 9$) ($F_{3,38} = 1.216$; $P > 0.05$).

Effects of the bilateral intra-amygdaloid administration of AVP on the behavior of rats in the Light-Dark Box

Although low values were found for the number of transitions and the time spent in the illuminated compartment of the Light-Dark Box in the control group, no effects of the bilateral intra-amygdaloid microinfusion of AVP and SSR149415 alone or in combination (1 ng/side + either 1 or10 ng/side) on the behavior of rats in the Light-Dark Box were observed in the latency ($F_{4,80} = 0.173$; $P > 0.05$) to enter into the dark side of the box, which is regarded as the most sensitive measure in this test (Fig. 3A), the time spent in the light side of the box ($F_{4,80} = 0.802$; $P > 0.05$) (Fig. 3B) or the number of transitions between light and dark compartments ($F_{4,80} = 1.267$; $P > 0.05$) (Fig. 3C). Likewise, no effects of any treatment were registered on locomotion in the Open-Field Test ($F_{4,80} = 0.089$; $P > 0.05$) (Fig. 3D).

Discussion

In this work, it was found that the bilateral intra-amygdaloid administration of AVP in male rats results in an increase in burying behavior, which is the most sensitive measure of anxiety in the Shock-Probe Burying Test (Treit et al. 1981; De Boer and Koolhaas 2003). In agreement with this, a trend for a decrease in freezing behavior was observed, suggesting that at least in this test and under the conditions employed (presence of bedding material), only active coping behaviors (De Boer and Koolhaas 2003) are modulated by AVP when administered into the amygdala.

Our results suggest that AVP has an anxiogenic role in the amygdaloid modulation of anxiety. The main finding of this work was however to disclose the involvement of AVP V1b receptor mechanisms in this modulation. Thus, it was found that the bilateral intra-CeA infusion of SSR149415, a selective AVP V1b receptor antagonist (Serradeil-Le Gal et al. 2002),

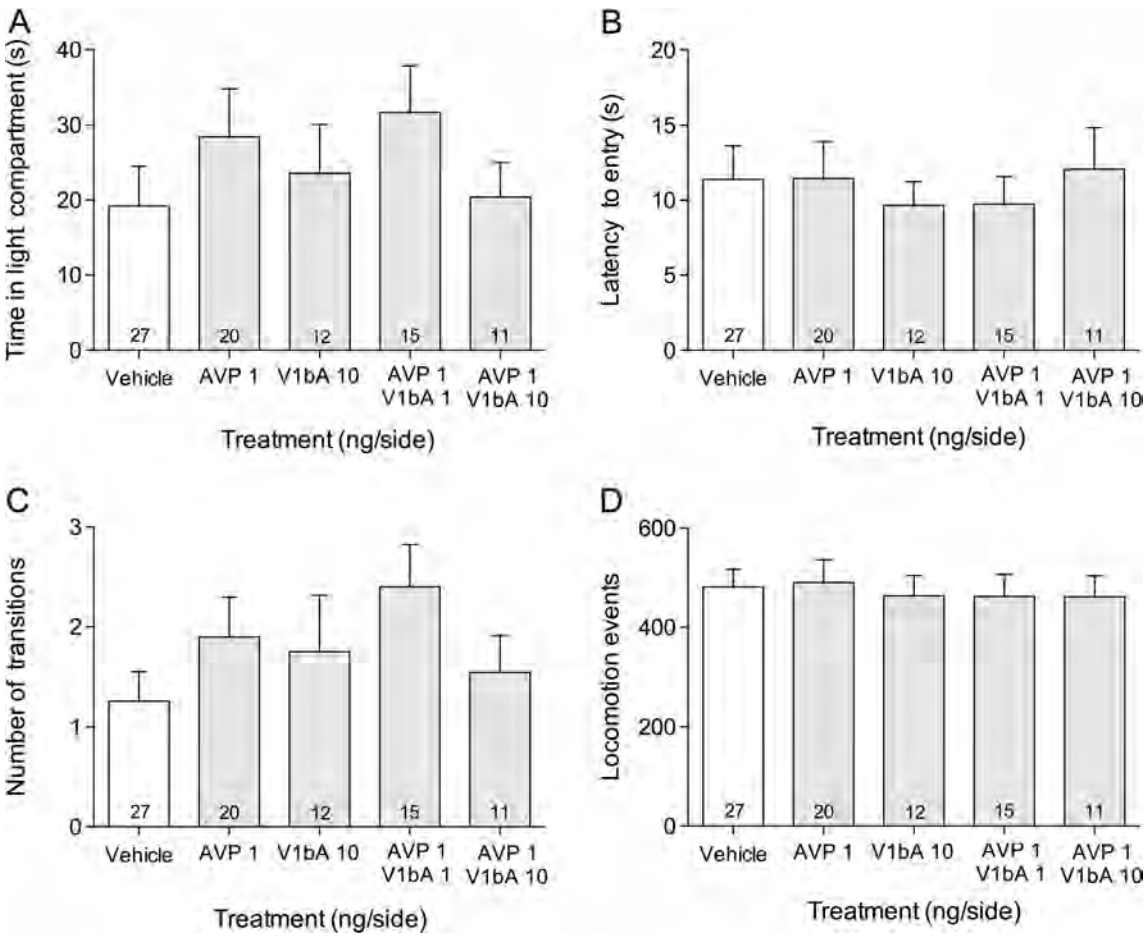

Fig. 3 Effects of the bilateral administration of arginine-vasopressin (AVP) and SSR149415 (AVP V1b antagonist) in the CeA on the behavior of rats in the Light-Dark Box. Both AVP and SSR149415 either alone or in combination failed to affect both time in the light compartment (**a**), latency to enter into the light compartment (**b**), and number of transitions between light and dark compartments (**c**). Similarly, no effects were observed for any treatment in the locomotion in the Open-Field (**d**). Results are expressed as means ± SEM. *N* values are depicted inside each bar. * $P < 0.05$. One-way ANOVA was followed by Dunnett's test

fully prevented the AVP-induced effects on anxiety-like behavior. The effects of SSR149415 on AVP-induced anxiety-like behavior seem to have been produced by a blocking effect on agonist activated AVP V1b receptors. In agreement with Salomé et al. (2006), no effects were produced by its intra-amygdaloid administration alone indicating a lack of an AVP tone within the CeA under the current basal conditions. Furthermore, its blocking effects on anxiety-like behavior seem to be rather specific since its intra-amygdaloid administration did not elicit any changes on both number of shocks received during the Shock-Probe Burying Test and locomotion in the Open-Field. Likewise, no effects were observed in the Dark-Light Box.

In support of the above, $(d(CH_2)_5{}^1,Tyr(Me)^2,Arg^8)$-vasopressin (Manning compound), a selective AVP V1a receptor antagonist (Manning et al. 1992), was unable to block the AVP-induced anxiogenic-like effects when it was bilaterally administered together with AVP into the CeA. The possibility that the lack of effects of this antagonist was due to the fact that they were administered simultaneously is unlikely for the reasons indicated under "Drug administration" and because SSR149415 injected under the same conditions was effective in blocking the anxiogenic effects of AVP. Furthermore, it can be argued that the Manning compound failed to block AVP V1a receptors because of the simultaneous presence of AVP in the infused fluid. However, this is unlikely since AVP has, as a matter of fact, a lower affinity than the Manning compound for the AVP V1a receptor (AVP Ki 2.6 nM (Manning et al. 2008) vs Manning compound (Ki 0.7 nM (Thibonnier et al. 1997). Furthermore, this antagonist was administered at higher doses than AVP (10- and 30-ng Manning compound vs 1-ng AVP).

The effects of AVP on anxiety-like behavior were only observed at low doses (1 ng/side) and disappeared when the dose was increased to 10 ng/side. These effects may indicate that at this high dose, AVP may have bound and activated codistributed (Huber et al. 2005; Mouillac et al. 1995) oxytocin receptors, which exert anxiolytic-like effects (Bale et al. 2001; De la Mora et al. 2016; Laszlo et al. 2016). Their activation may have dampened the anxiogenic AVP effects. In support of this possibility, recent work of Song et al. (2014, 2016) has convincingly demonstrated that, at least in Syrian hamsters, there exists a cross-talk between OT-AVP receptors and AVP-OT receptors.

The significant increase in latency observed with 1-ng AVP in the Shock-Probe Burying Test in one experiment along with several trends for the same effect observed in other experiments is quite surprising in spite of the fact that is not the main measure of anxiety in this test. Thus, one should expect a negative correlation between burying time and latency to bury (De Boer and Koolhaas 2003). However, latency to bury can be linked to the reactivity of the animal rather than to anxiety and a diminished orofacial sensitivity has been reported following the intra-CeA infusion of AVP (Ahn et al. 2001). Therefore, the increase in the latency observed in the current work may be related to a decreased sensitivity to the electrical shock received for the first time by the rats. In agreement with this suggestion, a trend for an increase in the number of shocks received during this test was recorded in our experiments. Alternatively, it may be feasible that the latency to bury is not sensitive to the administration of AVP in the present study.

In line with the anxiogenic effects of AVP with the low dose in the Shock-Probe Burying Test, an increased exploration of the open arms in the Elevated-Plus Maze was reported by Salomé et al. (2006) upon the intra-amygdaloid blockade of AVP V1b receptors with SSR149415. This also suggests that AVP is playing an anxiogenic role in its modulation of the amygdaloid activity.

In addition, our results also suggest that AVP does not exert a global effect in the amygdaloid modulation of anxiety but rather that its effects depend on the specific aspects of the anxiety modeled by each test. Thus, in contrast to the effects of AVP in the Shock-Probe Burying Test reported in this work and by Salomé et al. (2006) in the Elevated Plus-Maze, no effects of this neurotransmitter were observed in the "Light-Dark Box," another anxiety paradigm (Costall et al. 1989; Misslin et al. 1989). The reason for these differences is not clear as this test has also been successively used to detect both anxiogenic and anxiolytic effects (Hughes and Hancock 2016; Onaivi and Martin 1989). In consequence, the lack of effects of AVP neurotransmission in this test may be explained by the presence of significant dissimilarities between the aspects of anxiety modeled in each unconditioned anxiety paradigm (Spooren et al. 2000) undermining any global effect of AVP neurotransmission in anxiety. Interestingly, in line with our results, no differences in anxiety-like behavior were observed when AVP V1b KO and wild-type mice were evaluated in the Light-Dark Box (Egashira et al. 2005) and the EPM (Wersinger et al. 2002). It remains for the future to clarify whether or not different unconditioned anxiety features, as modeled in different unconditioned paradigms, are indeed differentially modulated by different AVP V1 receptor subtypes.

Since small infusion volumes (250 nl/side) and low-pressure flows (50 nl/min) were used to microinfuse all compounds used in this work, it is possible that the effects of AVP and all antagonists infused have taken place very near the site of their injection. In support of this, when [FITC][Ahx]CYIQNCPLG[amide], a fluorescent oxytocin analog, having a similar molecular weight and amino acid composition to AVP, was administered into the amygdala under the same experimental conditions used in this work, it was found to remain near the cannulae tip and radially diffusing no more than 0.38 mm^3 from the site of its injection. Moreover, previous results from our (de la Perez et al. 2006) and other laboratories (James and Starr 1978; Peterson 1988) have shown that several other compounds having a considerably lower

molecular weight than oxytocin and AVP also remain near their site of injection when they were microinfused under the conditions used in this work.

Most of the cannulae tips in this work were found within CeA and there exists evidence of a dense vasopressinergic innervation of CeA from the hypothalamic PVN nucleus (Hernández et al. 2016) and of the presence in the same area of AVP 1b receptors (Corbani et al. 2017; Hernando et al. 2001; Stemmelin et al. 2005; Young et al. 2006). It is therefore reasonable to suggest that the anxiogenic AVP effects reported in this work as well as their blockade by SSR149415 may have occurred somewhere within the CeA. It is however rather intriguing that while in the Salomé et al. (2006) study in agreement with our work, SSR149415 did not show any effects after being infused into the CeA, it had anxiolytic effects following its administration into the BLA. This suggests the existence of a selective AVP V1b-mediated AVP endogenous tone, which was blocked by the local SSR149415 administration. It will remain for the future to establish whether such a selective tone may in fact exist within the BLA.

A high density of AVP V1a receptors has been described located on CeA output neurons (Huber et al. 2005) presumably together with AVP V1b receptors (Corbani et al. 2017). It seems therefore likely that AVP infused into CeA in our experiments binds and activates both AVP V1a and presumably V1b receptors and at high concentrations, even OT receptors (Song et al. 2014). This broadens the modulatory features of the actions of AVP neurotransmission on the anxiogenic output of amygdala. In this context, it is important to note that GPCR iso- and heteroreceptor complexes are frequently formed representing a novel integrative mechanism in the CNS of high interest (Borroto-Escuela et al. 2014, 2016; Fuxe et al. 2013, 2014; de la Mora et al. 2016), including heteromerization of AVP V1a receptors with oxytocin receptors (Terrillon et al. 2003, 2004). The mechanisms by which AVP V1b receptors in CeA increase anxiety and neuronal excitability (Lu et al. 1997; Raggenbass 2008) could also involve actions at AVP V1b receptor protomers in putative AVP V1b-AVP V1a heterodimers modulating the AVP V1a receptor protomer signaling. Therefore, it is proposed that the anxiogenic effects of AVP in the Shock-Probe Burying Test can be a result of activation of AVP V1b receptor monomers/homomers and/or of AVP V1b-AVP V1a and AVP V1b-oxytocin heteroreceptor complexes altering their signaling in at least a pool of medial CeA anxiogenic output neurons.

The results of this work underline the role of AVP neurotransmission in the amygdaloid modulation of anxiety and open the possibility that not only AVP V1a but also the AVP V1b receptor contribute to this modulation. Furthermore, our results also suggest that AVP does not have a global modulatory effect on the amygdaloid regulation of anxiety but its modulatory action is dependent upon specific features of this response modeled by each paradigm of anxiety. It remains for the future to study whether and how both AVP receptor subtypes differentially participate in the modulation of specific features of unconditioned anxiety and their signaling integrated through allosteric mechanisms in putative AVP V1a-AVP V1b and/or AVP V1b-oxytocin heteroreceptor complexes of the medial CeA projection neurons.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Ahn DK, Kim KH, Ju JS, Kwon S, Park JS (2001) Microinjection of arginine vasopressin into the central nucleus of amygdala suppressed nociceptive jaw opening reflex in freely moving rats. Brain Res Bull 55(1):117–121. https://doi.org/10.1016/S0361-9230(01)00493-2

Appenrodt E, Schwarzberg H (2000) Central vasopressin administration failed to influence anxiety behavior after pinealectomy in rats. Physiol Behav 103:393–403 68: 735-9

Bale TL, Davis AM, Auger AP, Dorsa DM, McCarthy MM (2001) CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior. J Neurosci 21(7):2546–2552

Barberis C, Morin D, Durroux T, Mouillac B, Guillon G, Hibert M, Tribollet E, Manning M (1999) Molecular pharmacology of vasopressin and oxytocin receptors and therapeutic potential. Manning Drug News Perspect 12(5):279–292. https://doi.org/10.1358/dnp.1999.12.5.863621

Bielsky IF, Hu SB, Szegda KL, Westphal H, Young LJ (2004) Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacology 29(3):483–493. https://doi.org/10.1038/sj.npp.1300360

Borroto-Escuela DO, Brito I, Romero-Fernandez W, Di Palma M, Oflijan J, Skieterska K, Duchou J, Van Craenenbroeck K, Suárez-Boomgaard D, Rivera A, Guidolin D, Agnati LF, Fuxe K (2014) The G protein-coupled receptor heterodimer network (GPCR-HetNet) and its hub components. Int J Mol Sci 15(5):8570–8590. https://doi.org/10.3390/ijms15058570

Borroto-Escuela DO, Tarakanov AO, Fuxe K (2016) FGFR1-5-HT1A heteroreceptor complexes: implications for understanding and treating major depression. Trends Neurosci 39(1):5–15. https://doi.org/10.1016/j.tins.2015.11.003

Bosch OJ, Sartori SB, Singewald N, Neumann ID (2007) Extracellular amino acid levels in the paraventricular nucleus and the central amygdala in high- and low-anxiety dams rats during maternal aggression: regulation by oxytocin. Stress 10(3):261–270. https://doi.org/10.1080/10253890701223197

Buijs RM (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. Cell Tissue Res 192:423–435

Buijs RM (1980) Immunocytochemical demonstration of vasopressin and oxytocin in the rat brain by light and electron microscopy. J Histochem Cytochem 28(4):357–360. https://doi.org/10.1177/28.4.6989899

Buijs RM, Hermes MLHJ, Kalsbeek A, Vanderwoude T, Vanheerikhuize JJ (1991) Vasopressin distribution, origin and functions in the central-nervous-system. Colloq Inserm 208:149–158

Caffe AR, van Leeuwen FW, Luiten PG (1987) Vasopressin cells in the medial amygdala of the rat project to the lateral septum and ventral hippocampus. J Comp Neurol 261(2):237–252. https://doi.org/10.1002/cne.902610206

Corbani M, Marir R, Trueba M, Chafai M, Vincent A, Borie AM, Desarmenien MG, Ueta Y, Tomboly C, Olma A, Manning M, Guillon G (2017) Neuroanatomical distribution and function of the vasopressin V1B receptor in the rat brain deciphered using specific fluorescent ligands. Gen Comp Endocrinol. https://doi.org/10.1016/j.ygcen.2017.10.011

Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM (1989) Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol Biochem Behav 32(3):777–785. https://doi.org/10.1016/0091-3057(89)90033-6

Davis M, Whalen PJ (2001) The amygdala: vigilance and emotion. Mol Psychiatry 6(1):13–34. https://doi.org/10.1038/sj.mp.4000812

De Boer SF, Koolhaas JM (2003) Defensive burying in rodents: ethology, neurobiology and psychopharmacology. Eur J Pharmacol 463(1-3):145–161. https://doi.org/10.1016/S0014-2999(03)01278-0

de la Mora MP, Cardenas-Cachon L, Vazquez-Garcia M, Crespo-Ramirez M, Jacobsen K, Hoistad M, Agnati L, Fuxe K (2005) Anxiolytic effects of intra-amygdaloid injection of the D1 antagonist SCH23390 in the rat. Neurosci Lett 377(2):101–105. https://doi.org/10.1016/j.neulet.2004.11.079

de la Mora MP, Gallegos-Cari A, Arizmendi-García Y, Marcellino D, Fuxe K (2010) Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: structural and functional analysis. Prog Neurobiol 90(2):198–216. https://doi.org/10.1016/j.pneurobio.2009.10.010

de la Mora MP, Pérez-Carrera D, Crespo-Ramírez M, Tarakanov A, Fuxe K, Borroto-Escuela DO (2016) Signaling in dopamine D2 receptor-oxytocin receptor heterocomplexes and its relevance for the anxiolytic effects of dopamine and oxytocin interactions in the amygdala of the rat. Biochim Biophys Acta 1862(11):2075–2085. https://doi.org/10.1016/j.bbadis.2016.07.004

de la Perez M, Lara-García D, Jacobsen KX, Vázquez-García M, Crespo-Ramírez M, Flores-Gracia C, Escamilla-Marvan E, Fuxe K (2006) Anxiolytic-like effects of the selective metabotropic glutamate receptor 5 antagonist MPEP after its intra-amygdaloid microinjection in three different non-conditioned rat models of anxiety. Eur J Neurosci 23:2749–2759

De Vries GJ, Buijs RM, Van Leeuwen FW, Caffe AR, Swaab DF (1985) The vasopressinergic innervation of the brain in normal and castrated rats. J Comp Neurol 233:236–254

Di Benedictis BT, Nussbaum ER, Cheung HK, Veenema AH (2017) Quantitative mapping reveals age and sex differences in vasopressin, but not oxytocin, immunoreactivity in the rat social behavior neural network. J Comp Neurol 525(11):2549–2570. https://doi.org/10.1002/cne.24216

Dumais KM, Veenema AH (2016) Vasopressin and oxytocin receptor systems in the brain: sex differences and sex-specific regulation of social behavior. Front Neuroendocrinol 40:1–23. https://doi.org/10.1016/j.yfrne.2015.04.003

Dumais KM, Bredewold R, Mayer TE, Veenema AH (2013) Sex differences in oxytocin receptor binding in forebrain regions: correlations with social interest in brain region- and sex-specific ways. Horm Behav 64(4):693–701. https://doi.org/10.1016/j.yhbeh.2013.08.012

Ebner K, Wotjak CT, Holsboer F, Landgraf R, Engelmann M (1999) Vasopressin released within the septal brain area during swim stress modulates the behavioural stress response in rats. Eur J Neurosci 11(3):997–1002. https://doi.org/10.1046/j.1460-9568.1999.00508.x

Ebner K, Wotjak CT, Landgraf R, Engelmann M (2002) Forced swimming triggers vasopressin release within the amygdala to modulate stress-coping strategies in rats. Eur J Neurosci 15(2):384–388. https://doi.org/10.1046/j.0953-816x.2001.01869.x

Egashira N, Tanoue A, Higashihara F, Fuchigami H, Sano K, Mishima K, Fukue Y, Nagai H, Takano Y, Tsujimoto G, Stemmelin J, Griebel G, Iwasaki K, Ikeda T, Nishimura R, Fujiwara M (2005) Disruption of the prepulse inhibition of the startle reflex in vasopressin V1b receptor knockout mice: reversal by antipsychotic drugs. Neuropsychopharmacology 30(11):1996–2005. https://doi.org/10.1038/sj.npp.1300784

Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herry C, Lüthi A (2009) Amygdala inhibitory circuits and the control of fear memory. Neuron 62(6):757–771. https://doi.org/10.1016/j.neuron.2009.05.026

Engin E, Treit D (2008) Dissociation of the anxiolytic-like effects of Avpr1a and Avpr1b receptor antagonists in the dorsal and ventral hippocampus. Neuropeptides 42(4):411–421. https://doi.org/10.1016/j.npep.2008.04.004

Fernandez-Guasti A, Ferreira A, Picazo O (2001) Diazepam, but not buspirone, induces similar anxiolytic-like actions in lactating and ovariectomized Wistar rats. Pharmacol Biochem Behav 70(1):85–93. https://doi.org/10.1016/S0091-3057(01)00586-X

Fuxe K, Borroto-Escuela DO, Tarakanov A, Fernandez WR, Manger P, Rivera A, Van Craenenbroeck K, Skieterska K, Diaz-Cabiale Z, Filip M, Ferraro L, Tanganelli S, Guidolin D, Cullheim S, de la Mora MP, Agnati LF (2013) Understanding the balance and integration of volume and synaptic transmission. Relevance for psychiatry. Neurol Psychiatry Brain Res 19(4):141–158. https://doi.org/10.1016/j.npbr.2013.10.002

Fuxe K, Borroto-Escuela DO, Romero-Fernandez W, Palkovits M, Tarakanov AO, Ciruela F, Agnati LF (2014) Moonlighting proteins and protein-protein interactions as neurotherapeutic targets in the G protein-coupled receptor field. Neuropsychopharmacology 39(1):131–155. https://doi.org/10.1038/npp.2013.242

Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B, Maffrand JP, Soubrie P (2002) Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. Proc Natl Acad Sci U S A 99(9):6370–6375. https://doi.org/10.1073/pnas.092012099

Henniger MS, Ohl F, Holter SM, Weissenbacher P, Toschi N, Lorscher P, Wigger A, Spanagel R, Landgraf R (2000) Unconditioned anxiety and social behaviour in two rat lines selectively bred for high and low anxiety-related behaviour. Behav Brain Res 111(1-2):153–163. https://doi.org/10.1016/S0166-4328(00)00151-0

Hernández VS, Hernández OR, Perez de la Mora M, Gómora MJ, Fuxe K, Eiden LE, Zhang L (2016) Hypothalamic vasopressinergic projections innervate central amygdala GABAergic neurons: implications for anxiety and stress coping. Front Neural Circuits 10(92):1–19. https://doi.org/10.3389/fncir.2016.00092

Hernando F, Schoots O, Lolait SJ, Burbach JPH (2001) Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: anatomical support for its involvement in the central effects of vasopressin. Endocrinology 142(4):1659–1668. https://doi.org/10.1210/endo.142.4.8067

Hodgson RA, Higgins GA, Guthrie DH, Lu SX, Pond AJ, Mullins DE, Guzzi MF, Parker EM, Varty GB (2007) Comparison of the V1b antagonist, SSR149415, and the CRF1 antagonist, CP-154,526, in

rodent models of anxiety and depression. Pharmacol Biochem Behav 86(3):431–440. https://doi.org/10.1016/j.pbb.2006.12.021

Huber D, Veinante P, Stoop R (2005) Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. Science 308(5719):245–248. https://doi.org/10.1126/science.1105636

Hughes RN, Hancock NJ (2016) Strain-dependent effects of acute caffeine on anxiety-related behavior in PVG/c, Long-Evans and Wistar rats. Pharmacol Biochem Behav 140:51–61. https://doi.org/10.1016/j.pbb.2015.11.005

James TA, Starr MS (1978) Effects and the rate and volume of injection of the pharmacological response elicited by intranigral microinjection of drugs. J Pharmacol Methods 1(3):197–202. https://doi.org/10.1016/0160-5402(78)90049-9

Keck ME, Wigger A, Welt T, Muller MB, Gesing A, Reul JM, Holsboer F, Landgraf R, Neumann ID (2002) Vasopressin mediates the response of the combined dexamethasone/CRH test in hyperanxious rats: implications for pathogenesis of affective disorders. Neuropsychopharmacology 26(1):94–105. https://doi.org/10.1016/S0893-133X(01)00351-7

Landgraf R, Kubota M, Holsboer F, Wotjak CT (1995a) Release of vasopressin and oxytocin within the brain and into blood: microdialysis and antisense targeting. Int Congr Ser 1098:243–256

Landgraf R, Gerstberger R, Montkowski A, Probst JC, Wotjak CT, Holsboer F, Engelmann M (1995b) V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. J Neurosci 15(6):4250–4258

Laszlo K, Kovács A, Zagoracz O, Ollmann T, Péczely L, Kertes E, Lacy DG, Lénárd L (2016) Positive reinforcing effect of oxytocin microinjection in the rat central nucleus of amygdala. Behav Brain Res 296:279–285. https://doi.org/10.1016/j.bbr.2015.09.021

Le Doux J (2007) The amygdala. Curr Biol 17:R868–R874

Liebsch G, Wotjak CT, Landgraf R, Engelmann M (1996) Septal vasopressin modulates anxiety-related behaviour in rats. Neurosci Lett 217(2-3):101–104. https://doi.org/10.1016/0304-3940(96)13069-X

Litvin Y, Murakami G, Pfaff DW (2011) Effects of chronic social defeat on behavioral and neural correlates of sociality: vasopressin, oxytocin and the vasopressinergic V1b receptor. Physiol Behav 103(3-4):393–403. https://doi.org/10.1016/j.physbeh.2011.03.007

Lolait SJ, O'Carroll AM, Mahan LC, Felder CC, Button DC, Young WS 3rd, Mezey E, Brownstein MJ (1995) Extrapituitary expression of the rat V1b vasopressin receptor gene. Proc Natl Acad Sci U S A 92(15):6783–6787. https://doi.org/10.1073/pnas.92.15.6783

Lu YF, Moriwaki A, Tomizawa K, Onuma H, Cai XH, Matsui H (1997) Effects of vasopressin and involvement of receptor subtypes in the rat central amygdaloid nucleus in vitro. Brain Res 768(1-2):266–272. https://doi.org/10.1016/S0006-8993(97)00655-0

Manning M, Stoev S, Bankowski K, Misicka A, Lammek B, Wo NC, Sawyer WH (1992) Synthesis and some pharmacological properties of potent and selective antagonists of the vasopressor (V1-receptor) response to arginine-vasopressin. J Med Chem 35(2):382–388

Manning M, Stoev S, Chini B, Durroux T, Mouillac B, Guillon G (2008) Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents. Prog Brain Res 170:473–512. https://doi.org/10.1016/S0079-6123(08)00437-8

Misslin R, Belzung C, Vogel E (1989) Behavioural validation of a light/dark choice procedure for testing anti-anxiety agents. Behav Process 18(1-3):119–132. https://doi.org/10.1016/S0376-6357(89)80010-5

Mouillac B, Chini B, Balestre MN, Jard S, Barberis C, Manning M, Tribollet E, Trumpp-Kallmeyer S, Hoflack J, Elands J (1995) Identification of agonist binding sites of vasopressin and oxytocin receptors. Adv Exp Med Biol 395:301–310

Onaivi ES, Martin BR (1989) Neuropharmacological and physiological validation of a computer-controlled two-compartment black and white box for the assessment of anxiety. Prog Neuro-Psychopharmacol Biol Psychiatry 13(6):963–976. https://doi.org/10.1016/0278-5846(89)90047-X

Ostrowski NL, Lolait SJ, Young WS (1994) Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135(4):1511–1528. https://doi.org/10.1210/endo.135.4.7925112

Overstreet DH, Griebel G (2005) Antidepressant-like effects of the vasopressin V1b receptor antagonist SSR149415 in the Flinders Sensitive Line rat. Pharmacol Biochem Behav 82(1):223–227. https://doi.org/10.1016/j.pbb.2005.07.021

Pare D, Quirk GJ, LeDoux JE (2004) New vistas on amygdala networks in conditioned fear. J Neurophysiol 92(1):1–9. https://doi.org/10.1152/jn.00153.2004

Paxinos G, Watson C (1986) The rat brain in stereotaxical coordinates, 4th edn. Academic Press, San Diego

Pesold C, Treit D (1995) The central and basolateral amygdala differentially mediate the anxiolytic effects of benzodiazepines. Brain Res 671(2):213–221. https://doi.org/10.1016/0006-8993(94)01318-C

Peterson SL (1988) Drug microinjection in discrete brain regions. KOPF Carrier 50:1–6

Raggenbass M (2008) Overview of cellular electrophysiological actions of vasopressin. Eur J Pharmacol 583(2–3):243–254. https://doi.org/10.1016/j.ejphar.2007.11.074

Roozendaal B, Wiersma A, Driscoll P, Koolhaas JM, Bohus B (1992) Vasopressinergic modulation of stress responses in the central amygdala of the Roman high-avoidance and low-avoidance rat. Brain Res 596(1-2):35–40. https://doi.org/10.1016/0006-8993(92)91529-N

Salomé N, Stemmelin J, Cohen C, Griebel G (2006) Differential roles of amygdaloid nuclei in the anxiolytic- and antidepressant-like effects of the V1b receptor antagonist, SSR149415, in rats. Psychopharmacology 187(2):237–244. https://doi.org/10.1007/s00213-006-0424-1

Scott LV, Dinan TG (1998) Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: implications for the pathophysiology of depression. Life Sci 62(22):1985–1998. https://doi.org/10.1016/S0024-3205(98)00027-7

Serradeil-Le Gal C, Wagnon J, Simiand J, Griebel G, Lacour C, Guillon G, Barberis C, Brossard G, Soubrie P, Nisato D, Pascal M, Pruss R, Scatton B, Maffrand JP, Le Fur G (2002) Characterization of (2S, 4R)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamide (SSR149415), a selective and orally active vasopressin V1b receptor antagonist. J Pharmacol Exp Ther 300(3):1122–1130

Shimazaki T, Iijima M, Chaki S (2006) The pituitary mediates the anxiolytic-like effects of the vasopressin V1B receptor antagonist, SSR149415, in a social interaction test in rats. Eur J Pharmacol 543(1-3):63–67. https://doi.org/10.1016/j.ejphar.2006.06.032

Song Z, McCann KE, McNeill JK, Larkin TE 2nd, Huhman KL, Albers HE (2014) Oxcytocin induces social communication by activating arginine-vassopressin V1a receptors and not oxytocin receptors. Psychoneuroendocrinology. https://doi.org/10.1016/jpsyneuen.20014.08.005

Song Z, Larkin TE, Malley MO, Albers HE (2016) Oxytocin (OT) and arinine vasopressin (AVP) act on OT receptors an not AVP V1a receptors to enhance social recognition in adult Syrian hamsters (*Mesocricetus aureatus*). Horm Behav. https://doi.org/10.1016/j.yhbeh.2016.02.004

Spooren WP, Vassout A, Neijt HC, Kuhn R, Gasparini F, Roux S, Porsolt RD, Gentsch C (2000) Anxiolytic-like effects of the prototypical metabotropic glutamate receptor 5 antagonist 2-methyl-6-(phenylethynyl)pyridine in rodents. J Pharmacol Exp Ther 295(3):1267–1275

Stemmelin J, Lukovic L, Salomé N, Griebel G (2005) Evidence that the lateral septum is involved in the antidepressant-like effects of the vasopressin V1b receptor antagonist, SSR149415.

Neuropsychopharmacology 30(1):35–42. https://doi.org/10.1038/sj.npp.1300562

Szot P, Bale TL, Dorsa DM (1994) Distribution of messenger RNA for the vasopressin V1a receptor in the CNS of male and female rats. Brain Res Mol Brain Res 24(1-4):1–10. https://doi.org/10.1016/0169-328X(94)90111-2

Terrillon S, Durroux T, Mouillac B, Breit A, Ayoub MA, Taulan M, Jockers R, Barberis C, Bouvier M (2003) Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis. Mol Endocrinol 17(4):677–691. https://doi.org/10.1210/me.2002-0222

Terrillon S, Barberis C, Bouvier M (2004) Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. Proc Natl Acad Sci U S A 101(6):1548–1553. https://doi.org/10.1073/pnas.0305322101

Thibonnier M, Preston JA, Dulin N, Wilkins PL, Berti-Mattera LN, Mattera R (1997) The human V3 pituitary vasopressin receptor: ligand binding profile and density-dependent signaling pathways. Endocrinology 138(10):4109–4122. https://doi.org/10.1210/endo.138.10.5432

Treit D, Pinel JP, Fibiger HC (1981) Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. Pharmacol Biochem Behav 15(4):619–626. https://doi.org/10.1016/0091-3057(81)90219-7

Wersinger SR, Ginns EI, O'Carroll AM, Lolait SJ, Young WS (2002) Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Mol Psychiatry 7(9):975–984. https://doi.org/10.1038/sj.mp.4001195

Wigger A, Sanchez MM, Mathys KC, Ebner K, Frank E, Liu D, Kresse A, Neumann ID, Holsboer F, Plotsky PM, Landgrafl R (2004) Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. Neuropsychopharmacology 29(1):1–14. https://doi.org/10.1038/sj.npp.1300290

Wotjak CT, Kubota M, Liebsch G, Montkowski A, Holsboer F, Neumann I, Landgraf R (1996) Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J Neurosci 16(23):7725–7732

Wotjak CT, Ludwig M, Ebner K, Russell JA, Singewald N, Landgraf R, Engelmann M (2002) Vasopressin from hypothalamic magnocellular neurons has opposite actions at the adenohypophysis and in the supraoptic nucleus on ACTH secretion. Eur J Neurosci 16(3):477–485. https://doi.org/10.1046/j.1460-9568.2002.02101.x

Young SW, Li J, Wersninger SR, Palkovits M (2006) The vasopressin 1b receptor is prominent in the hippocampal area CA2 where its unaffected by restraint stress or adrenalectomy. Neuroscience 143(4):1031–1039. https://doi.org/10.1016/j.neuroscience.2006.08.040

Zelena D (2012) Vasopressin in health and disease with a focus on affective disorders. Cent Nerv Syst Agents Med Chem 12(4):286–303. https://doi.org/10.2174/187152412803760609

Zhang L, Medina MP, Hernandez VS, Estrada FS, Vega-Gonzalez A (2010) Vasopressinergic network abnormalities potentiate conditioned anxious state of rats subjected to maternal hyperthyroidism. Neuroscience 168(2):416–428. https://doi.org/10.1016/j.neuroscience.2010.03.059

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Hypothalamic Vasopressinergic Projections Innervate Central Amygdala GABAergic Neurons: Implications for Anxiety and Stress Coping

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The arginine-vasopressin (AVP)-containing hypothalamic magnocellular neurosecretory neurons (VPMNNs) are known for their role in hydro-electrolytic balance control via their projections to the neurohypophysis. Recently, projections from these same neurons to hippocampus, habenula and other brain regions in which vasopressin infusion modulates contingent social and emotionally-affected behaviors, have been reported. Here, we present evidence that VPMNN collaterals also project to the amygdaloid complex, and establish synaptic connections with neurons in central amygdala (CeA). The density of AVP innervation in amygdala was substantially increased in adult rats that had experienced neonatal maternal separation (MS), consistent with our previous observations that MS enhances VPMNN number in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. In the CeA, V1a AVP receptor mRNA was only observed in GABAergic neurons, demonstrated by complete co-localization of V1a transcripts in neurons expressing Gad1 and Gad2 transcripts in CeA using the RNAscope method. V1b and V2 receptor mRNAs were not detected, using the same method. Water-deprivation (WD) for 24 h, which increased the metabolic activity of VPMNNs, also increased anxiety-like behavior measured using the elevated plus maze (EPM) test, and this effect was mimicked by bilateral microinfusion of AVP into the CeA. Anxious behavior induced by either WD or AVP infusion was reversed by CeA infusion of V1a antagonist. VPMNNs are thus a newly discovered source of CeA inhibitory circuit modulation, through which both early-life and adult stress coping signals are conveyed from the hypothalamus to the amygdala.

Keywords: magnocellular, synapses, RNAscope, Gad1, Gad2, V1a, microinjection, maternal separation

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INTRODUCTION

Arginine-vasopressin (AVP)-containing magnocellular neurosecretory neurons (VPMNNs) have a critical role in the control of water-electrolyte balance via AVP release from the neurohypophysis, and also influence fundamental behaviors important for survival, presumably at the level of the brain itself (Greving, 1923, 1928; Harris, 1948; de Wied, 1979; Koob et al., 1981; Landgraf and Neumann, 2004). However, despite the profound behavioral and physiological effects of central AVP (Mühlethaler et al., 1982; Landgraf and Neumann, 2004), and early attempts to characterize the central projections of the VPMNN (Buijs, 1978, 1980; DeVries et al., 1985), we remain relatively uninformed about the central organization of the underlying AVP neuronal connections within the central nervous system (CNS): i.e., physiological circuit interactions, synaptic innervation patterns, and the identity of post-synaptic neuronal targets.

One area of the brain in which AVP likely exerts profound behavioral effects is the amygdala. The amygdala is a complex structure involved in anxiety and fear processing (Davis and Whalen, 2001; LeDoux, 2007). In particular, the central amygdala (CeA) is thought to be critical for emotional processing of environmental input, especially in regard to plasticity of behavioral responses to stress (Penzo et al., 2015). A potential target for AVP input to CeA are its numerous GABAergic neurons (Sun and Cassell, 1993; Swanson and Petrovich, 1998), which are important for expression of anxiogenic responses (Sun et al., 1994; Pará et al., 2004; Huber et al., 2005).

There are well-documented AVP projections to amygdala (Buijs, 1978, 1980; Buijs and Swaab, 1979; Buijs et al., 1983; Caffé and van Leeuwen, 1983; Rood and De Vries, 2011; Zhang and Hernández, 2013), and AVP elevation in CeA is correlated with maternal aggression and other stress-related behaviors (Landgraf and Neumann, 2004). However, the origin, distribution, and co-localization with classical neurotransmitters of these fibers, and behavioral consequences of activation of AVP release from these fibers, especially during development, have received little attention. In particular, it has only been demonstrated recently that dynamic regulation of VPMNNs of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei may convey stressor response signals to non-neurohypophyseal limbic regions (Hernández and Zhang, 2012; Zhang et al., 2012; Zhang and Hernández, 2013; Hernández et al., 2015). Thus, we have now begun to characterize the VP-MNN projections to the amygdala, and investigate their involvement in developmentally-related and adult stress responses.

Perinatal stress in rodents, such as maternal separation (MS), fragmented maternal care, and maternal endocrine dysfunction, has been used to examine the causal relationship between early life stress and later stress overreactivity and susceptibility to affective disorders (Zhang et al., 2010, 2012; Lukas et al., 2011). AVP is among several neurotransmitter systems reported to be altered as a consequence of early life stress (Veenema et al., 2006, 2007; Lukas et al., 2011), and therefore is among the neurotransmitter candidates for mediating the reported later-life behavioral hyperreactivity associated with early life stress. We have reported that the AVP system is activated as a consequence of stress during the early life period, with increased VPMNN PVN and SON cell number, enlargement of Herring body densities, and increased number of reciprocal synaptic connections between vasopressinergic and corticotropin releasing factor (CRF) containing neurons. This neuronal plasticity is associated with increased sensitivity to acute stressors, and anxiogenic conditions in adulthood (Zhang et al., 2010, 2012).

In the present study, we have combined immunohistochemistry with antero- and retrograde tracing, electron microscopy and anatomical analysis, to show that AVP innervation of the amygdaloid complex has at least dual origins: thin fibers likely arising from a small population of neurons localized in the bed nucleus of stria terminalis intra-amygdaloid division (STIA), likely to establish Gray type II synapses; and thick fibers originating from hypothalamic PVN and SON, positive for vGluT2 and establishing Gray type I synapses. Semiquantitative anatomical analysis in combination with AVP-IHC showed that the medial amygdala (MeA) was the region with the densest AVP innervation followed by the rostral part of the CeA, where V1a receptor-positive GABAergic neurons were located. MS produced a substantial increase in AVP immunopositive fibers, especially in the central and medial amygdala. We also found that in the CeA the V1a receptors were only expressed in GABAergic neurons. Finally, physiological and pharmacological manipulations of vasopressinergic neurotransmission from PVN to CeA were used to show the functional importance of this projection to the modulation of anxious behavior in the rat. In this regard, we examined the effects on vasopressinergic projections from the hypothalamic VPMNNs to the amygdala, of at least one physiological condition, water deprivation (WD), for which neurohypophyseal AVP (hormone) release is a known homeostatic response, in order to determine if there is a corresponding allostatic response to WD in the amygdala. Here, we report that this is indeed the case, and discuss its implications for dual vasopressinergic homeostatic regulation via the neurohypophysis, and allostatic regulation via VPMNN projections to extrahypothalamic brain, including the amygdala.

MATERIALS AND METHODS

Animals

Adult male Wistar rats of 280 ± 20 g were obtained from the local animal facility. Animals were housed three per cage under controlled temperature and illumination (12 h/12 h, inverted to the natural light/dark cycle) with water and food *ad libitum*. All animal procedures were approved by the Comision de investigacion y Etica de la Facultad de Medicina, Universidad Nacional Autonoma de Mexico (approval number: CIEFM-086-2013).

Maternal Separation Protocol

The MS (3 h daily, MS 3 h) procedure is described in detail elsewhere (Zhang et al., 2012). Briefly, female

and male adult rats were mated for 2 days. During the last week of gestation, female rats were single-housed in standard rat Plexiglas cages and maintained under standard laboratory conditions. On the day after parturition, postnatal day (PND) 2, each litter was culled to 7–8 pups, in which 5–6 were males. During the period from PND 2–PND 16, the pups were separated daily from their dams, and placed into an incubator at 29°C ± 1°C, between 09:00 h and 12:00 h. After this period rats were returned to their home cages. After ending the MS protocol, animals were left undisturbed until the weaning at PND 28, when male and female rats were separated. Animals were then left undisturbed until PND 90 when the elevated plus maze (EPM) was performed during their activity period. Animal facility reared (AFR) rats were treated in the same conditions as described above except that these animals were left undisturbed in their cages during the period when MS 3 h rats were separated from their dam. Bedding was changed twice a week for both groups, with minimum disturbance to the rats.

Immunohistochemistry

Rats were deeply anesthetized with an overdose of sodium pentobarbital (63 mg/kg, Sedalpharma, México) and perfused transaortically with 0.9% saline followed by cold fixative containing 4% of paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4) plus 15% v/v saturated picric acid for 15 min (for the immunoreaction using monoclonal antibody against GABA, 0.1% glutaraldehyde was also added to the fixative solution). Brains were immediately removed, blocked, and then thoroughly rinsed with PB. Brains were sectioned soon after perfusion using a Leica VT 1000S vibratome. Freshly-cut freely-floating sections were blocked with 20% normal donkey serum (NDS) in Tris-buffered (0.05 M, pH 7.4) saline (0.9%) plus 0.3% of Triton X-100 (TBST) for 1 h at room temperature and incubated with the primary antibodies listed in **Table 1** (for antibody specificity see "Supplementary Material Table S1"). For light microscopical immunohistochemistry, Vectastain Elite ABC Kit was used (Vector Labs, Burlingame, CA, USA) followed by DAB-peroxidase reaction, while for immunofluorescence reactions, sections were incubated with the corresponding fluorochrome-conjugated secondary antibodies. For immunoreaction visualization, a Nikon Eclipse 50i light microscope and a Leica TCS-SP5 confocal microscope were used.

Anatomical Assessment of AVP Innervation Density in Amygdala Subfields and Comparison Between MS and AFR Rats

Observations were made under light microscopy. Anatomical nomenclature, especially on amygdala subfields, and regional delineation were according to Paxinos and Watson (2006). To evaluate the densities of vasopressin-containing axon projections in amygdala, observations were made under a light microscope (Nikon Eclise, 50i, with 40× objective) and tracing was performed with the help of a drawing tube attached to the microscope (amplification at 10.6×). A square equivalent to 0.04 mm^2 of the brain tissue section was placed under the drawing tube projecting to the visual field coinciding with the anatomical regions of interests. AVPir+ fibers inside the projected square were manually traced. Calculation of fiber length in each square was performed using ImageJ (open image > make binary > measure). The value of the area in the results window corresponds to the number of pixels in the image. Since the process of skeletonization converts every trace into a one pixel thick trace, the number of pixels equals the summed length of the traces in pixels. The real length is converted to millimetre taking into account the correspondence between pixels and a known scale value. AVPir+ fiber length from postero-dorsal medial amygdala (MePD) from coronal section (Bregma. −2.7 mm) was 3618 mm and was assigned as 100%. Fiber lengths falling in the range of 100%–76% were assigned as ++++; 75–51% as +++; 50–26%: as ++ and 25–1% +.

Neuronal Activation Through Fos-Expression Assessment

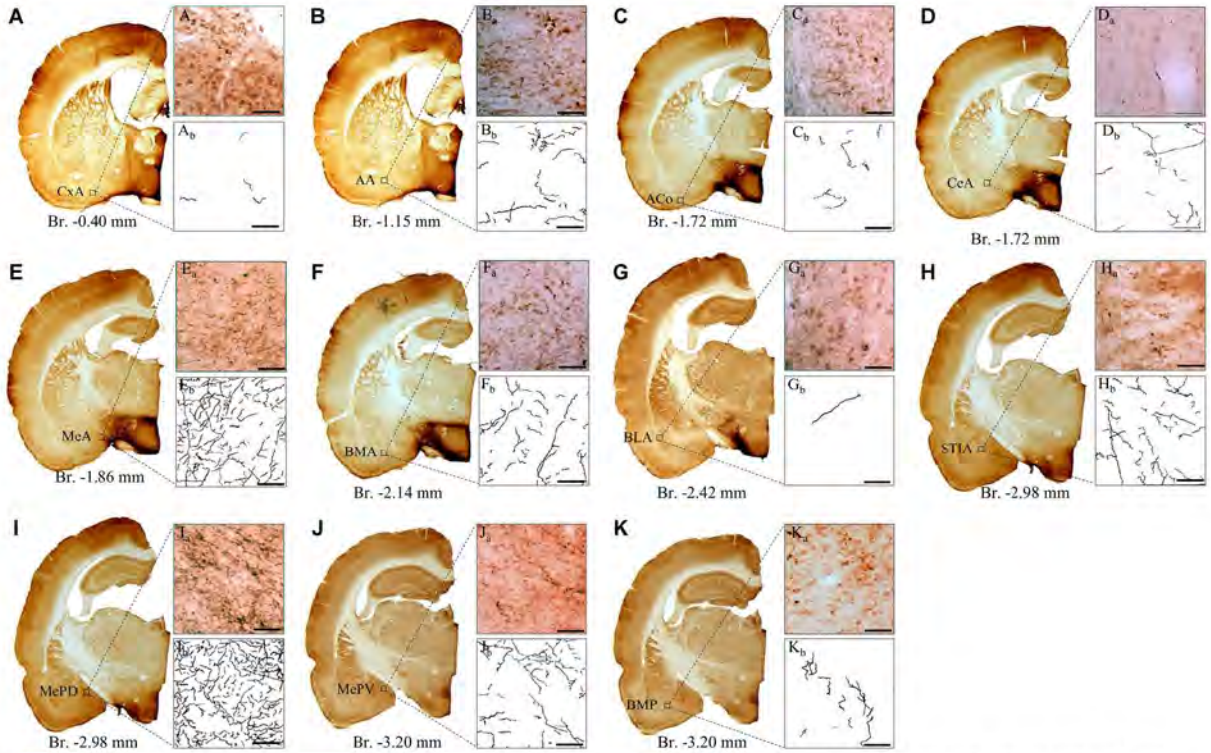
The pattern of amygdala neuronal activation after the EPM test was evaluated in four animals for each of the cannulated rat groups, i.e., those infused with NaCl 0.9%, with AVP 1 ng, or with AVP 1 ng + V1a antagonist 10 ng. The number of Fos+ nuclei in an area of 0.2 mm^2 was counted in the central (CeA), basolateral (BLA) and MeA, around Bregma −2.12 mm. A two way analysis of variance (ANOVA) was used to evaluate if the differences between the NaCl, AVP 1 ng and AVP 1 ng + V1a antagonist groups were statistically significant.

Immunohistochemistry for Transmission Electron Microscopy (TEM)

Immuno-electron microscopy procedures were performed as reported previously (Zhang and Hernández, 2013). Briefly,

TABLE 1 | Primary antibodies used in this study.

Molecule	Host	Dilution	Source	Source code
[Arg8]-vasopressin	Rabbit	1:5000	Peninsula-Bachem Americas, Inc., CA, USA (www.bachem.com)	T-4563
[Arg8]-vasopressin	Rabbit	1:2000	Prof. R.M. Buijs, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, UNAM	–
c-Fos	Rabbit	1:2000	Santa Cruz Biotechnology, Dallas, TX, USA (www.scbt.com)	SC-52
Vesicular glutamate transporter 2	Guinea pig	1:1000	Frontier Institute Co., Ltd., Hokkaido, Japan (www.frontier-institute.com)	GP-AF240-1
Gamma-aminobutyric Acid (GABA)	Mouse	1:1000	Sigma-Aldrich Corporation, St. Louis, MO, USA (www.sigmaaldrich.com)	A031
Vasopressin V1A Receptor	Rabbit	1:2000	Alomone Labs., Jerusalem, Israel (www.alomone.com/)	AVR-010



Bregma	CxA	AA	ACo	MeA	BMA	CeA	BLA	STIA	MePD	MePV	BMP
-0.12	+										
-0.26	+	+									
-0.40	+	+									
-0.54	+	+	++								
-0.72	+	++	++								
-0.86		+++	++								
-1.15		++	++	++++							
-1.30		+++	++	++++	+++						
-1.44		+++	+	+++	++	+	+				
-1.58		++	+	+++	+	+	+				
-1.72			+	+++	+	+	+				
-1.86			+	+++	+	+	+				
-2.00			+	+++	++	+	+				
-2.14			+	++	++	+	+				
-2.28			+	++	++	+	+	++			
-2.42			+	++	++	+	+	++			
-2.56			+	++	+++	+	+	++			
-2.70					++	+	+	++	++++	+	+
-2.84						+	+	+++	++++	++	+
-2.98						+	+	+++	++++	++	+
-3.20						+	+	+++	++++	++	+
-3.26						+	+	+++	++++	+	+
-3.40									+++	++	+

FIGURE 1 | Distribution of vasopressin-immunostained fibers through the rostrocaudal extent of amygdala in the rat. Low **(A–K)** and high power **(A_a–K_a)** photomicrographs and tracings of arginine-vasopressin (AVP) labeled fibers **(A_b–K_b)** in coronal sections, illustrating the heterogeneous distribution in the density of AVP fibers among different amygdalar regions. The highest density was found in the MePD region at Bregma level −2.70 mm (panel **I**, and table). Each of the panels from **(A–K)** corresponds to a representative example at the bregma level indicated in the table gray boxes. For the semiquantitative analysis in the table, the summed length (calculated with ImageJ) of fibers in a 0.04 mm^2 area, inside this highest density region was assigned as 100%, regions with summed lengths between 76–100% were assigned as ++++; between 51–75% : +++; between 26–50%: ++ and between 1–25%: +.

(Continued)

FIGURE 1 | Continued
Anatomical maps and nuclei nomenclature are from the rat atlas of Swanson (2003). CxA, cortico-amygdalar transition zone; AA, anterior amygdala area; Aco, anterior-cortical amygdaloid nucleus; MeA, medial anterior amygdaloid nucleus; BMA, basomedial anterior amygdaloid nucleus; CeA, central nucleus of amygdala; BLA, basolateral amygdala; STIA, stria terminalis intra-amygdaloid division; MePD, medial postero-dorsal amygdaloid nucleus; MePV, medial postero-ventral amygdaloid nucleus; BMP, basomedial posterior amygdaloid nucleus. Scale bars in (**A_a–K_a, A_b–K_b**) 50 μm, Bregma levels are indicated below each low power photomicrograph. Squared region indicates the 200 μm area used for calculation of summed length for each amygdala region.

two male rats were perfused with NaCl 0.9%, and fixed with ice-cold 4% paraformaldehyde (Sigma P6148) + 15% v/v saturated picric acid solution (Fermont) + 0.05% glutaraldehyde (Sigma, G5882). Sections 70 μm-thick containing CeA were processed with conventional IHC for TEM (Zhang and Hernández, 2013). Sections were then osmificated in 1% OsO_4 for 1 h and dehydrated in a graded series of ethanol and embedded in synthetic resin (Epon). Ultrathin sections were then cut, counterstained with lead citrate (Electron Microscopy) and examined with a Philips CM 100 transmission electron microscope and photographed with a digital micrograph 3.4 camera (Gatan Inc., Pleasanton, CA, USA).

Fluoro-Gold Tracing Experiments

Anesthesia was induced and maintained with xylazine (Procin, Mexico; 20 mg/ml) and ketamine (Inoketam, Virbac, Mexico; 100 mg/ml) mixed in a 1:1 volume ratio and administered intramuscularly with a dose of 1 ml/kg body weight. Rats were fixed in a stereotaxic apparatus and were injected in the CeA (Bregma −2.12 mm, medio-lateral 4.2 mm, dorsoventral 7.7 mm (Paxinos and Watson, 2006)) with the retrograde tracer Fluoro-Gold (FG, Fluorochrome, LLC, Denver, CO, USA), dissolved 1% in 0.2 M sodium cacodylate buffer (pH 7.5). The FG was delivered iontophoretically using an iontophoresis pump (Value Kation Sci VAB-500) through a stereotaxically positioned glass micropipette with an inner tip diameter of approximately 40 μm, by applying current pulses of 0.1 μA, at 0.2 Hz, with a 50% duty cycle, for 20 min. The micropipette was left in place for an additional 10 min to prevent backflow of the tracer up the injection track after each injection. After completing the surgery, rats received 0.4 mg/kg *i.p*. Ketorolac (Apotex, Mexico) and 50 mg/kg *i.p* ceftriaxone (Kendric, Mexico) as analgesic/anti-inflammatory and antibiotic agents, respectively. Three to four weeks after the FG injections, the rats were perfused as previously described (Zhang and Hernández, 2013). Coronal and sagittal sections of 70 μm were obtained, and AVP IHC was performed to evaluate if the SON and PVN AVP+ neurons were labeled with FG. Observations were made under light (Nikon ECLIPSE 50i with B-2A long-pass emission filter) and confocal microscopy (Leica TCS-SP5).

ISH Assays With Radio-Probes

Rats were deeply anesthetized with sodium pentobarbital (Sedalpharma, México, 63 mg/kg b.w., i.p.) and perfused via ascending aorta with 0.9% saline followed by cold fixative containing 4% paraformaldehyde in 0.1 M PB, pH 7.4. Brains were post-fixed with 1% paraformaldehyde in PB and kept at 4°C until use. All solutions used had been diethyl pyrocarbonate (DEPC)-treated (0.1% v/v with gentle stirring for at least 4 h at room temperature) to inactivate any residual RNase, and then autoclaved to inactivate the traces of DEPC.

Two days before the start of sectioning, the brains were moved to 18% sucrose in RNase free PB + NaN3. Another change was done 1 day before the sectioning and a third change with a fresh sucrose solution was done 1 h before the sectioning. Serial coronal sections between Bregma −0.36 mm and Bregma −3.00 mm of 12 μm of thickness were made using a Leica CM1950 cryostat (Leica Microsystem, Wetzlar, 35578, Germany). Sections were collected on Leica glass insert and then transferred to a 24-well tissue culture plate with PB.

In situ hybridization (ISH) was performed in one out of six coronal sections as previously described (Morales and Bloom, 1997) using ^{35}S- and ^{33}P-UTP-labeled ribonucleotide probes. The pT7T3D-PacI plasmid (accession number: AI072073, clone ID: 1786383 Thermo Scientific) containing rat vasopressin receptor V1a cDNA (Morel et al., 1992) was linearized with EcoRI and then transcribed *in vitro* with T3 RNA polymerase to yield antisense complementary RNA probe. The construct was verified by sequencing. The radioactivity was adjusted to 10^7 cpm per ml hybridization buffer. Sections were mounted on coated slides and air-dried. Slides were exposed to autoradiography film and analyzed on a phosphorimager (Fuji BAS5000, Tokyo, Japan).

RNAscope ISH Assays

Two rats were deeply anesthetized and perfused via the ascending aorta with saline. Whole brain tissues were removed and rapidly frozen on Dry Ice. The fresh-frozen tissue sections (thickness: 20 μm) were mounted on positively charged microscopic glass slides (Fisher Scientific, Pittsburgh, PA, USA). The Gad1, Gad2 and V1a specific RNA probes (Rn-Gad1, 316401-C1; Rn-Gad2, 435801-C2; Rn-Avp1a, 402531-C3) were designed and provided by Advanced Cell Diagnostics (Hayward, CA, USA). All staining steps were performed following RNAscope protocols. Stained slides were cover-slipped with fluorescent mounting medium (ProLong Gold Antifade Reagent P36930; Life Technologies, Carlsbad, CA, USA) and examined with a confocal microscope (Leica TCS-SP5).

Intra-Amygdaloid Microinjection

For the implantation of permanent guide cannulas into the amygdala, rats were anesthetized with ketamine hydrochloride (170 mg/kg, i.p.) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) with the incisor bar set at −3.3 mm. Body temperature was maintained at 37°C using a CMA/150 temperature controller (CMA/Microdialysis, Stockholm, Sweden). Bilateral stainless steel cannulae of 0.46 mm outer diameter (C315G, Plastics One, Roanoke, VA, USA) were aimed to CeA (Bregma −1.7 mm, mediolateral ± 4.2 mm,

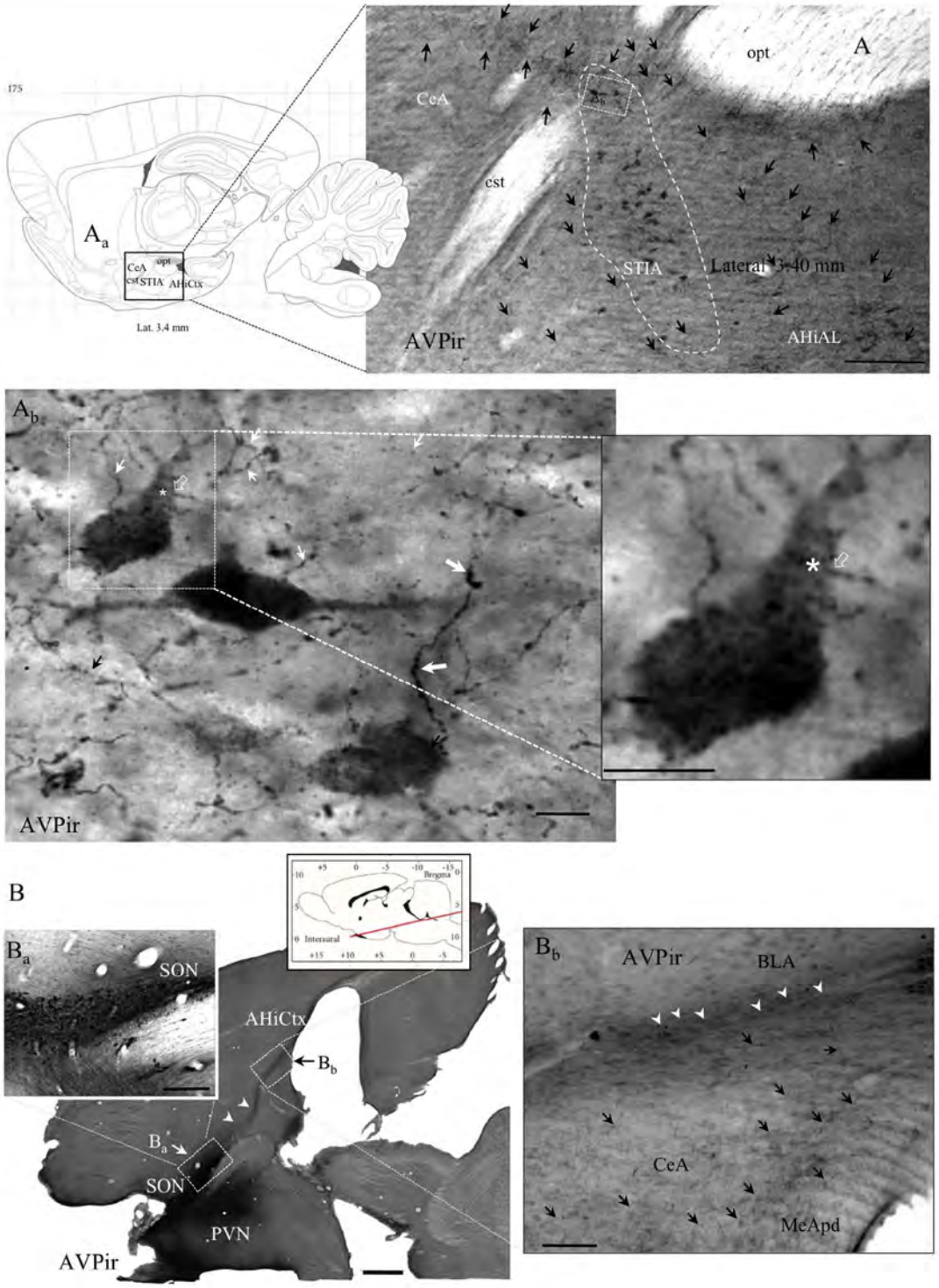


FIGURE 2 | Anatomo-immunohistochemical features of vasopressin immuno-reactive (AVPir) somata and fibers in the amygdaloidal complex suggest that AVP modulates distinct neuronal circuits. (A) Photomicrograph of AVPir in a sagittal section of rat amygdala at lateral 3.40 mm (**A_a**, inset: sagittal view of amygdaloid complex, squared region, from the Rat Brain Atlas). Note that the AVP immunopositive fibers (indicated by black arrows) are heterogeneously distributed in the whole amygdala complex and numerous AVP+ cells were inside the bed nucleus of *stria terminalis*, intra-amygdaloid division (STIA; circumscribed with white dashed line). **(A_b)** Amplification of a region inside the STIA. Note that there are two types of AVPir+ fibers, regarding their thickness and spatial frequency of varicosities: the type I fibers have thick diameter and frequent varicosities (thick white arrows); the type II fibers are thin and had less frequent varicosities (thin white arrows). Both types are intermingled in the same region. The asterisk indicates the site where one thin-type axon (the axon initial segment was indicated with

(Continued)

FIGURE 2 | Continued
a hollow arrow, white in both **(A_b)** and its inset) originated (asterisk) from one proximal dendrite of the respective neuron. **(B)** A semi-horizontal section of rat brain showing the vicinity of the hypothalamic AVP+ supraoptic (SON) and paraventricular (PVN) nuclei and the massive projection of AVPir fibers toward amygdala and amygdala-hippocampal cortex (AHiCtx; white arrowheads in **B_a,B_b**). The red line of the atlas inset symbolizes the level and the inclination of the section plan. **(B_a)** Amplification of the corresponding squared region showing the SON is seen from the semi-horizontal view. **(B_b)** Amplification of the corresponding squared region of amygdaloid complex. Note the heterogeneous presence of the AVPir fibers in the amygdaloid subdivisions. MeApd, medial amygdala postero-dorsal; CeA, central amygdala; BLA, basolateral amygdala. Scale bars: **(A)** 500 μm; **(A_b)** and Inset: 20 μm; **(B)** 500 μm; **(B_a)** 100 μm; **(B_b)** 100 μm.

dorsoventral −7.7 mm from skull surface, according to the atlas of Paxinos and Watson (2006). Guide cannulas were fixed with stainless-steel screws and dental acrylic cement (Laboratorios Arias, Mexico City, Mexico) and sealed with dummy cannulae (C315DC, Plastics One). Benzylpenicillin benzathine (Estrepto benzetacil V-Fortificado (Fort Dodge Animal Health Laboratories, Mexico City, Mexico)) was given to prevent infection. The animals were housed in individual cages post-surgery. A total of 30 rats were employed for microinjection experiments.

Beginning on the fourth day post-surgery, rats were handled once a day for 5 min for three consecutive days, and behavioral tests were performed the next day (seventh day post-surgery).

On the day of the experiment, saline alone (250 nl per side), AVP (Sigma V9879, 1 ng in 250 nl per side of NaCl 0.9%, (Appenrodt and Schwarzberg, 2000) or V1a antagonist ($d(CH_2)_5$ 1, Tyr(Me) 2, Arg^8)-Vasopressin trifluoroacetate salt, Manning compound; 10 ng in 250 nl per side), depending the experimental groups, were injected bilaterally via an injection cannula (0.20 mm outer diameter, C315I, Plastics One), which protruded 1 mm beyond the end of the guide cannula, over a period of 5 min (Pérez de la Mora et al., 2006), using two CMA/Microdialysis pumps (CMA/Microdialysis, Stockholm, Sweden). The cannulae were kept in place for 1 min after the injection to prevent backflow and to allow for diffusion. Behavioral observations were started 15 min after microinjections.

Elevated Plus Maze

The EPM was made locally (for details see Zhang et al., 2010, 2012). The test was performed during the early activity period under dim red light and video recorded. Briefly, testing is performed by placing the rat in the center of the maze facing towards an open arm, and free exploratory activity is observed for 5 min. An entry into an open arm was counted when the four paws of the rat were placed within that arm. The time spent in the open arms, as percentage of total time (300 s), was analyzed off-line. For each treatment, the person administering the drug was aware of the animal's status (i.e., control-reared or MS-reared). The persons who performed behavioral assessments were unaware of either the rearing or treatment status of each assessed animal.

Two sets of experiments ("Experiment 1" and "Experiment 2") were performed. Experiment 1: MS (n = 10) and AFR (n = 10) rats underwent a 24 h water-deprivation period (WD 24 h) previous to the EPM test. The rationale of this set of experiments was that WD 24 h potently up-regulates the metabolic activity of the hypothalamic VPMNNs but with only a modest increase in plasma osmolarity (Dunn et al., 1973; Zhang et al., 2012). Moreover, we have previously reported that MS rats possess a potentiated VPMNN system in adulthood, with enhanced anxiety state as measured by the Vogel conflict test, after WD 24 h (Zhang et al., 2012). Experiment 2: three experimental groups of AFR rats were assessed under EPM paradigm. Group A: microinjection with saline; group B, microinjection with AVP and group C, microinjection with AVP and the V1a specific antagonist.

Statistical Analysis

Quantitative results were expressed as mean ± SEM. Groups were tested for normality with a D'Agostino and Pearson test. Differences between means were evaluated by one-way ANOVA followed by the Bonferroni test. All computations were done using Prism (GraphPad Software, Version 6, San Diego, CA, USA). Differences in all cases were considered statistically significant when $P < 0.05$.

RESULTS

Anatomical Features of Arginine-Vasopressin Containing Somata and Fibers in the Amygdaloidal Complex as Revealed by Immune-Reaction (AVPir)

We used an AVPir-optimized protocol previously designed by us (Zhang and Hernández, 2013) to reveal the AVP-containing fibers and somata distribution. We observed, as shown in **Figure 1**, a heterogeneous distribution of fibers in the whole amygdaloid complex, with the medial MePD, the MeA (rostral to MePD), the bed nucleus of STIA and the anterior amygdala (AA, rostral to CeA) being the most densely innervated regions.

AVPir somata were observed in the STIA and surrounding regions (**Figure 2A** dashed line circumscribed region). These cells were sparsely distributed and had pale AVPir labeling. AVPir + fibers all over amygdala showed either a strong-dark labeling or a weak-pale labeling pattern. Two kinds of fibers were observed. The first kind of fiber was varicose, and was the most frequently observed fiber type (**Figure $2A_b$**). The second kind was rarer, and less varicose. We will refer to these as Type I and Type II fibers, respectively, in accordance with the previous nomenclature that we used for hippocampal AVP innervation (Zhang and Hernández, 2013). Some type II axon proximal segments could be found leaving AVPir somata (**Figure $2A_b$** inset). Using semi-horizontal sections, we could clearly appreciate the limits between the hypothalamic PVN, SON and amygdala. **Figures 2Bs** show the massive projection and the heterogeneous distribution patterns of AVPir fibers towards amygdala/amygdalo-hippocampal cortex.

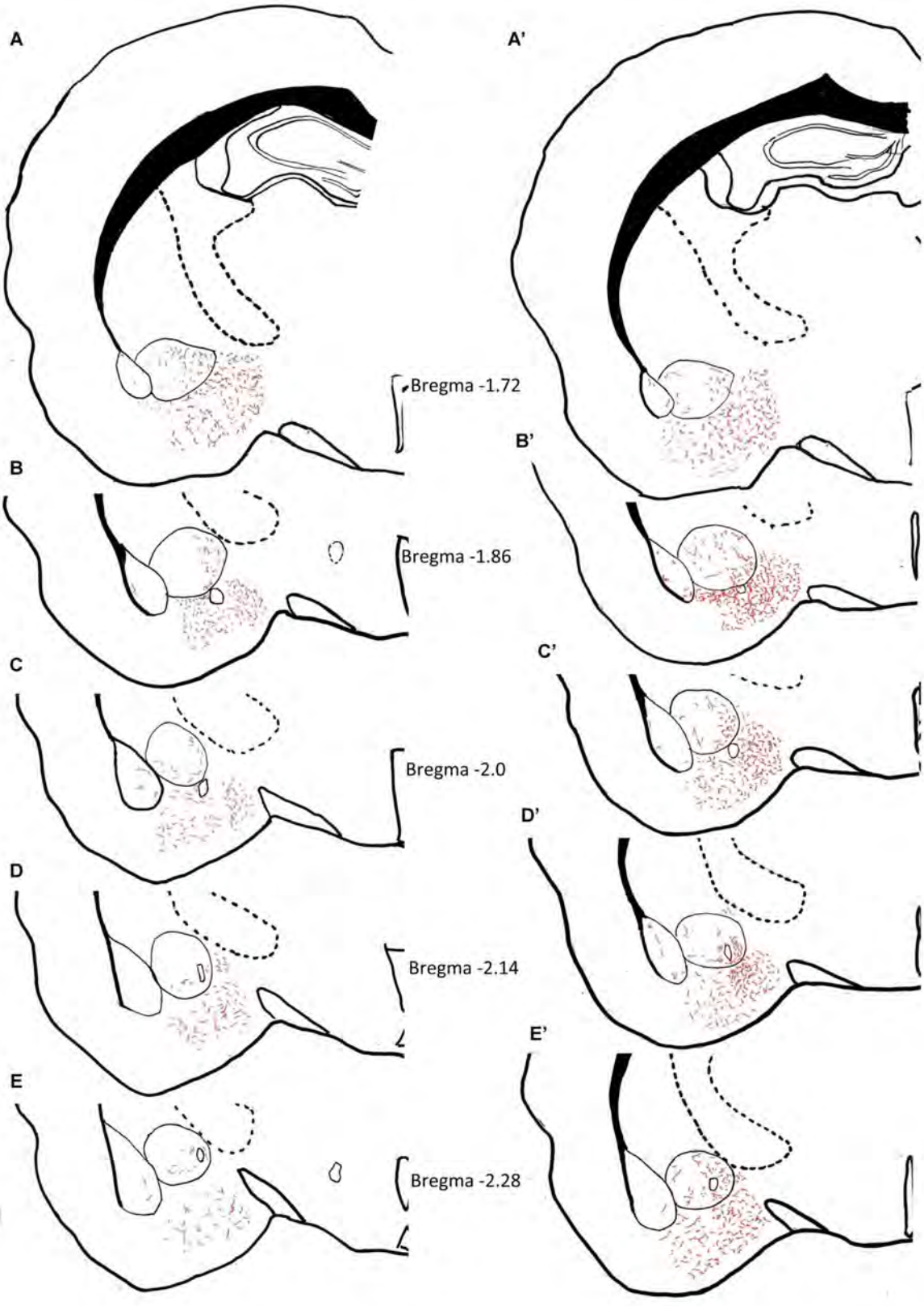


FIGURE 3 | Continued

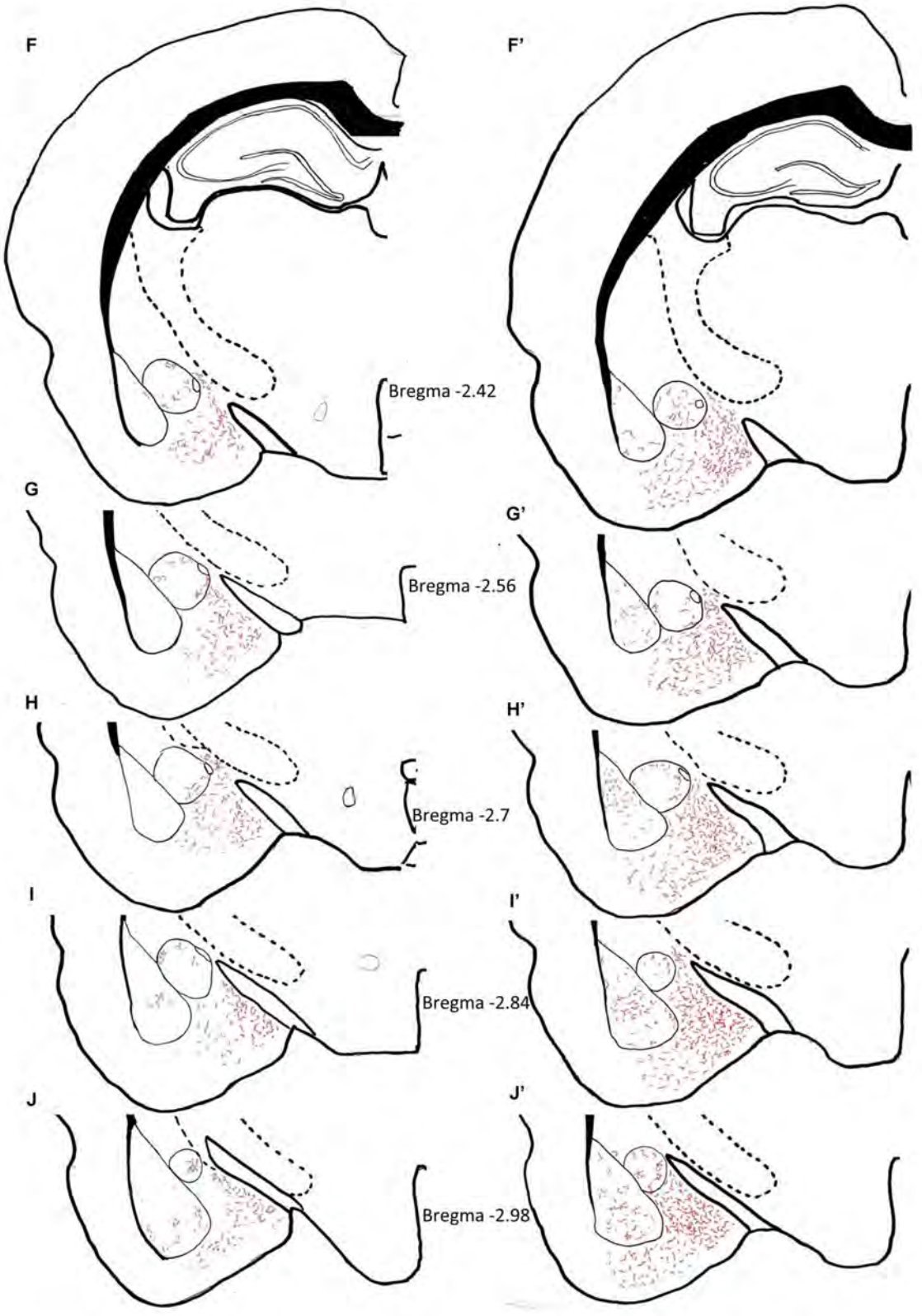


FIGURE 3 | Anatomical charting of AVPir+ fiber distribution in amygdala in both control (A–J) and maternal separation (MS) male adult rat (MS, **A'–J'**). Chartings of coronal sections at 10 rostro-caudal levels with line drawings referenced with microscopic observation, representing AVP fiber distribution through the entire amygdaloidal complex. Note the remarkable increase in AVP innervation densities in all regions of the amygdala as a function of MS.

TABLE 2 | Vasopressin fiber density distribution in animal facility reared (AFR) and MS rats.

Control				Maternal separation			
Bregma (mm)	BLA	CeA	MeA	Bregma (mm)	BLA	CeA	MeA
−1.72	+	++	+++	−1.72	+	++	++++
−1.86	+	++	+++	−1.86	+	++	++++
−2	+	++	+++	−2	+	++	++++
−2.14	−	+	+++	−2.14	+	++	++++
−2.28	+	+	+++	−2.28	+	++	++++
−2.42	+	+	+++	−2.42	+	++	++++
−2.56	−	+	+++	−2.56	++	+	++++
−2.7	−	+	+++	−2.7	++	+	++++
−2.84	+	+	+++	−2.84	++	++	++++
−2.98	+	++	+++	−2.98	++	+	++++

Tracing was performed under camera lucida and calculation of axonal length in a determined region was calculated using ImageJ. Axon length value from Medial Amygdala (MeA) at Bregma level −1.86 mm in maternal separation (MS), was asigned to 100% (++++) = MAX value. For each region, ++++, +++, ++ and + semi-quantitative values were assigned for fiber length values 76–100%, 51–75%, 26–50% and 1–25% of the MAX value.

Effect of Maternal Separation on Density of AVP Innervation in Amygdala

The density of AVP innervation in amygdala was substantially increased in adult rats who had experienced neonatal MS, consistent with our previous observations that MS enhances VPMNN number in the PVN and SON nuclei of the hypothalamus. Using anatomical analysis with the help of light microscope and a drawing tube, we produced a coordinate-detailed anatomical chartings of AVP innervation in adult male rat amygdala (**Figures 3A–J**). The density of AVP innervation in amygdala was substantially increased in adult rats that had experienced neonatal MS (**Figures 3A'–J'** and **Table 2**), consistent with our previous observations that MS enhances VPMNN number in the PVN of the hypothalamus.

Immuno-Electron Microscopical Evidence of Synaptic Innervation by Thick and Thin AVP-Containing Fibers onto Central Amygdala (CeA) Neurons, Establishing either Gray Type I (Asymmetric; Thick) or Gray Type II (Symmetric; Thin) Synapses

Ultrastructural analysis of the AVP innervation showed that thin and thick AVP immunopositive fibers, with dense core vesicles (dcv, AVPir+), made synapses onto dendrites in the CeA. The axon terminals (AT) of the thin fibers presented pleomorphic clear vesicles characteristic of GABAergic AT and preferentially made presumptive inhibitory (GABAergic) symmetric "Gray type II" synapses, while the thick fibers made presumptive excitatory (glutamatergic) asymmetric "Gray type I" synapses (**Figures 4A–D**). To further confirm the excitatory nature of the thick fibers in amygdala, double immunohistochemistry against AVP and the vesicular glutamate transporter (VGLUT2) was performed. Confocal microscopical analysis in the CeA showed that thick varicose axonal fibers containing AVP co-expressed VGLUT2 (**Figures 4E–H**).

Anterograde and Retrograde Tracing Revealed that PVN and SON Magnocellular Vasopressinergic Neurons Innervate the Central Amygdala

The presence of two AVP+ fiber populations, with distinct diameters and synaptic profiles, suggested that the AVP innervation in amygdala could have different origins. To assess this, fluorogold (FG) was stereotaxically injected in the CeA. Three to four weeks following the FG injection, rats were perfused, brains were sectioned, immunohistochemistry against vasopressin was performed, and areas of interest were evaluated under a confocal microscope. Cases in which the injection area was sufficiently focused (<500 μm in diameter), and localization of the injection site to CeA was achieved, were evaluated. A number of AVP+ neurons in the PVN and SON showed clear FG labeling, mainly accumulated in the perikarya of lysosome-like granules (**Figures 5B–D**), which is a prominent characteristic reported for successful long-term FG labeling (Schmued and Fallon, 1986; Wessendorf, 1991; Persson and Havton, 2009).

These results confirmed previous observations of our group (Hernández et al., 2015), in which some axons of an *in vivo* juxtacellularly labeled AVP+ magnocellular neuron from hypothalamic PVN nucleus, reconstructed under a camera lucida, were seen reaching the CeA and MeA (**Figure 5A**).

An Increase in Anxious Behavior Observed After Water Deprivation of 24 h (WD 24 h) and Further Significantly Increased in MS Subjects

Anxiety was assessed using the EPM. To evaluate whether up-regulation of the VPMNN system could influence anxiety as measured in the EPM test, and if MS, which had resulted in a hyper-innervation pattern of AVP in the amygdala, could further influence anxiety in adults, we performed this behavior test with the following four groups: AFR basal, MS basal, AFR with 24 h of WD (AFR WD 24 h) and MS with 24 h of WD (MS WD 24 h; **Figure 6**). One-way ANOVA showed that the differences between means were statistically significant ($p < 0.001$, $F_{(3,36)} = 26.44$). *Post hoc* Bonferroni's multiple comparisons test showed that under basal conditions no significant difference was seen between the percentage of time spent in the open arms by the AFR (29.75% ± 3.51%) and MS (29.13% ± 2.65%) rats ($p > 0.05$). WD significantly decreased the time spent in the open arms in both groups ($p < 0.001$, when compared with the corresponding basal condition) with WD 24 h reducing open arm time to 14.08% ± 2.45% in the AFR group, and to 4.67% ± 0.93% in the MS group. There was a significantly greater reduction in time spent in open arm following WD 24 h in the MS, than in the AFR group ($p < 0.001$).

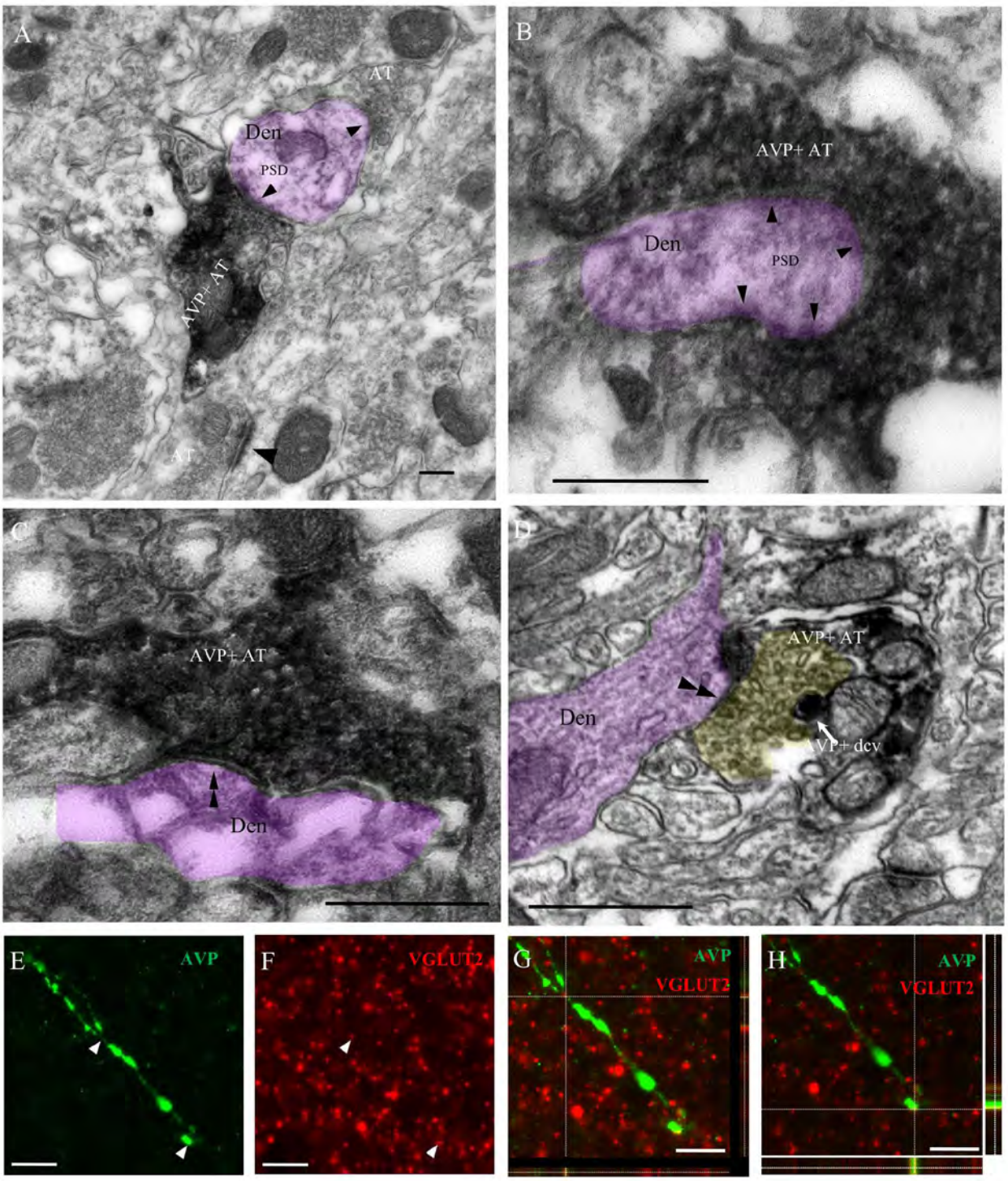


FIGURE 4 | Immuno-electron microscopical evidence of synaptic innervation by thick and thin AVP-containing fibers onto central amygdala (CeA) neurons, establishing either Gray type I (asymmetric; thick) or Gray type II (symmetric; thin) synapses. Examples of thick axon terminals (AT) **(A,B)** making Gray type I synapses (the presence of postsynaptic density, PSD, are indicated by single arrowheads) and thin AT **(C,D)** making Gray type II synapses (the absence of PSD are indicated by double arrowhead) onto CeA neurons' dendrites (Den, pink shaded). Note that the yellow shaded presynaptic active zone of an axon terminal of a thin fiber contained pleomorphic small clear vesicles, which is a remarkable feature of a GABAergic AT; it also contained AVPir+ dense core vesicle (dcv). Thick AVPir fibers in the CeA contain vesicular glutamate transporter (VGLUT2)-positive varicosities. Confocal microscopy analysis of CeA showed thick varicose fibers immunopositive for AVP **(E)** and multiple VGLUT2-positive terminals **(F)** with co-expression of VGLUT2 and AVP in a representative thick fiber **(G,H)**. For **(G,H)**, the *x–y* (center), *x–z* (box below) and *y–z* (right box) images are shown. Scale bars: **(A–D)** 500 nm; **(E–H)** 10 μm.

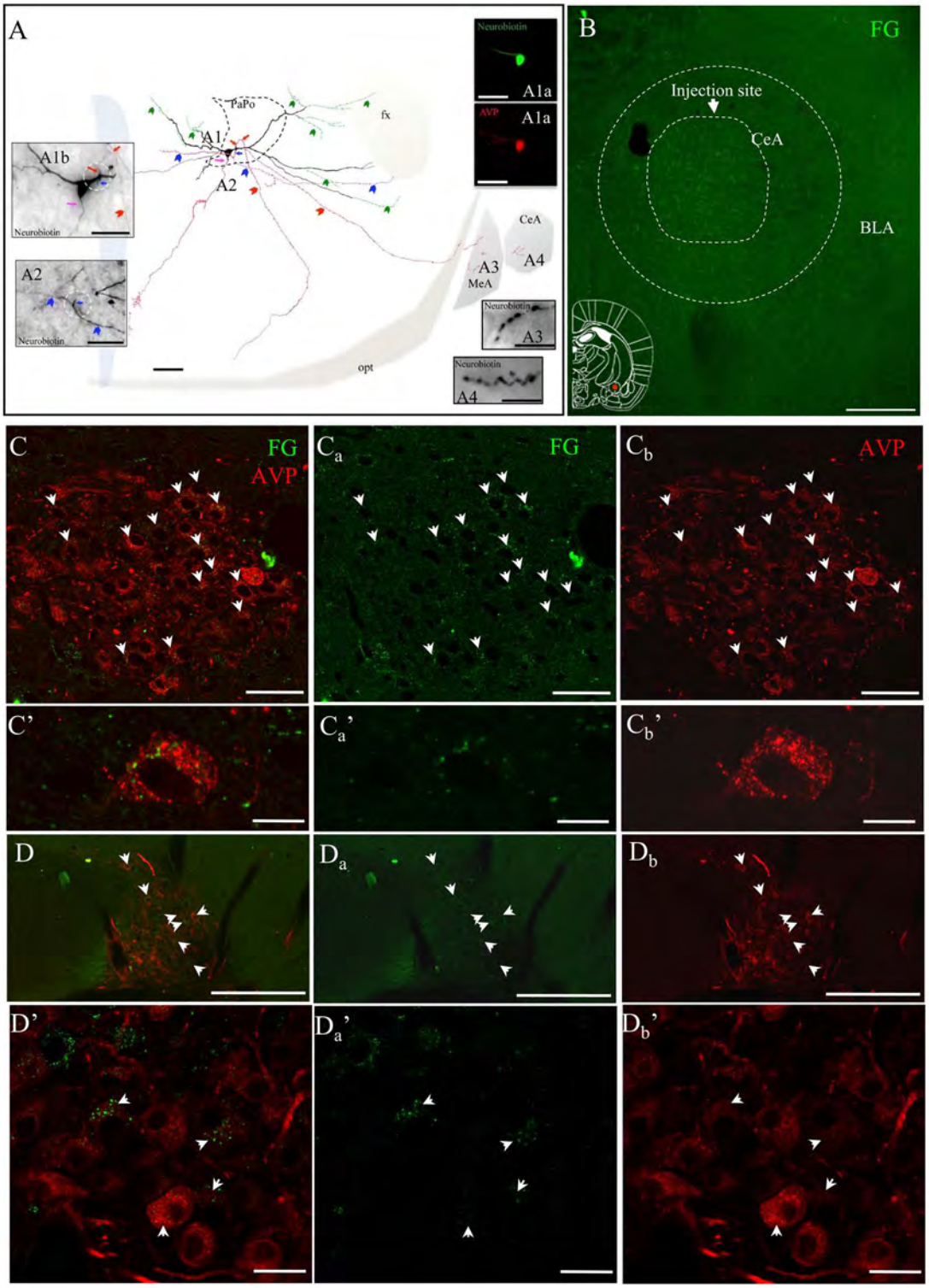


FIGURE 5 | AVP-containing magnocellular neurons innervate the CeA. (A) Camera lucida reconstruction of the coronal projection and corresponding photomicrographs (insets) showing the dendritic and axonal patterns of an anterogradely labeled AVP-containing magnocellular neurosecretory neuron sending axon collaterals to the medial and CeA. This panel was modified from Hernández et al. (2015), see the original publication for method descriptions. Red, blue and pink arrows indicate the origins of three parent-axons. Red and blue arrowheads indicate the subsequent axonal branches. The green arrowheads indicate the beaded processes originated from the dendritic processes, some of them entering the fornix (green arrowheads). Such processes were described in an early study and were considered as axonal processes (Sofroniew and Glasmann, 1981). **(A1a)** AVP-containing nature was ascertained by AVP immunoreaction;

(Continued)

FIGURE 5 | Continued
(A1b) neurobiotin-peroxidase-AB complex histochemical reaction showing the morphology of the filled AVPir+ neuron's soma and its three parental axons emitted from proximal dendrites (pink, red and blue arrows indicate the sites where the axons were originated). Note that two of those axons were emitted from the same proximal dendritic point (red and blue arrows but coursed to opposite directions). **(A2)** Photomicrograph of the section contiguous caudally to the section of soma **(A1b)** showing the branching of the axon collaterals (colors are aimed to encode each group of collaterals originated from the same parental main axon). **(A3,A4)** show axonal processes found in amygdala. Scale bars: 100 μm for **(A)** 50 μm for all photomicrographs. MeA, medial amygdala; CeA, central amygdala; opt, optical tract; fx, fornix. Panel **(B)** Fluorogold (FG; retrograde neural tracer) was iontophoretically injected into CeA. **(Cs)** panels correspond to the retrograde labeling images of the PVN with FG, AVPir and overlapping of the two channels respectively. **(C's)** show an example of a magnocellular AVPir+ neuron in high magnification. Note that the green dots, which correspond to FG, are peri-nuclear which likely are intra-lisosomal. **(D,D')** are the corresponding images for supraoptic nuclear labeling. Scale bar: 50 μm.

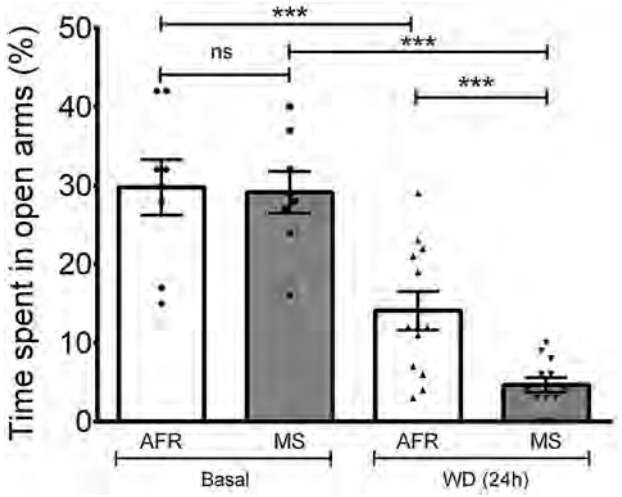


FIGURE 6 | Elevated plus maze (EPM) performance after AVP magnocellular system upregulation induced by 24-h water deprivation (WD, 24 h). Under basal conditions, no differences in the time spent in the open arms were observed between MS and control (animal facility reared, AFR) rats. One way ANOVA with Bonferroni *Post hoc* test, showed that WD 24 h induced a significant reduction in the time spent in the open arm in both AFR and MS groups (***$p < 0.001$ for WD (24 h) compared to basal, for each of the two groups AFR and MS). Reduction in time spent in open arms following WD (24 h) was significantly greater in the MS compared to the AFR group (***$p < 0.001$).

V1A Receptor mRNA Expression in Anterior and Central Amygdala

V1a receptor mRNA is strongly expressed in AA and CeA while V1b and V2 mRNA were not detectable using the RNAscope method. The V1a mRNA expression had a complete co-localization with Gad1 and Gad2 mRNA in CeA. Based on the foregoing findings, we hypothesized that the some or all the currently identified GPCRs for vasopressin, namely the V1a, V1b and V2 receptors, were involved in the anxiogenic action of vasopressin during the WD 24 h. We first successfully developed a V1a riboprobe based on the procedures of Morel et al. (1992) and we used the traditional ISH method to examine the V1a mRNA in the whole amygdaloidal complex (including rostro-caudal span). Phospho-imager detection showed strong and localized expression of V1a mRNA in the AA and CeA from Bregma −0.36 mm to −2.52 mm (**Figures 7As**). To confirm this finding, we used another highly-sensitive RNAscope ISH method, which allowed us to simultaneously detect V1a mRNA together with Gad1 and Gad2 mRNA in CeA. **Figures 7B–D's** shows representative confocal images under low and high magnification, illustrating the complete co-localization of V1a mRNA with the Gad1 and Gad2 mRNA. V1b and V2 probes for RNAscope, which gave positive signals in anterior pituitary gland and renal tissues, did not yield any positive labeling in the amygdaloidal complex (data not shown).

Using immunohistochemistry, we observed AVPir+ fibers surrounding the GABAir+ soma in the CeA (**Figure 8A**). **Figure 8Bs** shows an example taken from CeA of a rat perfused 90 min after the EPM test from the group of MS/WD 24 h, where all the GABAir+ neurons exhibited nuclear Fos expression, a marker for neuronal activation, and also had membrane V1a labeling.

AVP Infusion into CeA Induced Anxiogenesis via a V1aR Pathway

To further evaluate the effect on anxiety of increased vasopressin levels in amygdala, vasopressin (1 ng), vasopressin ((1 ng) + the vasopressin V1a receptor antagonist Manning compound (10 ng)) or vehicle (NaCl 0.9%) was infused at each of two sites 15 min before the EPM test, via cannulas stereotaxically placed in the CeA (**Figure 9A**). One-way ANOVA showed that the differences between means were statistically significant ($p < 0.0001$, $F_{(2,32)} = 15$). *Post hoc* Bonferroni's multiple comparisons test showed that vasopressin infusion significantly ($p < 0.001$) diminished the percentage of time spent in the open arms (12.38% ± 1.48%) with respect to the vehicle group (24.33% ± 1.16%). The AVP + V1a antagonist group showed a significant increase in this value (29.75% ± 3.51%) with respect to the AVP group ($p < 0.01$) and was no different than the vehicle treated group (**Figure 9B**).

Vasopressin Infusion Enhanced the Neuronal Activation in CeA and BLA After the EPM

To evaluate the effect of the infusion of vasopressin, vasopressin + the V1a receptor antagonist (Manning compound) or vehicle in the number of neurons activated by the EPM in the CeA, BLA and MeA, we perfused the rats 90 min after finishing the EPM test, sliced the brains, and performed immunohistochemistry against c-Fos. Five brains, in which correct cannula placement in CeA was confirmed, were selected to count the number of Fos+ nuclei in an area of 0.2 mm^2, inside the CeA, BLA and MeA in 5–6 slices around the injection site (**Figure 9C**). Panels (D–F) and inserts of **Figure 9** show examples of these immunoreactions in the vehicle, AVP and AVP + V1a antagonist treated rats.

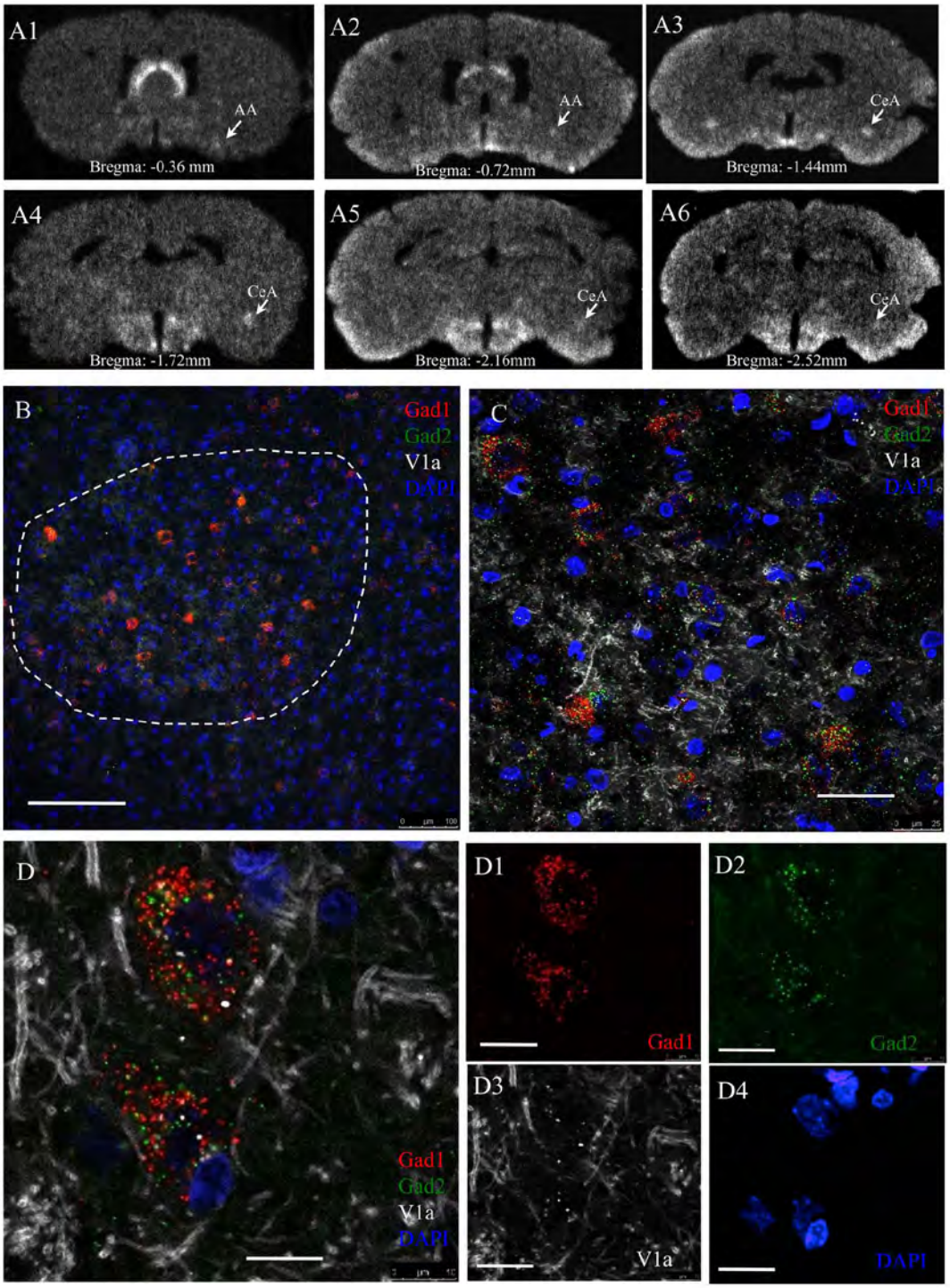


FIGURE 7 | AVP receptor V1a mRNA expression in GABAergic neurons in the CeA. Panel **(A's)** sequential coronal sections were hybridized with antisense radioactive AVP V1a riboprobe. Autoradiographs with overnight-exposure were read by phosphorimager. Rostro-caudal coordinates were indicated according to Rat Brain Atlas (Paxinos and Watson, 2006). AA, Anterior amygdala; CeA, central amygdala. **(B–D)** *In situ* hybridization using RNAscope-multiplex method targeting the Gad-1, Gad-2 and V1a mRNAs in CeA. V1a mRNA is particularly expressed by Gad-1 and Gad-2 positive neurons. **(B)** Low magnification showing the selective location of Gad1 + Gad2 expressing cell population in the CeA (dash-line circumscribed region). **(C)** Further magnifications showing the exclusive expression of V1a mRNA in Gad1 and Gad2 positive cells (100%, $n = 98$ neurons analyzed). Optical section thickness 1 μm. **(D's)** Confocal 1 μm optical slice of two cells in CeA expressing Gad1 (red), Gad2 (green) and V1a receptor (white) mRNA. Using the same method for V1b mRNA, we did not find any positive labeling. This data is not shown. The positive control for this latter experiment was done using adenohypophysis tissue. Scale bars: **(B)** 100 mm; **(C)** 20 μm; **(D)** 10 μm.

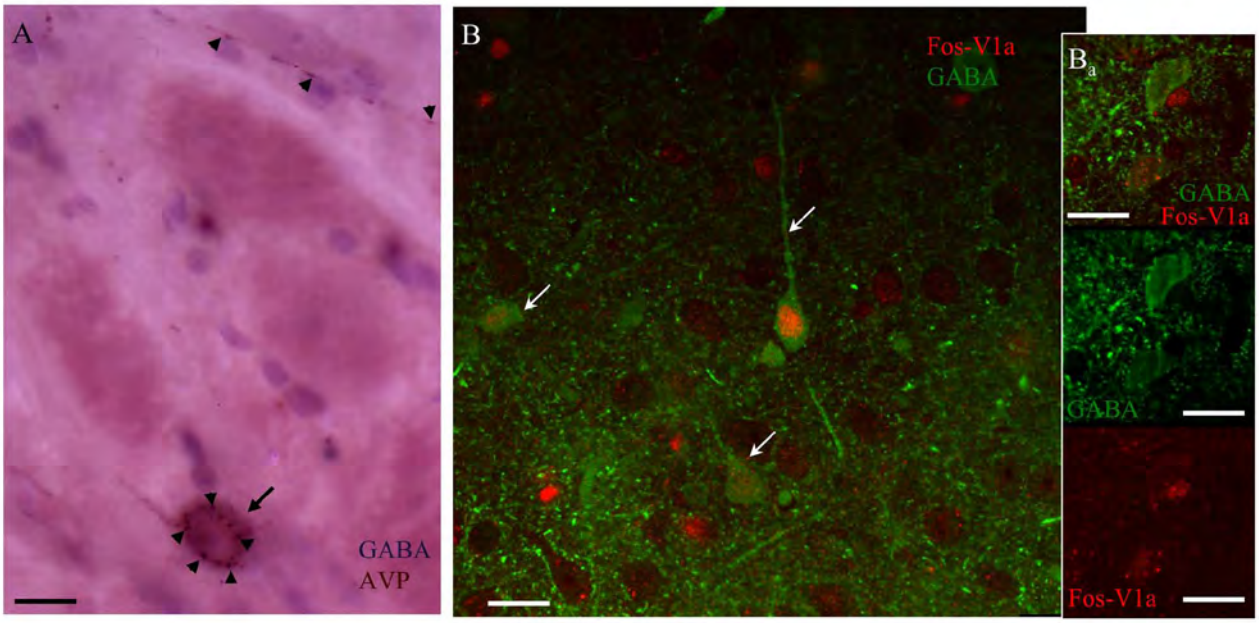


FIGURE 8 | AVP containing fibers in CeA synapse upon V1a receptor-positive GABAergic neurons activated after EPM. (A) Immunohistochemistry against AVP (arrowheads) and GABA (arrow) showing punctate AVP fibers terminals in close apposition with the GABA-labeled soma. **(B)** Confocal image of CeA immunoreacted against FOS (red nuclei indicated by arrows); V1a receptor (red punctuated label, indicated by arrowheads) and GABA (green cells). The immunohistochemistry was performed 90 min after the EPM in an animal infused with vasopressin in the CeA. **(B_a)** A different optical section of the same region showing two GABA+ neurons, one of them negative for V1a receptor showing no expression of Fos, the other one expressing the V1a receptor being Fos+. Scale bars: 10 μm.

Using a two-way ANOVA analysis (factors: region and treatment), we observed a significant effect for region: $F_{(2,225)} = 28.9$, $p < 0.0001$; treatment: $F_{(2,225)} = 285.5$, $p < 0.001$ and their interaction: $F_{(4,225)} = 67.2$, $p < 0.001$. Tukey's multiple comparisons test showed significant differences in the CeA between the vehicle and AVP infused groups: $F_{(2,225)} = 27.95$, $p < 0.001$ and between the AVP and AVP + V1a antagonist: $F_{(2,225)} = 24.68$, $p < 0.001$; no difference between vehicle and AVP + V1a antagonist was found: $F_{(2,225)} = 0.035$, $p > 0.05$. In the BLA region a similar pattern was found, with significant differences between the vehicle and AVP infused groups: $F_{(2,225)} = 21.26$, $p < 0.001$ and between the AVP and AVP + V1a antagonist: $F_{(2,225)} = 18.07$, $p < 0.001$; no difference between vehicle and AVP + V1a antagonist was found: $F_{(2,225)} = 0.75$, $p > 0.05$. With respect to the MeA, no significant differences were found between any of the treatment groups.

DISCUSSION

In this study, we investigated vasopressinergic innervation of the rat amygdaloid complex, and its functional implications. We quantified the density of AVP innervation in the subnuclei of the amygdaloid complex and demonstrated that in parallel to the AVP containing neurons in the STIA, previously described (Caffé and van Leeuwen, 1983; Plumari et al., 2002; Rood and De Vries, 2011), which are likely to be GABAergic neurons (Dabrowska et al., 2013), there exist a direct AVP-containing glutamatergic pathway, from VPMNN axon collaterals, synaptically targeting the CeA. We report that early life stress (MS) increased the density, and altered the pattern, of AVP innervation to amygdala in agreement with the enlarged PVN and SON previously reported (Zhang et al., 2012). In CeA, mRNA encoding the V1a receptor was detected as highly expressed and, using the newly developed RNAscope method, we could demonstrate that all the cells expressing V1a mRNA in CeA were Gad1- and Gad2-expressing cells. Using immunohistochemistry, we observed that GABAergic neurons inside the CeA expressed V1a receptor protein on their plasma membrane. Physiological up-regulation of the VPMNN system by WD 24 was associated with higher expression of anxiety-like behavior, and increased Fos expression in the CeA, mainly within GABA-positive neurons. The effects of WD 24 were mimicked by AVP local micro-infusion into the CeA, and these effects, and those of WD 24 itself, were attenuated by local infusion of Manning compound a V1a receptor specific antagonist-into the CeA.

A significant body of literature implicates AVP as a transmitter subserving stress responses in adult rodents without a history of stressor exposure, and in adult rodents with a history of stressor exposure during early post-natal life (Veenema and Neumann, 2008; Zhang et al., 2010, 2012, 2016; Zhang and Hernández, 2013; Hernández et al., 2015). A potential linkage between these two AVP-related types of stress response is found in the observations that early-life stress, in rats, leads to reorganization of vasopressinergic neurons of the PVN (Zhang

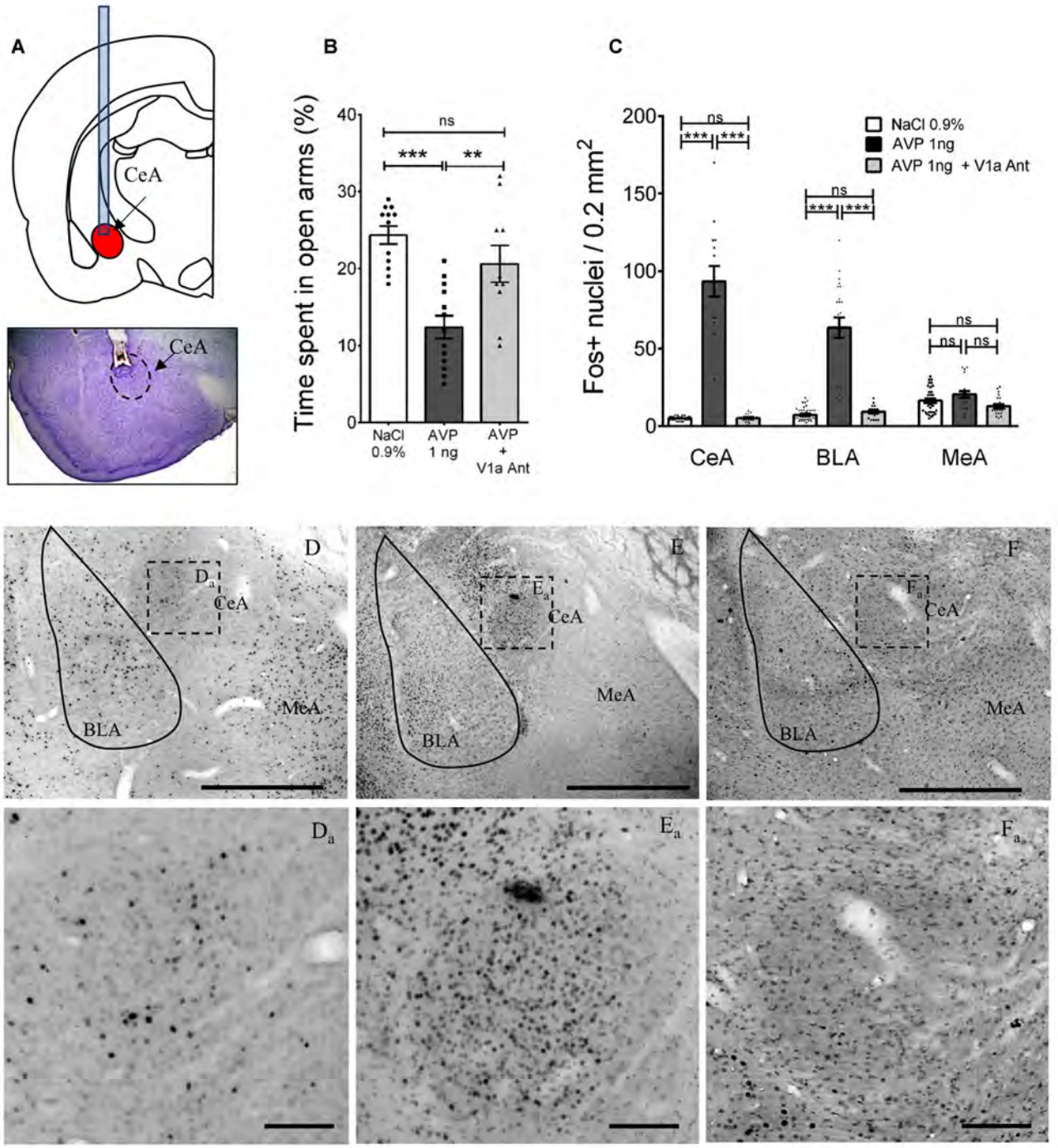


FIGURE 9 | Altered anxious behavior and Fos expression after AVP infusion into CeA in AFR rats and reversal by co-infusion of V1a antagonist (Manning compound). (A) Upper panel depicts positioning of cannulae in CeA for infusion of vehicle, AVP, or AVP plus V1a antagonist (Manning compound); lower panel is a representative photomicrograph showing the trajectory of the cannula toward the CeA. **(B)** Bar graph showing the time spent in the open arms of the EPM in animals infused bilaterally with vehicle (NaCl 0.9%), AVP or AVP + V1a antagonist (Manning compound). Infusion of AVP into CeA produced anxiety-like behavior shown as a reduction of the time spent in the open arms (**$p < 0.01$, ***$p < 0.001$) compared with vehicle-treated rats. Infusion of AVP + V1a antagonist reversed anxious behavior (decreased time spent in open arms) to control levels (i.e., indistinguishable from vehicle-treated rats). **(C)** Bar graph showing the quantification of neurons activated (measured by Fos expression) after EPM in the CeA, BLA and MeA in vehicle, AVP, or AVP plus V1a antagonist infused groups. AVP produced a significant increase in the Fos expression in the central (CeA, $p < 0.001$) and basolateral nuclei (BLA, $p < 0.001$) that was blunted in the AVP + V1a antagonist group. No effect of the treatments was observed in the MeA. **(D–F)** Photomicrographs showing the Fos expression patterns seen in the three infusion groups after the EPM, **(D_a–F_a)** are magnifications of the squared regions in **(D–F)**. BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala. Scale Bars: **(D–F)** 1 mm; **(D_a–F_a)** 0.1 mm.

et al., 2012). However, an anatomical connection between effects of AVP on behavioral responses to stress, mainly associated with AVP receptors resident in the amygdala, and specific projections from paraventricular VPMNNs, has not yet been made. As we have previously identified axonal projections from VPMNNs to various extrahypothalamic brain regions (Hernández et al., 2015), including those putatively involved in stress responding (Zhang and Hernández, 2013; Zhang et al., 2016), we hypothesized that VPMNN projections to the amygdala might modulate both adult stress/anxiogenic circuits, and provide an anatomical substrate for adult stress response conditioning occurring during the neonatal period. Accordingly, we designed and carried out a functional neuroanatomical study of AVP VPMNNs projections to amygdala, their putative neurons of origin, and the potential role of these AVP neurons in stress response modulation in both acute adult, and developmentally determined, behavior contexts.

In brief (*vida supra*), our data suggest that vasopressinergic projections, originating from magnocellular soma of the PVN (VPMNNs), synapse upon GABAergic neurons of the CeA which contain V1a receptors; that the CeA projections of these neurons are increased by early-life stress (MS); that activation of these neurons via WD in adulthood is anxiogenic; that the degree of anxiogenesis is correlated with the number and AVP content of these projections; and that anxiogenesis correlated with the dynamic changes of VPMNN system projecting to the CeA can be mimicked by CeA infusion of AVP, and blocked by CeA infusion of a V1a antagonist. What are the implications of these findings?

First, the localization of AVP-dependent stress responding to VPMNNs of the PVN, in both adult stress responding and in developmental shaping of the stress response, allows the testing of further important hypotheses about the amygdalar microcircuitry of stress, and the extent of AVP involvement in this circuitry. Thus, we may now hypothesize that the locus of action, or at least one anatomical target, of psychogenic stressors such as MS in early life, that affect long-term neuroplasticity of the VPMNN population, is at synapses on GABAergic neurons of the CeA. It is noteworthy that MS does not cause increased adult anxious behavior on its own, but rather increases the penetrance of other stressors (thirst, this study; electrical shock in previous studies—see Zhang et al., 2012) in a way that requires AVP transmission within the CeA. Examination of the corresponding dependence on AVP release within the CeA, as probed with local infusion of V1A antagonist, of other adult and anxiogenic stressors, including restraint, social defeat, and social subordination, appears highly warranted by the results presented here.

Second, the localization to GABAergic neurons of CeA of these vasopressinergic effects on stress responding raises the important question of where these circuits ultimately find expression in altered exploratory and other anxiety-associated behaviors in the rodent. The recent expansion of detailed knowledge about GABAergic neurons in CeA involved in mediating the salience of negative (fear- and anxiety-inducing) environmental stimuli now allows more anatomically detailed inquiry into the precise role of VPMNN projections in this circuitry (for example see Haubensak et al., 2010).

Finally, this work exposes a new opportunity to test the relative specificities of various neuropeptides in modulation of the stress response. The metabolic activation of VPMNNs following 24 h of thirst is itself a stressor, but a very specific paraphysiological one, that has allowed a clear linkage between VPMNN activation and anxiogenesis to be revealed (vide supra). However this work also allows the testing of AVP and VPMNN dependence of behavioral responses to other stressors, as well as exploring whether or not AVP release onto GABAergic synapses in CeA is a general feature of amygdala-mediated modulation of emotional responses to stress, or is reserved to specific subtypes of stress responding, with other neuropeptides mediating different and equally specific modes of stress response modulation.

AUTHOR CONTRIBUTIONS

LZ and VSH: conceived and designed the experiments. LZ, VSH, ORH and MJG: performed the experiments. LZ, VSH, ORH, MPM, KF and LEE: analyzed the data. LZ, LEE and MPM: contributed equipment/reagents/materials/analysis tools. LZ, VSH and LEE: wrote the article. VSH, ORH, MPM, MJG, KF, LEE and LZ: revised the manuscript critically for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncir.2016.00092/full#supplementary-material

REFERENCES

Appenrodt, E., and Schwarzberg, H. (2000). Central vasopressin administration failed to influence anxiety behavior after pinealectomy in rats. *Physiol. Behav.* 68, 735–739. doi: 10.1016/s0031-9384(99)00237-1

Buijs, R. M. (1978). Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. *Cell Tissue Res.* 192, 423–435. doi: 10.1007/bf00212323

Buijs, R. M. (1980). Immunocytochemical demonstration of vasopressin and oxytocin in the rat brain by light and electron microscopy. *J. Histochem. Cytochem.* 28, 357–360. doi: 10.1177/28.4.6989899

Buijs, R. M., De Vries, G. J., Van Leeuwen, F. W., and Swaab, D. F. (1983). Vasopressin and oxytocin: distribution and putative functions in the brain. *Prog. Brain Res.* 60, 115–122. doi: 10.1016/s0079-6123(08)64379-4

Buijs, R. M., and Swaab, D. F. (1979). Immuno-electron microscopical demonstration of vasopressin and oxytocin synapses in the limbic system of the rat. *Cell Tissue Res.* 204, 355–365. doi: 10.1007/bf00233648

Caffé, A. R., and van Leeuwen, F. W. (1983). Vasopressin-immunoreactive cells in the dorsomedial hypothalamic region, medial amygdaloid nucleus and locus coeruleus of the rat. *Cell Tissue Res.* 233, 23–33. doi: 10.1007/bf00222229

Dabrowska, J., Hazra, R., Guo, J. D., Dewitt, S., and Rainnie, D. G. (2013). Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. *Front. Neurosci.* 7:156. doi: 10.3389/fnins.2013.00156

Davis, M., and Whalen, P. J. (2001). The amygdala: vigilance and emotion. *Mol. Psychiatry* 6, 13–34. doi: 10.1038/sj.mp.4000812

DeVries, G. J., Buijs, R. M., Van Leeuwen, F. W., Caffe, A. R., and Swaab, D. F. (1985). The vasopressinergic innervation of the brain in normal and castrated rats. *J. Comp. Neurol.* 233, 236–254. doi: 10.1002/cne.902330206

de Wied, D. (1979). Neuropeptides and behavior. The neuropeptide concept. *Ned. Tijdschr. Geneeskd.* 123, 1589–1590.

Dunn, F. L., Brennan, T. J., Nelson, A. E., and Robertson, G. L. (1973). The role of blood osmolality and volume in regulating vasopressin secretion in the rat. *J. Clin. Invest.* 52, 3212–3219. doi: 10.1172/jci107521

Greving, R. (1923). Zur anatomie, physiologie und pathologie del der vegetativen zentren im zwischenhirn. *Ergebn. Anat. EntwGesch.* 24, 348–413.

Greving, R. (1928). Das Zwischenhirn-hypophysensystem. Seine morphologie, phylogenese und klinische bedeutung. *Klin. Wochenschr.* 7, 734–737. doi: 10.1007/bf01732231

Harris, G. W. (1948). Neural control of the pituitary gland. *Physiol. Rev.* 28, 139–179.

Haubensak, W., Kunwar, P. S., Cai, H., Ciocchi, S., Wall, N. R., Ponnusamy, R., et al. (2010). Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature* 468, 270–276. doi: 10.1038/nature09553

Hernández, V., Vázquez-Juárez, E., Márquez, M. M., Jáuregui-Huerta, J. H., Barrio, R., and Zhang, L. (2015). Extra-neurohypophyseal axonal projections from individual vasopressin-containing magnocellular neurons in rat hypothalamus. *Front. Neuroanat.* 9:130. doi: 10.3389/fnana.2015.00130

Hernández, V. S., and Zhang, L. (2012). "Vasopressin containing fibers distribution and synaptic innervation in the medial and central amygdala: an immunohistochemical study using light and electron microscopy," in *Work Presented at SFN Annual Meeting*, (New Orleans, LA).

Huber, D., Veinante, P., and Stoop, R. (2005). Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. *Science* 308, 245–248. doi: 10.1126/science.1105636

Koob, G. F., Le Moal, M., Gaffori, O., Manning, M., Sawyer, W. H., Rivier, J., et al. (1981). Arginine vasopressin and a vasopressin antagonist peptide: opposite effects on extinction of active avoidance in rats. *Regul. Pept.* 2, 153–163. doi: 10.1016/0167-0115(81)90009-4

Landgraf, R., and Neumann, I. D. (2004). Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Front. Neuroendocrinol.* 25, 150–176. doi: 10.1016/j.yfrne.2004.05.001

LeDoux, J. (2007). The amygdala. *Curr. Biol.* 17, R868–R874. doi: 10.1016/j.cub.2007.08.005

Lukas, M., Bredewold, R., Landgraf, R., Neumann, I. D., and Veenema, A. H. (2011). Early life stress impairs social recognition due to a blunted response of vasopressin release within the septum of adult male rats. *Psychoneuroendocrinology* 36, 843–853. doi: 10.1016/j.psyneuen.2010.11.007

Morales, M., and Bloom, F. E. (1997). The 5-HT3 receptor is present in different subpopulations of GABAergic neurons in the rat telencephalon. *J. Neurosci.* 17, 3157–3167.

Morel, A., O'Carroll, A. M., Brownstein, M. J., and Lolait, S. J. (1992). Molecular cloning and expression of a rat V1a arginine vasopressin receptor. *Nature* 356, 523–526. doi: 10.1038/356523a0

Mühlethaler, M., Dreifuss, J. J., and Gähwiler, B. H. (1982). Vasopressin excites hippocampal neurones. *Nature* 296, 749–751. doi: 10.1038/296749a0

Pará, D., Quirk, G. J., and Ledoux, J. E. (2004). New vistas on amygdala networks in conditioned fear. *J. Neurophysiol.* 92, 1–9. doi: 10.1152/jn.00153.2004

Paxinos, G., and Watson, C. (2006). *The Rat Brain in Stereotaxic Coordinates*, 6th Edn. Elsevier: Amsterdam.

Penzo, M. A., Robert, V., Tucciarone, J., De Bundel, D., Wang, M., Van Aelst, L., et al. (2015). The paraventricular thalamus controls a central amygdala fear circuit. *Nature* 519, 455–459. doi: 10.1038/nature13978

Pérez de la Mora, M., Lara-García, D., Jacobsen, K. X., Vázquez-García, M., Crespo-Ramírez, M., Flores-Gracia, C., et al. (2006). Anxiolytic-like effects of the selective metabotropic glutamate receptor 5 antagonist MPEP after its intra-amygdaloid microinjection in three different non-conditioned rat models of anxiety. *Eur. J. Neurosci.* 23, 2749–2759. doi: 10.1111/j.1460-9568.2006.04798.x

Persson, S., and Havton, L. A. (2009). Retrogradely transported fluorogold accumulates in lysosomes of neurons and is detectable ultrastructurally using post-embedding immuno-gold methods. *J. Neurosci. Methods* 184, 42–47. doi: 10.1016/j.jneumeth.2009.07.017

Plumari, L., Viglietti-Panzica, C., Allieri, F., Honda, S., Harada, N., Absil, P., et al. (2002). Changes in the arginine-vasopressin immunoreactive systems in male mice lacking a functional aromatase gene. *J. Neuroendocrinol.* 14, 971–978. doi: 10.1046/j.1365-2826.2002.00866.x

Rood, B. D., and De Vries, G. J. (2011). Vasopressin innervation of the mouse (Mus musculus) brain and spinal cord. *J. Comp. Neurol.* 519, 2434–2474. doi: 10.1002/cne.22635

Schmued, L. C., and Fallon, J. H. (1986). Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Res.* 377, 147–154. doi: 10.1016/0006-8993(86)91199-6

Sofroniew, M. V., and Glasmann, W. (1981). Golgi-like immunoperoxidase staining of hypothalamic magnocellular neurons that contain vasopressin, oxytocin or neurophysin in the rat. *Neuroscience* 6, 619–643. doi: 10.1016/0306-4522(81)90147-0

Sun, N., and Cassell, M. D. (1993). Intrinsic GABAergic neurons in the rat central extended amygdala. *J. Comp. Neurol.* 330, 381–404. doi: 10.1002/cne.903300308

Sun, N., Yi, H., and Cassell, M. D. (1994). Evidence for a GABAergic interface between cortical afferents and brainstem projection neurons in the rat central extended amygdala. *J. Comp. Neurol.* 340, 43–64. doi: 10.1002/cne.903400105

Swanson, L. W. (2003). *Brain Maps: Structure of the Rat Brain*, 3rd Edn. Elsevier: Amsterdam.

Swanson, L. W., and Petrovich, G. D. (1998). What is the amygdala? *Trends Neurosci.* 21, 323–331. doi: 10.1016/S0166-2236(98)01265-X

Veenema, A. H., Blume, A., Niederle, D., Buwalda, B., and Neumann, I. D. (2006). Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. *Eur. J. Neurosci.* 24, 1711–1720. doi: 10.1111/j.1460-9568.2006.05045.x

Veenema, A. H., Bredewold, R., and Neumann, I. D. (2007). Opposite effects of maternal separation on intermale and maternal aggression in C57BL/6 mice:

link to hypothalamic vasopressin and oxytocin immunoreactivity. *Psychoneuroendocrinology* 32, 437–450. doi: 10.1016/j.psyneuen.2007.02.008

Veenema, A. H., and Neumann, I. D. (2008). Central vasopressin and oxytocin release: regulation of complex social behaviours. *Prog. Brain Res.* 170, 261–276. doi: 10.1016/S0079-6123(08)00422-6

Wessendorf, M. W. (1991). Fluoro-Gold: composition and mechanism of uptake. *Brain Res.* 553, 135–148. doi: 10.1016/0006-8993(91)90241-m

Zhang, L., and Hernández, V. S. (2013). Synaptic innervation to rat hippocampus by vasopressin-immuno-positive fibres from the hypothalamic supraoptic and paraventricular nuclei. *Neuroscience* 228, 139–162. doi: 10.1016/j.neuroscience.2012.10.010

Zhang, L., Hernández, V. S., Liu, B., Medina, M. P., Nava-Kopp, A. T., Irles, C., et al. (2012). Hypothalamic vasopressin system regulation by maternal separation: its impact on anxiety in rats. *Neuroscience* 215, 135–148. doi: 10.1016/j.neuroscience.2012.03.046

Zhang, L., Hernández, V. S., Vázquez-Juárez, E., Chay, F. K., and Barrio, R. A. (2016). Thirst is associated with suppression of habenula output and active stress coping: is there a role for a non-canonical vasopressin-glutamate pathway? *Front. Neural Circuits* 10:13. doi: 10.3389/fncir.2016.00013

Zhang, L., Medina, M. P., Hernández, V. S., Estrada, F. S., and Vega-González, A. (2010). Vasopressinergic network abnormalities potentiate conditioned anxious state of rats subjected to maternal hyperthyroidism. *Neuroscience* 168, 416–428. doi: 10.1016/j.neuroscience.2010.03.059

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BRAIN RESEARCH

Review

The intercalated paracapsular islands as a module for integration of signals regulating anxiety in the amygdala

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ABSTRACT

The intercalated paracapsular (IPC) islands are clusters of dopamine-D1-and μ-opioid 1-receptor rich GABAergic neurons which surround the rostral half of the basolateral complex of the amygdala (BLA) giving rise to several subgroups which can be further subdivided. IPC cells are small-sized and have an axonal and dendritic pattern which differs according to the group they belong. Functionally, IPC neurons are endowed with unique properties that set them apart from other amygdaloid interneurons and allow them to participate in integrative functions. Consistent with this role IPC cells usually remain confined within the amygdala where they receive BLA and cortical inputs and interact synaptically with each other. They project into both the central (CeA) and medial (MeA) amygdaloid nuclei. Their main effect at the network level seems to control the trafficking of nerve impulses to the main input (BLA) and output (CeA) stations of the amygdala. Such a task seems to be accomplished by providing feedforward inhibition to BLA neurons from putative inputs of the medial prefrontal cortex (mPFC) and to CeA from both mPFC and BLA projections. Current experimental evidence will be discussed suggesting that through feedforward inhibitory effects on specific amygdaloid nuclei IPC neurons participate in the maintenance of basal anxiety as well as in the modulation of unconditioned and conditioned fear, and in the process of fear extinction.

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Abbreviations: BA, basal nucleus; BL, basolateral nucleus; BLA, amygdaloid basolateral complex; BM, basomedial nucleus; CCK, cholecystokinin; CeA, amygdaloid central nucleus; CeL, centrolateral nucleus; CeM, centromedial nucleus; CS, conditioned stimuli; EPSPs, excitatory postsynaptic potencials; GAD, glutamic acid decarboxylase; GIRK channels, G-protein-mediated activation of inwardly rectifying K^+ channels; Iap, anterior paracapsular cluster; IL, infralimbic cortex; IM, main intercalated island; Imp, medial paracapsular cluster; IPAC, interstitial nucleus of the posterior limb of the anterior commisure; IPC, intercalated paracapsular; IPSCs, Inhibitory postsynaptic currents; LA, lateral nucleus; MeA, amygdaloid medial nucleus; mPFC, medial prefrontal cortex; PL, prelimbic cortex; US, unconditioned stimuli; VT, volume transmission; VTA, ventral tegmental area

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Contents

1. Introduction

Anxiety is an adaptive response which detects and protects an individual against danger. Although many brain regions have been found to participate in its regulation it is commonly accepted that among them the amygdala plays a prominent role (Canteras et al., 2010; Davis and Whalen, 2001; Ehrlich et al., 2009; Engin and Treit, 2008; Everitt et al., 2003; Herry et al., 2010; LeDoux, 2000, 2007; McNally et al., 2011; Pape and Paré, 2010; Paré et al., 2004; Pérez de la Mora et al., 2008, 2010; Roozendaal et al., 2009). Thus, according to the most prevalent theory (LeDoux, 2000, 2007) emotionally significant stimuli from the environment reach the amygdaloid basolateral complex (BLA) via thalamic and cortical afferents and, after local processing, the new information is conveyed to the amygdaloid central nucleus (CeA) and particularly to its medial subdivision (CeM) where a proper anxiogenic response is implemented. In support of this view, it has been demonstrated, using conditioned models of fear/anxiety, that afferent information on conditioned (CS) and unconditioned (US) stimuli gathers within BLA (LeDoux et al., 1990; McDonald, 1998; Romanski and LeDoux, 1993) and that efferent fibers from CeM reach important hypothalamic and brain stem targets (Cassell et al., 1986; Gray, 1989; Hopkins and Holstege, 1978; Krettek and Price,

1978; Sun et al., 1994; Veeinig et al., 1984) involved in the hormonal and autonomic anxiogenic response. Furthermore, important experimental evidence points out BLA and particularly its lateral nucleus (LA) as an important plasticity center involved in fear conditioning (Blair et al., 2001; Gewirtz and Davis, 1997; Goosens and Maren, 2003; Miserendino et al., 1990; Quirk et al., 1995; Rodrigues et al., 2004). Besides this linear information processing, recent work has demonstrated that a horizontal information processing may also operate whereby information reaching either CeA or BLA alone may elicit anxiogenic responses (Ciocchi et al., 2010; Corbit and Balleine, 2005; Holland et al., 2001; Killcross et al., 1997). See also Pérez de la Mora et al., 2010, 2012.

In spite of the importance of BLA and CeA in the amygdaloid modulation of fear/anxiety accumulating evidence has indicated that clusters of GABAergic neurons forming the main intercalated island (IM) and the intercalated paracapsular (IPC) islands which in the rat are either located ventral to CeA or surrounding the BLA respectively also have a paramount importance in the amygdaloid modulation of fear/anxiety. As it will be discussed below, as a consequence of the scarceness of connections between BLA and CeM, IPC neurons seem to form a link between them providing an inhibitory interface which will control the trafficking of nerve impulses between both nuclei (Royer et al., 1999) thereby contributing to modulate the output of the amygdala. On the other hand, IPC neurons by providing feedforward inhibition to BLA, the main input station of the amygdala (Marowsky et al., 2005), will also be able to modulate the strong inhibitory brake imposed to this structure by the activity of cortical neurons (Lang and Paré, 1998; Quirk et al., 2003; Rosenkranz and Grace, 2001, 2002). The aim of this paper will be to review the structural and functional characteristics of IPC neurons and to discuss their functional impact on the amygdaloid modulation of fear/anxiety.

2. Anatomical studies

2.1. *Brain distribution*

A common feature of mammalian amygdala is the presence of numerous dense Golgi stained cell clusters forming a distinct pattern of cell masses or islands within a landscape of fiber tracts and major amygdaloid nuclei. Thin cell strands seem to connect individual islands forming a complex insular system (Millhouse, 1986). Such islands have been described in a variety of mammal species (Berkelbach van der Sprengel, 1926; Fox, 1940; Humphrey, 1936; Paré and Smith, 1993a, 1994) including rodents (Brodal, 1947; Fuxe et al., 2003; Geracitano et al., 2007; Gurdijan, 1928; Millhouse, 1986; Nitecka and Ben-Ari, 1987; Spiegel, 1919) non human primates (Lauer, 1945; McDonald and Augustine, 1993; Pitkänen and Amaral, 1994; Völsch, 1910) and humans (Braak et al., 1994; Crosby and Humphrey, 1941; Sims and Williams, 1990). It is interesting that although these islands were recognized long ago their neuronal nature was not accepted until recently (Hall, 1972; Kamal and Tömböl, 1975; Tömböl and Szafrańska-Kosmal, 1972).

The IPC islands seem to represent the ventral extension of the striatum (Busti et al., 2011a; Millhouse, 1986) since as we will see below they are populated by GABAergic medium spiny neurons which receive cortical glutamatergic afferents and project to large pallidal like neurons enriched in mGluR1 α receptors (Ferraguti et al., 2008). In support of this, developmental studies have indicated that although both the IPC islands and CeA have a subpallial origen the IPC islands and at least a part of the CeA derive from progenitors located in the dorsolateral and ventrolateral ganglionic eminence respectively (Waclaw et al., 2010).

IPC islands surround the rostral half of BLA and stretch across the anterior amygdala in the direction to the amygdaloid medial nucleus (MeA) and rostrally they reach the interstitial nucleus of the posterior limb of the anterior commisura (IPAC) (Fuxe et al., 2003; Millhouse, 1986) (Fig. 1). According to their situation within the amygdala it is possible to distinguish a medial IPC insular group interposed within the BLA and CeA (Fuxe et al., 2003; Geracitano et al., 2007; Millhouse, 1986; Paré and Smith, 1993a, 1993b) and a lateral IPC insular group situated laterally to the BLA along the fiber bundles of the external capsule (Marowsky et al., 2005; Millhouse, 1986). In addition, a large cell cluster located ventral to BLA and CeA has been designated IM. Finally, a group of large islands lying around the rostral pole of BLA within the striato-pallidal area (Millhouse, 1986; Paré and Smith, 1994) gives rise to the anterior IPC insular group.

2.1.1. *Medial IPC islands subdivision*

On the basis of their relative situation within the intermediate capsule and their connectivity medial IPC islands in the cat and guinea pig have been further subdivided into groups of lateral and medial islands (Royer et al., 1999, 2000a, 2000b). These cell groups owing to the different orientation of the main nuclei of the amygdala in the rat in which BLA is located in a lateral position with respect to CeA instead to lie ventrally to this nucleus as in cat or guinea pig correspond to the dorsal and ventral IPC clusters described by Amir et al. (2011) within the intermediate capsule of the rat. In turn, Busti et al. (2011a) have recognized in their 3D reconstruction of the mouse insular system the presence of two cell clusters, medial (Imp) and anterior (Iap) paracapsular clusters which may correspond to the dorsal and ventral clusters described by Amir et al. (2011) in the rat.

2.1.2. *Lateral IPC islands subdivision*

Although less studied than the medial IPC island subdivision the studies of Amir et al. (2011) in the rat indicate the presence of a wide dorsal cluster and several thinner and elongated clusters surrounding the BLA (see Fig. 1a in this paper taken from Fuxe et al., 2003 and Pérez de la Mora et al., 2006, 2007). Interestingly, reconstruction studies in mice by Busti et al. (2011a) and Marowsky et al. (2005, supplementary material) indicate that lateral IPC cells in the mouse may form a single large island.

2.2. *Cytological features*

Since the detailed and pioneer Golgi studies of Millhouse (1986) it was clear that although cells within the IPC islands shared many cytological features they were not forming a homogeneous population of neurons since many of them were found to differ in either size, shape or in their dendritic and axonal

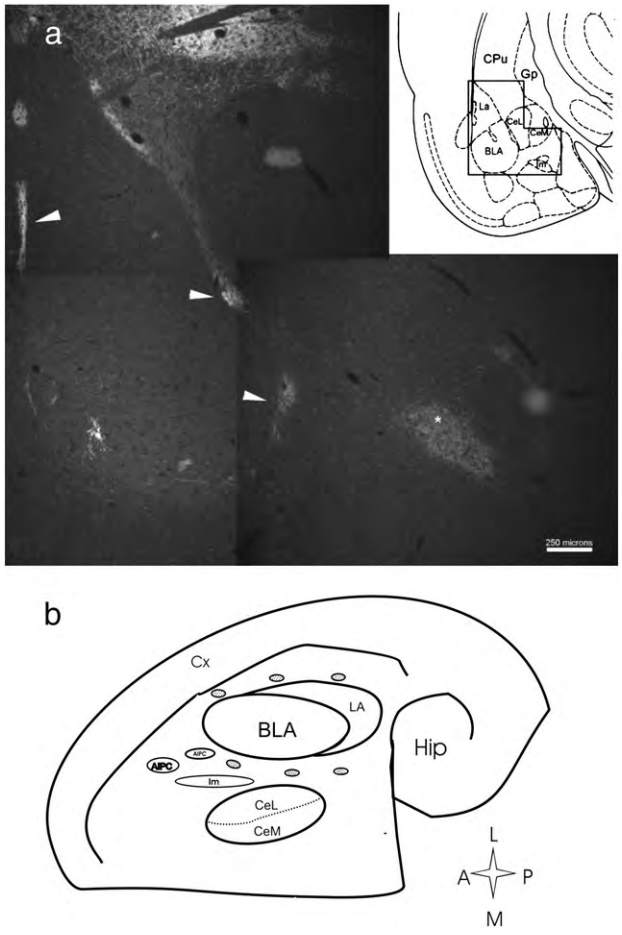

Fig. 1 – Anatomical distribution of intercalated paracapsular (IPC) islands. a. Digital photo-montage showing the distribution of D1 receptor immunoreactivity within the amygdala. The region depicted is shown in the inset from the atlas of Paxinos and Watson (1986) at Br level –2.56. Note that whereas the IPC islands (arrows) are distributed along the lateral and medial IPC group of islands BLA borders the main intercalated island (asterisk) located ventrally to both BLA, and central nuclei. b. Position of the IPC cell groups in relation to the BLA and CeM and CeL nuclei in a schematic horizontal representation based in part on the atlas of Paxinos and Watson (1986) at level Br –7.80. Note the close relationship between the anterior IPC islands and the main intercalated island. The orientation of the scheme is indicated by the cross (A, anterior; P, posterior; L, lateral; M, medial). Abreviattions: BLA, amygdaloid basolateral complex; CeL, centrolateral nucleus; CeM, centro medial nucleus; CPu, caudate putamen; Gp, globus pallidus; Hip, hippocampus; IM, main intercalated island of the amygdala; LA, lateral nucleus.
Panel a. Taken from Fuxe et al. (2003) with permission from Elsevier.

patterns. However, as demonstrated by Millhouse (1986) the vast majority of IPC neurons, irrespective of the identity of their group represent medium-spiny neurons. They are round or fusiform and have a size from 7 to 12 μm. However, in addition to these cells, large fusiform or oval neurons (15–30 μm) comprising no more than 5% of the total neuronal population were also found along the edges of the islands (see also Busti et al., 2011b). Medium-sized neurons as also described by Millhouse (1986) bear thick (3–5 μm) and short (5–15 μm) dendrites containing on their surface numerous spines which extend no more than 100–300 μm from the soma. Although the complexity of the dendritic tree of these neurons varies considerably among them it is relatively simple. Neurons originate 2 to 4 primary dendrites that divide into a couple of secondary dendrites which in turn divide only once. The direction of the axons seems instead to be dependent on the location of the neuron. In any case, irrespective of their location axons from medial and lateral IPC cells send collaterals which make *bouteaux en passant* with dendrites of neighboring IPC neurons and enter CeA and BLA. Axons from IM cells send instead collaterals which enter the internal capsule (Millhouse, 1986) or reach either the BLA, all CeA subdivisions or both the lateral and medial IPC islands (Manko et al., 2011). Albeit not so frequently found, a very large spiny or aspiny cell type interspersed with medium spiny cells was also found by Millhouse (1986) in all insular locations. The cell bodies of these large cells are bigger than 15 μm wide and 40–60 μm long. They are round within IM whereas in the medial and lateral IPC islands they adopt a fusiform shape. Their dendrites give branches that remain locally or enter short distances into the BLA or the CeA whereas their axons give collaterals which contact nearby medium spiny cells (see Section 2.4.5: Large IPC neurons).

2.3. *Axonal and dendritic patterns*

IPC neurons display a considerable variability in their dendritic and axonal patterns. Such variability seems to depend among other factors on the subset of IPC neurons to which they belong their intended functional task in amygdala function and their topographical position in relation with the BLA and CeA nuclei.

Medial IPC neurons seem to be endowed with the most complex dendritic tree of all IPC cell groups in terms of size, branching and number of spines. Its dendritic tree stretches along the intermediate capsule, it is plane and adopts a bipolar disposition around the soma (Geracitano et al., 2007; Millhouse, 1986; Royer et al., 1999, 2000a). In contrast, IM neurons display poorly branched dendrites which fan out in all directions but remain confined to the limits of the island (Manko et al., 2011). The dendritic pattern of the lateral IPC cell group albeit much less complex that of the medial IPC cell group shares with this group many of its features including its general shape and its disposition along fiber bundles i.e. external capsule (Marowsky et al., 2005). IM neurons have in turn a poorly branched dendritic tree which remains confined within the IM and possesses a long axon which interacts with cells of all other insular systems and provides innervation to many amygdaloid nuclei including CeA and BLA and the extended amygdala (Manko et al., 2011).

Elegant and well conducted electrophysiological studies by Paré and colaborators (Royer et al., 1999, 2000a, 2000b) have shown that in guinea pig interactions between medially located IPC islands are topographically polarized occurring in a lateromedial direction and such groups of islands seem to form an inhibitory interphase which controls the trafficking of impulses from BLA to CeA. In keeping with these functional features morphological studies carried out by the same group (Royer et al., 2000a) have shown that medial IPC neurons have

an asymmetric axonal and dendritic pattern. Thus, whereas their laterally-oriented dendrites are longer than those directed medially their axonal arborization tends to distribute predominantly in a lateromedial direction. Medially directed axons are long, extremely varicose and give rise to dorsal collaterals which ramify extensively within the CeA. Laterally directed axons are short and innervate predominantly neurons within the same island (Paré and Smith, 1993a; Royer et al., 1999, 2000a).

In spite that detailed morphometric studies similar to those made in the guinea pig (Royer et al., 2000a) for the length and complexity of the dendritic tree and axonal arborization of medial IPC neurons are lacking in other species it seems that at least for the rat the same general topographic principle can be applied (Amir et al., 2011). One has; however, to be aware that owing to the different orientation of the main amygdaloid nuclei in the rat as compared to the guinea pig or the cat the flow of information in the rat between islands of the medial IPC group follows a dorso-medial direction (Amir et al., 2011).

2.4. *Connectivity*

Consistent with the role of IPC islands as inhibitory interfaces gating the flow of impulses to and between some of the major amygdaloid nuclei, afferents to the IPC neurons come from both extra and intra-amygdaloid regions and their efferents reach mostly intra-amygdaloid targets. In addition, in keeping with their role as integrative interfaces a thorough information exchange among IPC neurons from the same or different clusters or groups will be needed. For a summary on IPC islands connectivity see Fig. 2.

2.4.1. *Afferents to IPC islands*

In view that the origin and termination of most fibers belonging to the internal and external capsules are not known, the list of afferents to the IPC groups of islands remains largely incomplete. Since IPC neurons are critically involved in information transfer between medial prefrontal cortex (mPFC) and most probably all major amygdaloid nuclei as well as from BLA to CeA it is not surprising that inputs from these regions will reach IPC neurons. BLA afferents were the first to be identified (Millhouse, 1986). These afferents are glutamatergic (Royer et al., 1999; 2000a) and reach mainly medial IPC islands (Millhouse, 1986; Royer et al., 1999) although the IM and anterior IPC islands are also reached (Manko et al., 2011; Millhouse, 1986). In addition, studies from several laboratories (Ghashghaei and Barbas, 2002; Marowsky et al., 2005) have shown that glutamatergic fibers from the mPFC and particularly from the infralimbic cortex (IL) (Berretta et al., 2005; Manko et al., 2011; Pinto and Sesack, 2008; Quirk et al., 2003) and insular cortices (McDonald et al., 1996) provide a strong innervation most probably to all IPC cell groups. Hippocampal afferents have also been found to innervate IPC islands (Canteras and Swanson, 1992; Kishi et al., 2006). Especially important for IPC island function is its dopaminergic innervation. Consequently, a dense dopaminergic innervation (reviewed in Pérez de la Mora et al., 2010) to IPC islands comes from the ventral tegmental area (VTA). It is interesting to this regard that though all IPC cell groups are innervated by the VTA the degree of its innervation varies according to its actual target. Thus, it has been found both in rat (Fuxe et al., 2003) and mice (Busti et al., 2011a, 2011b) that whereas the rostrolateral portion of the IM receives a substantial dopaminergic innervation, its medial and caudal portions are barely innervated by dopaminergic terminals. Likewise, caudally located medial IPC clusters receive less dopaminergic terminals than rostrally located IPC islands (Fuxe et al., 2003). Similarly, IPC islands are also reached by noradrenergic (Fuxe et al., 2003) and serotonergic (Bauman and Amaral, 2005) afferents though in considerably less density.

2.4.2. *Efferents from medial IPC islands*

Consistent with its role as an interface which controls the flow of impulses from both the mPFC and the BLA to the CeA, the medial IPC neurons receive BLA afferents and project to neighboring IPC neurons for information processing. Once the information has been locally processed it is relayed to CeA and perhaps in some cases to extra-amygdaloid targets. Accordingly, numerous efferent fibers from the medial IPC islands (Amir et al., 2011; Busti et al., 2011a; Geracitano et al., 2007; Royer et al., 1999, 2000a) innervate this nucleus.

The innervation provided by medial IPC islands is particularly interesting since it is topographically organized in such a way that not all the islands from this group project into the same target. In agreement studies by Busti et al. (2011a) in mice have found that particular cells located dorsally within the intermediate capsule belonging to Imp, which may correspond to the dorsal cluster described by Amir et al. (2011) in the rat, mainly project into centrolateral nucleus (CeL) and only occasionally to CeM. Furthermore, according to Paré and collaborators (Amir et al., 2011; Royer et al., 1999, 2000a) dorsal islands from this group (lateral islands in guinea pig) receive information from LA to be sent to CeM whereas the ventrally located islands (medial IPC islands in the guinea pig) receive information from basolateral nucleus (BL) and basomedial nucleus (BM) to be sent to CeM (Amir et al., 2011; Royer et al., 1999, 2000a).

In addition, axons from medial IPC neurons ramify extensively within the intermediate capsule (Amir et al., 2011; Busti et al., 2011a; Millhouse, 1986; Royer et al., 2000a) establishing contacts and functional monosynaptic synapses with dendrites of different IPC neurons and with cells from the IM (Busti et al., 2011a; Royer et al., 2000a). Most importantly evidence obtained in the guinea pig by Royer et al. (2000a) suggests that medial IPC islands are polarized in such a way that cells from dorsally located islands inhibit neurons from clusters lying in a more ventral position. Under these conditions, a complex inhibitory–disinhibitory–inhibitory interaction between IPC cells, reminiscent of a binary system, (0,1,0,1…) is created allowing a precise temporo-spatial processing of different inputs on their way to CeA.

Finally, medial IPC neurons besides to providing innervation to intra-amygdaloid regions also project into extra-amygdaloid targets. Specifically, it has been shown both in the rat (Amir et al., 2011) and the mouse (Busti et al., 2011a; Geracitano et al., 2007) that a distinct population of medial IPC neurons emit axons that without innervating the CeA ramify extensively within the intermediate capsule and reach extra-amygdaloid regions such as the amygdalostriatal transition area (Amir et al., 2011), the interstitial nucleus of the ansa lenticularis (Busti

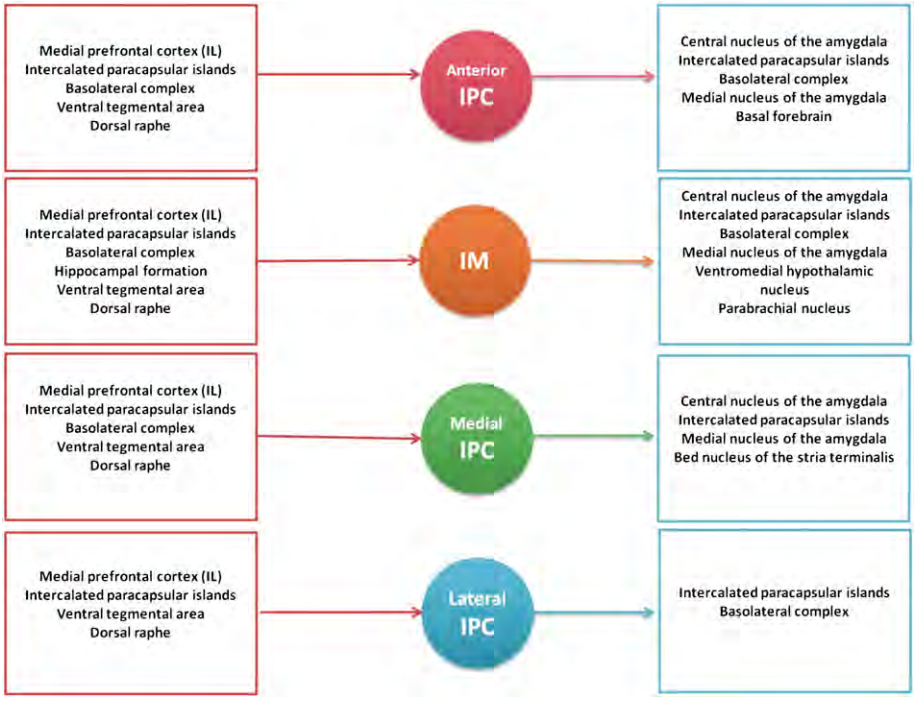

Fig. 2 – Connectivity of intercalated paracapsular (IPC) islands. As it is shown in the figure there is a thorough interaction between neurons of all IPC groups. Note that all insular groups are innervated by dopaminergic (less at caudal level) and possibly by serotonergic fibers from the ventral tegmental area and the dorsal raphe, respectively. They are also targeted, with the likely exception of the lateral IPC group of islands by glutamatergic inputs arriving from the basolateral complex. It is also supposed but not proven, with the exception of neurons from the medial group, that all intercalated cells are reached by afferents from the medial prefrontal cortex. In turn, all IPC groups of islands project to the medial and basolateral amygdala although the projection to the latter nucleus is rather scarce. It is not clear whether the central amygdaloid nucleus is innervated by the lateral IPC group of islands. Efferents from the anterior IPC group of islands and the main intercalated island to extra-amygdaloid targets are indicated at the respective places in the figure.

et al., 2011a) and parts of the extended amygdala (Geracitano et al., 2007).

2.4.3. *Efferents from lateral and anterior IPC islands*

Although there does not exist a systematic study indicating how lateral IPC neurons interact among each other and with cells from other IPC groups of islands the available evidence (Marowsky et al., 2005) suggests that they may keep the same dorso-ventral polarity that exists between medial IPC clusters. On the other hand, lateral IPC neurons project to and receive afferents from the IM (Manko et al., 2011) and may interact with medial IPC clusters through strands of interneurons which seem to link both groups of islands (Marowsky et al., 2005).

Convincing electrophysiological evidence from Marowsky et al. (2005) has shown that BLA projection neurons receive monosynaptic GABAergic innervation from lateral IPC neurons providing feedforward inhibition into this nucleus. On the other hand, in a series of experiments by Smith and Paré (1994) using retrograde and anterograde tracers combined with post-embedding GABA and glutamate immunocytochemistry, GABAergic projections from the anterior IPC islands were demonstrated in the basal forebrain. Likewise, studies using iontophoretic injections of cholera toxin B subunit within the MeA showed that the anterior IPC group contributes with the majority of fibers that reach this nucleus from all IPC cell groups (Paré and Smith, 1993a).

2.4.4. *Efferents from the main intercalated island*

Neurons from the IM keep also important interactions with other IPC cell groups (Fig. 2). Thus, it has been shown in the rat that medial IPC neurons belonging to the ventral clusters project to IM (Amir et al., 2011). In agreement with this finding, Busti et al. (2011a) showed that in the mouse a specific subset of neurons from their Imp sends fibers which form symmetric synapses with dendrites of the IM. Moreover, electrophysiological experiments from the same group also show that these synaptic contacts were monosynaptic and that the evoked inhibitory postsynaptic currents (IPSCs) were mediated by $GABA_A$ receptors (Busti et al., 2011a). In reciprocity, IM neurons project back to medial and lateral IPC cells from which they also receive GABAergic afferents (Manko et al., 2011). In addition, IM neurons provide GABAergic innervation to BLA, MeA, and all CeA divisions (Manko et al., 2011).

It is however rather interesting that IM neurons in spite of interacting extensively with cells of the lateral and medial IPC groups whose efferents are essentially intra-amygdaloid also behave as projection neurons. Consistent with this putative

role IM neurons are multipolar and exhibit long axons (Manko et al., 2011) which leave the amygdala to reach extra-amygdaloid targets such as the ventromedial hypothalamic (Luiten et al., 1983) and the parabrachial (Moga and Gray, 1985) nuclei. Finally, since both the anterior IPC clusters and the IM are located very close to each other, share a great deal of cytological features (Manko et al., 2011; Millhouse, 1986) and have projections to extra-amygdaloid targets it is tempting to suggest that these nuclei may in fact belong to the same group of IPC islands.

2.4.5. *Large IPC neurons*

In association with spiny-medium neurons, a population of large cells which differs widely in the number of dendritic spines, has been found in all IPC insular groups (Kamal and Tömböl, 1975; Millhouse, 1986; Tömböl and Szafrańska-Kosmal, 1972) and particularly within the medial IPC cluster (Millhouse, 1986). These cells tend to be located at the edges of the IPC islands (Busti et al., 2011b) and typically, as described by Millhouse (1986) display somata about 15 μm wide and 40–60 μm long. Their primary dendrites are rather thick (5–7 μm) and give rise to secondary branches which extend for 200–300 μm or more before they further divide. Terminal dendrites are thin and have numerous varicosities.

These cells have been found to interact with neighboring IPC neurons (Millhouse, 1986) through the formation of putative inhibitory synapses as shown by the presence of symmetric contacts between dendrites of these large cells and terminals (varicosities) arising from axons of the lateral IPC neurons (Busti et al., 2011b). Dendrites of these cells along with fibers from medium-spiny neurons follow either a ventral or a dorsal course squeezed between CeA and BLA or along the fibers of the external capsule giving rise to many collaterals during their course. Interestingly, it has been observed that in some cases dendrites of these large neurons distribute between the dorsal border of the CeA and the overlying striatum suggesting the possibility of synaptic interactions between them and striatal neurons (Millhouse, 1986). Rostrally, a population of large neurons extends beneath the anterior commisure and intermixes with large putative cholinergic neurons characteristic of the basal forebrain (Millhouse, 1986).

These large cells in spite of sharing their lodging with medium spiny neurons within the IPC islands belong to a different class of neuron since unlike the latter cells they are not GABAergic. Accordingly, they do not display GABA-immunoreactivity (Busti et al., 2011b; McDonald and Augustine, 1993; Nitecka and Ben-Ari, 1987; Paré and Smith, 1994) and no green fluorescence protein is expressed within them in transgenic mice (Busti et al., 2011a) expressing this protein under the control of the glutamic acid decarboxylase 65 (GAD_{65}) promoter. Synaptic targets for this particular set of neurons remain elusive yet a full tridimensional reconstruction of one of these neurons has shown that they innervate profusely the BLA and send efferents to both the endopeduncular nucleus and the enthorhinal cortex (Busti et al., 2011b). From a neurochemical point of view, recent studies by the same group (Busti et al., 2011a, 2011b) have shown that large neurons express parvalbumin, have receptors for neurokinin 1 and show an array of post (mGluR1α) and pre (mGlu7 and mGlu8) metabotropic glutamate receptors.

3. Electrophysiological properties of IPC neurons

3.1. *Intrinsic electrophysiological properties*

As shown in Table 1 mouse and rat IPC neurons display, irrespectively of the group they belong to, similar values for active and passive membrane electrophysiological parameters. However, a lower membrane capacitance and a higher firing frequency were observed in IM cells in comparison with medial and lateral IPC islands. Interestingly, similar values for most of these parameters have been reported in the guinea pig (Royer and Paré 2002, 2003; Royer et al., 1999; 2000a, 2000b). Consistent with its role as a specialized group of GABAergic cells IPC neurons also differ from typical BLA GABAergic interneurons and GABAergic capsular CeA neurons (Table 1). Acordingly, IPC cells display higher input resistance, lower membrane capacitance and wider action potentials. They also show lower firing frequency than BLA interneurons and capsular CeA cells.

3.2. *Slowly deactivating voltage-dependent K^+ conductance*

Work from Paré's group in the guinea pig (Royer et al., 2000b) has convincingly shown that IPC neurons are endowed with a voltage-dependent K^+ conductance which inactivates in response to suprathreshold depolarizations rendering these cells hyperexcitable. Thus, episodes of suprathreshold activity will keep IPC neurons in a state of high excitability associated with an increase in input resistance and membrane depolarization. Under these conditions ongoing synaptic activity will be able to trigger orthodromic action potentials or even generate sustained depolarizing plateau potentials capable of supporting long lasting tonic firing (Royer and Paré, 2003; Royer et al., 2000b). See also, Jahnsen and Llinás (1984). Such a K^+ conductance as we shall see below will be central for fear extinction as they may allow IPC neurons to overcome their mutual inhibitory interactions (see above) and elicit a sustained inhibitory activity on the amygdaloid output in response to CS-related inputs.

4. Chemical neuroanatomy of IPC neurons

4.1. *GABAergic system*

The distribution of chemical markers among the different groups of IPC islands is shown in Table 2. There is a common agreement that in all species studied so far IPC cells with the exception of the large IPC neurons discussed above are GABAergic since they are heavily stained with GABA antibodies (McDonald and Augustine, 1993; Nitecka and Ben-Ari, 1987; Paré and Smith, 1993a, 1994; Pitkänen and Amaral, 1994; Poulin et al., 2008; Smith and Paré, 1994) and seem to express the isoform 67 of the enzyme that synthesizes GABA, the GAD.

In view of the fact that IPC cells are GABAergic and interact among each other within and between IPC clusters it is not surprising that GABA receptors are expressed in the IPC insular system. Thus the presence of α-1 (McDonald and Mascagni, 2004), α-3 (Busti et al., 2011a; Marowsky et al., 2004) and β-2/3 (McDonald and Mascagni, 1996) $GABA_A$ receptor subunits has been demonstrated within the IPC islands by means of

Table 1 – Electrophysiological properties of intercalated paracapsular neurons.

Parameter	Medial IPC	Medial IPC	Medial IPC	Medial IPC	Lateral IPC	IM	BLA Interneurons [a]	Capsular CeA [a]
Passive membrane properties								
RMP (mV)	−86.7±1.2	–	−87.8±1.5	−78±1.6	−78±1.3	−71.2±1.1	−77±2.8	–
R_{in} (MΩ)	552±36	600±52	527+35	657±49	865±31	641+20	341+37	270±19
Membrane τ (ms)	–	27.2±1.8	32.4+2.3	–	–	20.3±0.8	–	22±2.4
C_m (pF)	–	62±1.3	70±0.10	59±3.1	57.1±1.2	30±2	93±7.2	83±7.1
Active membrane properties								
AP amplitude (mV)	–	78±1.6	79±2	68+1.4	67±1.4	75.7±1	70±1.9	82±3.3
AP width (ms)	1.1±0.05	0.84±0.039	0.78±0.04	1.2±0.03	1.7±0.032	0.8±0.05	1.2±0.09	0.60±0.097
Adaptation index	0.74±0.028	0.56±0.045	0.86±0.04	0.78±0.05	0.75±0.03	0.6±0.04	0.8±0.02	0.52±0.050
Max. firing (Hz)	–	28±2.7	29.3±1.9	24.8±1.9	19.9±1.2	37.3±2	45.4±4	25±4.4
Reference	Amir et al. (2011)	Busti et al. (2011a, 2011b)	Geracitano et al. (2007)	Marowsky et al. (2005)		Manko et al. (2011)	Marowsky et al. (2005)	Busti et al. (2011a, 2011b)

Mice were used in all studies excluding that of Amir et al. (2011) which was done in rats. Abbreviations: RMP, Resting membrane potential; R_{in}, input resistance; C_m, membrane capacitance; AP, action potential.
[a] Added for the sake of comparison.

immunohistochemical methods (Table 2). In agreement with these results, in-situ hybridization studies showed β-3 subunit mRNA expression within the IM (Zhang et al., 2004). Consistent with the presence of $GABA_A$ receptors, [^{3}H]flunitrazepam binding (Table 2) was observed to occur within the IPC islands (Zezula et al., 1988) suggesting the existence of benzodiazepine binding sites on at least a population of the $GABA_A$ receptors present in these locations.

In addition, Charles et al. (2001) have disclosed the existence of $GABA_B$ receptors in the IPC islands. Moreover, since IPC neurons might be involved in the anxiogenic effects resulting from the blockade of $GABA_AC$ receptors within the amygdala (Flores-Gracia et al., 2010), and IPSCs were found within CeL following medial IPC neurons stimulation (Delaney and Pankaj, 2001) it might be possible that $GABA_AC$ receptors may indeed exist within at least some IPC cell groups.

4.2. *Glutamatergic system*

A wealth of electrophysiological evidence indicates that IPC neurons are driven by glutamatergic afferents from thalamic (Bissière et al., 2003; Manko et al., 2011; Samson and Paré, 2005), cortical (Berretta et al., 2005; Ghashghaei and Barbas, 2002; Manko et al., 2011; Marowsky et al., 2005; Pinto and Sesack, 2008; Quirk et al., 2003) and perhaps from other unknown sources which activate both alpha-amino-3-hydroxy-5-methyl-4-isoxazilepropionate (AMPA), N-methyl -aspartate (NMDA) (Bissière et al., 2003; Manko et al., 2011; Royer and Paré, 2002, 2003) and metabotropic glutamate receptors (see Table 2).

It is rather interesting that, unlike AMPA and NMDA receptors which seem to be ubiquitously distributed among IPC cells, metabotropic glutamate receptors display a differential distribution (Table 2) indicating that this insular system is not homogeneous and suggesting that each insular group may subserve a distinct functional role within the amygdala. Thus, immunohistochemical work carried out within our group (Pérez de la Mora et al., 2007) has shown that mGluR5 receptors are restricted to the medial IPC islands. Likewise, Ohishi et al. (1995) have shown, using in situ hybridization methods that whereas there is a caudo-rostral gradient for the distribution of mGluR7 receptors in the IPC islands the mGluR4 receptors are mainly restricted to the rostral IPC cell groups.

4.3. *Catecholaminergic system*

IPC islands and IM (Table 2) contain within the amygdala the highest density of dopamine D1 receptors as shown by both immunocytochemistry (Fuxe et al., 2003; Pérez de la Mora et al., 2006, 2007) and receptor autoradiography (Dawson et al., 1988; Scibilia et al., 1992; Weiner et al., 1991), suggesting an important modulatory role of these receptors upon the activity of the whole insular system. Consistent with this role IPC islands are heavily innervated by dopaminergic fibers (Asan, 1993; Fallon and Ciofi, 1992; Fuxe, 1965a, 1965b; Fuxe et al., 2003; Pinard et al., 2008). Interestingly, although IPC islands contain a uniform density of D1 receptors their dopaminergic innervation is not uniform in all the components of the IPC insular system. Thus whereas it is very dense within the rostral and lateral portion of IM it is remarkably poor within its medial and caudal parts (Busti et al., 2011a; Fuxe et al., 2003). Additionally, rostrally located IPC islands are preferentially innervated by dopaminergic terminals (Fuxe et al., 2003). In contrast to the richness of the dopaminergic innervation, only few noradrenergic fibers have been observed targeting the IPC insular system. They seem restricted to the IM (Table 2) where they overlay this structure without forming real synaptic contacts (Fuxe et al., 2003).

4.4. *Serotonergic system*

In spite of the absence of $5HT_{2A}$ receptor immunoreactivity reported by Bombardi (2011) within IPC islands a moderate density of serotonergic axons (Bauman and Amaral, 2005) presumably bearing 5HT transporters (O'Rourke and Fudge, 2006) has been observed targeting all IPC clusters (Table 2). In view of the prominent role that IPC islands and serotonergic neurons (Akimova et al., 2009; Cools et al., 2008) play in the modulation of anxiety a thorough intra-amygdaloid mapping of 5HT receptor types paying attention to their distribution on IPC islands is warranted.

4.5. *Cholinergic system*

Choline acetyl transferase immunoreactive dendrites have been reported by Nitecka and Frotscher (1989) entering into IPC clusters. Furthermore, their electron microscopic studies have shown that these cholinergic-like dendrites make synaptic contacts with GAD-imunoreactive terminals of presumably IPC neurons (Nitecka and Frotscher, 1989). This finding, although still awaiting confirmation is of considerable physiological interest since it suggests that by this way IPC neurons may influence cognitive cortical activity. In line with this finding is the discovery by Paré and Smith (1994) that IPC neurons from the anterior group contribute fibers into the basal forebrain where they may interact with native cholinergic neurons.

4.6. *Neuropeptides*

Since considerable experimental evidence supports the notion that CeA and IPC islands represent the ventral extension of the dorsal striatum (Busti et al., 2011a; Kaoru et al., 2010; Millhouse, 1986; Swanson and Petrovich, 1998) it is not surprising that both IPC and CeA neurons will also express a complex neuropeptide array (see Cassell et al., 1999).

4.6.1. *Opioid peptides*

As in the case of GABA and dopamine D1 receptors, a high density of μ-opioid receptors has been demonstrated by both immunohistochemistry and in situ hybridization within all groups of IPC cells (Jacobsen et al., 2006; Poulin et al., 2006). In contrast, methionine and leucine- enkephalin immunoreactivities were found mainly restricted to the IM and to lateral IPC islands. β-endorphin was practically absent from IPC neurons (Jacobsen et al., 2006). Interestingly, a mismatch between enkephalin- and μ-opioid receptor-imunoreactivity was observed by Jacobsen et al. (2006) within the IM and the lateral IPC islands. Thus, whereas a strong μ opioid-1 receptor immunoreactivity was present throughout all the IPC insular system enkephalin-imunoreactive terminals were preferentially innervating the medial IPC islands and targeting the rostromedial portion of the IM.

4.6.2. *Cholecystokinin (CCK)*

As in other regions of the amygdala, CCK-8 is widely represented within the IPC neurons. In line with the presence of CCK-8-immunoreactive somata within the GABAergic IPC islands (Micevych et al., 1988) and the expression of CCK mRNA (Pu et al., 1994) in practically all IPC cell groups work from our group (Pérez de la Mora et al., 2007) has disclosed CCK/gastrin immunoreactive terminals targeting most, if not all, IPC islands. It is however not known whether dopamine and CCK/gastrin may coexist within the same dopamine terminals as described by Hökfelt et al. (1980, 1988) in CeA and nucleus accumbens.

4.6.3. *Other neuropeptides*

Evidence for the presence of enkephalin, neurotensin, somatostatin and corticotrophin releasing factor in IPC cells is now available suggesting the coexistence within these neurons of neuropeptides with GABA (Table 2). Particularly interesting in this regard are the studies of Moga and Gray (1985) who by using a combined retrograde transport-immunofluorescence method uncover the existence of peptidergic efferents from the anterior IPC neurons to the parabrachial neurons containing neurotensin, corticotrophin-releasing factor and somatostatin.

4.7. *Transcription factors*

Recent studies by Kaoru et al. (2010) have shown that IPC neurons are endowed with a specific set of transcription factors which support the idea that IPC islands represent a ventral extension of the dorsal striatum. In addition, they have also provided evidence which shows that IPC islands are heterogeneous demonstrating that one of those factors, Pax6, is only present within IM.

4.8. *Neurotransmitter-receptor mismatches within IPC neurons*

Mismatches between the anatomical distribution of a particular neurotransmitter and its receptor are very well established phenomena which have been analyzed in a number of cases (see Agnati and Fuxe, 2000; Elde et al., 1995; Fuxe and Agnati, 1991 for a review of this topic) and in particular for monoamine and peptide systems (Jansson et al., 2002). As indicated above, a mismatch between dopamine terminals and dopamine D1 receptor has been uncovered at the level of the IM where although dopamine D1 receptors are distributed throughout the IPC islands the dopaminergic innervation is mainly restricted to its rostrolateral part (Fuxe et al., 2003). In addition, it was also discovered that whereas the μ-opioid receptor-1 is present in all IPC cell groups enkephalin-immunoreactive terminals were only detected in the rostromedial portion of the IM, and the rostrolateral IPC masses. Under these conditions it is feasible that both the amygdaloid D1 mediated dopaminergic and the enkephalinergic effects on IPC islands function will occur through a concerted volume transmission (VT) including an extrasynaptic subtype and conventional synaptic transmission. In VT dopamine as a result of convective forces and concentration gradients can reach and activate extrasynaptic high-affinity DA receptors (Barbour and Häusser, 1997; Marcellino et al., 2011; Rice and Cragg, 2008; Zoli et al., 1999). In support of these results, microdialysis studies have shown that nmolar extracellular dopamine concentrations exist within the amygdala under basal conditions (Hori et al., 1993; Young and Rees, 1998) and its extracellular concentration is considerably increased under stress exposure (Yokoyama et al., 2005). Moreover, it would be conceivable that owing to the differential properties of both neurotransmitter modes (see Agnati et al., 2010; Fuxe et al., 2010), and in particular to their time effects and to their different type of connectivity that each mode of transmission would have a particular role in the modulation of fear and anxiety (Pérez de la Mora et al., 2008). Thus, whereas synaptic transmission would be responsible of immediate behavioral responses VT would be ideally suited to mediate long-term events like the maintenance of basal anxiety and the modulation of plasticity in the fear circuits. However, the extrasynaptic subtype of VT is more rapid and develops

Table 2 – Chemical neuroanatomy of IPC neurons [a].

Marker	Species	Cell group				Reference
		Medial	Lateral	Main island	Anterior	
GABA						
GABA-ir (somata and fibers)	Rat	+	+	+	+	Nitecka and Ben-Ari, 1987
	Cat	+	+	+	+	Paré and Smith, 1993a, 1993b, 1994
	Monkey	+	+	+	+	McDonald and Augustine, 1993
$GABA_A$ receptor subunit α_1-ir	Rat	–	–	+	–	McDonald and Mascagni, 2004
$GABA_A$ receptor subunit α_3-ir	Mice	+	+	+	–	Busti et al., 2011a; Marowsky et al., 2004
$GABA_A$ receptor subunit β_2/β_3-ir	Rat [b]	–	–	–	–	McDonald and Mascagni, 1996
	Monkey [b]	–	–	–	–	
$GABA_A$ receptor subunit β_3 (in situ hybridization)	Rat	–	–	+	–	Zhang et al., 2004
Benzodiazepine receptor [^{3}H]flunitrazepam binding	Human [b]	–	–	–	–	Zezula et al., 1988
$GABA_B$ receptor-ir	Rat [b]	–	–	–	–	Charles et al., 2001
Glutamate						
mGluR5-ir	Rat	+	–	–	–	Pérez de la Mora et al., 2006
MGluR4 mRNA (in situ hybridization)	Rat	–	–	–	+	Ohishi et al., 1995
MGluR7 mRNA (in situ hybridization)	Rat	+	+	+	+	Ohishi et al., 1995
Dopamine						
DA TH(+) DBH(–) ir (fibers and terminals)	Rat	+	+	+	+	Asan, 1993; Fallon and Ciofi, 1992; Fuxe, 1965a, 1965b; Fuxe et al., 2003; Pinard et al., 2008
D1 receptor binding	Rat	+	+	+	+	Dawson et al., 1988; Scibilia et al., 1992
D1 receptor-ir (fibers and terminals)	Rat	+	+	+	+	Fuxe et al., 2003
D1 mRNA (in situ hybridization)	Rat	+	+	+	+	Mansour and Watson, 1995; Weiner et al., 1991
D2 receptor binding	Rat	–	–	+	–	Scibilia et al., 1992
Noradrenaline						
DBH-ir (fibers and terminals)	Rat	–	–	+	–	Fuxe et al., 2003
Serotonin						
5-HT-ir (fibers and terminals)	Monkey	–	–	–	+	Bauman and Amaral, 2005
5-HT transporter-ir (fibers and terminals)	Monkey	+	+	+	+	O'Rourke and Fudge, 2006
5 HT_{2A}-ir (fibers and terminals)	Rat	–	–	–	–	Bombardi, 2011
Acetylcholine						
ChAT-ir (dendrites)	Rat	+	+	+	+	Nitecka and Frotscher, 1989
Cholecystokinin						
CCK/gastrin-ir (fibers and terminals)	Rat	+	+	+	?	Pérez de la Mora et al., 2007
CCK-ir (somata)	Rat	+	+	+	+	Micevych et al., 1988
CCK mRNA	Rat	–	–	–	+	Pu et al., 1994
Opioids						
Opioid receptor-ir	Rat	+	+	+	+	Jacobsen et al., 2006; Mansour and Watson, 1995
μ-Opioid mRNA (in situ hibridization)	Rat	+	+	+	+	Poulin et al., 2006
Enkephaline-ir	Rat	–	+	+	–	Jacobsen et al., 2006
β-Endorphin-ir	Rat	–	–	–	–	Jacobsen et al., 2006
Other neuropeptides						
[^{125}]Neurotensin	Human [b]	–	–	–	+?	Lantos et al., 1996
Neurotensin (retrograde transport-immunofluorescence)	Rat	–	–	–	+	Moga and Gray, 1985
CRF (retrograde transport-immunofluorescence)	Rat	–	–	–	+	Moga and Gray, 1985
Somatostatin (retrograde transport-immunofluorescence)	Rat	–	–	–	+	Moga and Gray, 1985

Table 2 (continued)

Marker	Species	Cell group				Reference
		Medial	Lateral	Main island	Anterior	
NPY	Monkey	–	–	–	–	McDonald et al., 1995
Calbindin 28K	Human	–	–	–	–	Sorvari et al., 1996
Transcription factors						
Dix 5; Foxp2; Pbx3; Meis2 mRNA (in situ hybridization)	Monkey	+	+	+	+	Kaoru et al., 2010
Pax6 mRNA (in situ hybridization)	Monkey	–	–	+	–	Kaoru et al., 2010
Ebfl; Nkx2; Foxp1 mRNA (in situ hybridization)	Monkey	–	–	–	–	Kaoru et al., 2010

[a] Based on information specifically stated by the authors or gathered from figures in the respective paper. A minus sign means non-detectable or very low density marker.
[b] No information available on differential distribution.

close to the synapses as a result of synaptic spillover and/or extrasynaptic release.

5. Heterogeneity of IPC cell groups

It is clear from the foregoing discussion that even though at the first glance, the IPC insular system seems rather homogeneous owing to the cytological features of their component neurons, their intrinsic electrophysiological properties, their common GABAergic nature and high density of dopamine D1 and μ-opioid receptors there exists a considerable heterogeneity among the different IPC insular groups and even between neurons from the same cluster.

Thus, from a structural point of view, although the vast majority of IPC cells within the medial and lateral groups are small-sized and have bipolar shape neurons within the IM and the anterior group display a multipolar appearance (Manko et al., 2011; Millhouse, 1986; Paré and Smith, 1994). Furthermore, within all IPC clusters, as discussed before, there exist intriguing large (Millhouse, 1986) non GABAergic neurons having a phenotype which differs markedly from that of all other IPC cells (Busti et al., 2011a, 2011b).

In addition, IPC neurons, apart from the large cells considered above, display some different biochemical markers (Table 2) and seem to differ from each other in their connectivity since whereas some of them remain immersed within the amygdala limits, interconnecting IPC cells and serving as inhibitory interfaces; others project into extra-amygdaloid targets (Busti et al., 2011a; Geracitano et al., 2007; Millhouse, 1986; Paré and Smith, 1994; Royer et al., 2000a).

Finally, from a functional point of view IPC neurons seem to differ also in displaying a different short-term plasticity. Paired recordings between visually identified neighboring IPC neurons within a single IPC cluster disclosed the presence of activity-dependent short-term plasticity within medial IPC islands (Geracitano et al., 2007). Thus, upon repeated stimulation within a frequency range similar to that found in vivo the probability of neurotransmitter release was either increased, decreased or remained constant as a consequence of a differential presynaptic activity. In the connectivity context it is rather interesting that a presynaptic neuron responsible of a given synaptic strength forms synaptic connections with different neurons and, conversely that the same postsynaptic neuron was contacted by different types of synaptic connections formed by different presynaptic cells. Furthermore, this functional heterogeneity is further supported by the finding that presynaptic neurons forming synapses having a different synaptic weights display a different axonal pattern. Thus, it was observed that axons from presynaptic neurons forming depressing synapses travel through or around the CeA toward the internal capsule or the stria terminalis. Axonal collaterals of these neurons seem to reach the IPAC and the striatum. CeA is instead innervated only by neurons forming facilitating and constant synapses. Axons forming all types of contacts send numerous collaterals in ventral and caudal directions, which interact with other IPC neurons located within the intermediate capsule. Axonal collaterals from neurons forming all three types of synapses reach the MeA but not the BLA, which was practically devoid of axonal collaterals. In addition, as we shall discuss below Busti et al. (2011a) taking advantage of the use of the early gene zif268 expression to signal activity in neurons have given evidence that whereas medial IPC neurons are activated during fear expression neurons from the IM enhance their activity upon extinction training.

6. Role of intercalated paracapsular islands in the oscillatory activity of the amygdala

Fear learning and extinction are strongly dependent upon a heavy depolarization capable to induce NMDA-dependent potentiation or depression of selected inputs within the amygdala including BLA inputs to IPC neurons (Royer and Paré, 2002).

However, as pointed out by Pape and Paré (2010), it is unlikely that such depolarizing conditions will be met in vivo during fear conditioning since the high depolarizing responses attained in LA during CS presentation (Quirk et al., 1995) become progressively attenuated reaching preconditioning levels at the moment of the US presentation. Nevertheless, as also suggested by these authors (Pape and Paré, 2010), the depolarization level required to induce this kind of long-term plasticity may arise as a consequence of the

induction of oscillatory activity within the BLA. Under these conditions the generation of short recurring periods of depolarization may allow the induction of NMDA-dependent plasticity without the need of sustained firing rates.

In support of this, it has been observed that BLA neurons have the intrinsic potential to generate rhythmic activity both in the theta and gamma frequency ranges (Pape et al., 1998) and that their discharges become synchronized in the theta frequency under anxiogenic conditions which involves the anticipation of noxious stimuli (Paré and Collins, 2000). Additionally synchronization of amygdalo-hippocampal activity at theta frequency has also been observed during conditioned fear responses (Narayanan et al., 2007; Pape et al., 2005; Seidenbecher et al., 2003).

IPC neurons might influence fear conditioning and fear extinction by affecting the induction of theta rhythm in the amygdala and its synchronization with other cortical pathways through a feedforward inhibition-dependent modulation of the time window within which the summation of excitatory stimuli elicits firing (Pouille and Scanziani, 2001). IPC neurons may also through the same mechanism contribute to the generation of faster rhythmic activity within the gamma frequency range in BLA neurons which may be instrumental for their own and their synchronization with intra- and extra-amygdaloid target neurons (Pape and Paré, 2010).

7. Role of IPC neurons in the amygdaloid modulation of fear and anxiety

7.1. *Role of IPC islands in conditioned fear*

The amygdala plays a role of paramount importance in the modulation of conditioned and unconditioned fear/anxiety. Conditioned fear is an experimental model developed by Pavlov whereby organisms learn to predict danger (Maren and Quirk, 2004). In this paradigm a previously neutral stimulus (CS) like a sound or a light acquires aversive properties when it is paired to a noxious stimulus (US), usually a footshock. Conditioned Freezing (Fanselow, 1980) and Fear Potentiated Startle (Davis, 1990) have been thoroughly used to study fear conditioning. In a related paradigm termed Fear Extinction the CS is presented repeatedly but without being coupled to the US. As a result, the animal stops associating CS to US and fear vanishes. It is worth mentioning that extinction training does not erase fear memories but creates a new extinction memory, which competes with fear memory for the control of behavior (for a review, see Herry et al., 2010).

As indicated before according to the prevalent theory on fear/anxiety (LeDoux, 2000, 2007) information on emotionally significant stimuli reaches BLA and particularly LA where the CS–US association takes place. The new information is subsequently conveyed into the CeM for the implementation of a proper anxiogenic response.

At the heart of this theory is the postulate that LA is the primary site of plasticity for fear conditioning since it is there where the CS and US meet and NMDA-dependent associative learning takes place (Goosens and Maren, 2004; LeDoux, 2000; Maren and Quirk, 2004; Paré and Collins, 2000). However, since anatomical studies have indicated that connections from LA to CeM are sparse (Krettek and Price, 1978; Pitkänen and Amaral, 1998; Pitkänen et al., 1995; Smith and Paré, 1994) and in all probability incapable of supporting direct information transfer from LA to CeM the communication between both nuclei should be indirect (Fig. 3).

To save this gap, IPC islands and specifically the medial group which is interposed between LA and CeA have been proposed as an interface between both nuclei (Ehrlich et al., 2009; LeDoux, 2007; Pape and Paré, 2010; Paré et al., 2004; Pérez de la Mora et al., 2008). As discussed above the connectivity of this group of islands is topographically organized in such a way that in the rat and mouse dorsally-located IPC islands receive glutamatergic afferents from LA and project to CeL eliciting feedforward inhibition upon local GABAergic neurons (Amir et al., 2011; Royer et al., 1999, 2000a). In contrast, ventrally-located islands are reached by BL afferents and evoke a tonic feedforward inhibition upon CeM output neurons (Amir et al., 2011; Royer et al., 1999, 2000a). Moreover, in guinea pig (Royer et al., 2000a) but also likely in the rat (Amir et al., 2011) and mouse (Busti et al., 2011a) IPC neurons are polarized in a dorso-ventral direction in such a way that dorsally located clusters (Fig. 3) would inhibit those islands located more ventrally (Amir et al., 2011) including the IM (Busti et al., 2011a; Manko et al., 2011). As a consequence of this arrangement (Fig. 3) dorsally located IPC islands will be able to set free CeM output neurons by releasing them of the inhibitory tonic constraint imposed to them by ventral IPC islands (Paré et al., 2004).

Since from an anatomical point of view the BM is the main LA target and BL is also receiving large number of afferents from this nucleus (Krettek and Price, 1978; Pitkänen et al., 1997; Smith and Paré, 1994) and both project into CeM (Amano et al., 2010; Paré et al., 1995; Pitkänen et al., 1997; Royer et al., 1999) their concurrent participation to fill up the gap between LA and CeM is a concurrent possibility. In support of this, BM and BL are activated during fear conditioning (Amano et al., 2011; Herry et al., 2008) and importantly BM responding outlasts for a significant period of time both the duration of the CS-evoked response in LA neurons which only last for 15 ms from the onset of the tone (Maren and Quirk, 2004) and the end of CS presentation which mirrors the time-course of the freezing response (Amano et al., 2011). This suggests that BM firing is sustained by a different intra- or extra-amygdaloid input. Experimental evidence suggests that a likely candidate for this response may be the prelimbic cortex (PL) of the mPFC since it keeps connections with BM (McDonald, 1998) and displays a CS-induced activation which also parallels in time the CS-induced freezing response (Burgos-Robles et al., 2009). A closer examination of the results from experiments in which fear conditioning was followed by its subsequent extinction disclosed a neural switch which allows changes between low and high states of anxiety within the basal nucleus (BA) (Herry et al., 2008). Thus, it was found that a subset of neurons ("fear neurons") which respond to CS with an increased firing during and after fear conditioning became inhibited following extinction training. In contrast, a separate cell population ("extinction neurons") remained silent during and after fear conditioning but was activated following extinction retrieval. Similar

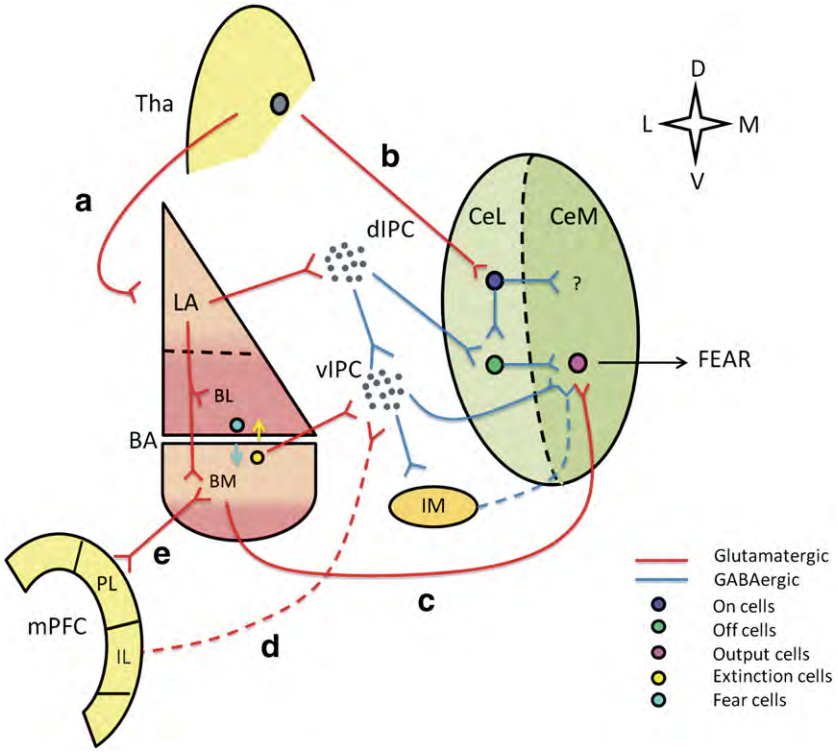

Fig. 3 – Schematic representation of the flow of information within the amygdala during fear conditioning and extinction. Although there are differences in the effects of BL and BM in both phenomena their contribution is depicted as resulting from the combined action of these nuclei. Either activation or dishinibition of CeM output neurons is considered to elicit axiogenic responses. GABAergic and glutamatergic pathways are depicted in blue and red respectively. Cells with a suggested particular function on fear/extinction are indicated as circles having different colors. Paths carrying information on fear conditioning and extinction are indicated by full and broken lines respectively. For facility the sequence of events during the description of the figure is indicated by small letters. The orientation of the scheme is indicated by the cross (A, anterior; P, posterior; L, lateral; M, medial). Putative thalamic glutamatergic afferents carrying information about the properties of CS and US reach LA (a) where their association will result in the formation of NMDA-dependent plasticity. Since afferents from LA to CeM are scarce the information resulting from the local plasticity will reach CeM output neurons via medial IPC cells distributed within the intermediate capsule in a dorsal and a ventral subgroup. As a result of this, dorsally located (dIPC) IPC islands receiving LA inputs will project to CeL inhibiting local GABAergic neurons and releasing in this way CeM output neurons. In addition, dorsal IPC islands may inhibit neurons from the ventral group thereby releasing also CeM output neurons. Thus, the net effect of LA-induced activation of dorsally located IPC neuron will be the production of anxiogenic responses. Alternatively, or in concert with these effects glutamatergic inputs from putative BA "fear neurons" which may have direct excitatory connections with CeM output cells (c) will also result in the production of anxiety. Finally, since NMDA-dependent plasticity has also been demonstrated in CeA, it is likely that information on CS and US association may reach CeL via thalamic afferents (d). Thus, following local CS–US associations, "on cell "activation will be followed by "off cell" inhibition and the ensuing release of CeM output neurons. After extinction training the concurrent interaction of infralimbic and BLA inputs on ventrally located medial IPC cells (d) will elicit feedforward inhibition of CeM output neurons with the subsequent suppression of anxiogenic responses. Activity in IM has been observed following extinction training. For the sake of clarity the anxiogenic PL–BA interactions (e) have only been indicated. Abbreviations: BA, basal nucleus; BL, basolateral nucleus; BM, basomedial nucleus; CeL, centrolatera nucleus; CeM, centromedial nucleus; CS, conditioned stimuli; US, unconditioned stimuli; dIPC, dorsal intercalated paracapsular islands; IL, infralimbic cortex, IM, main intercalated island; mPFC, medial prefrontal cortex; PL, prelimbic cortex; Tha, thalamus; vIPC, ventral intercalated paracapsular islands.

neuronal populations were also found in the rat BM albeit the number of "extinction neurons" was found to be lower (Amano et al., 2011). On these grounds, "fear neurons" might be responsible to activate CeM output neurons following associative learning within the BLA or after PL stimulation. However, evidence for an anatomical link between both LA and PL inputs to these neurons is still lacking.

Finally and interestingly, convincing studies from Ciocchi et al. (2010) have shown that 24 h after fear conditioning a subset of CeL GABA neurons (CEL_{on}) acquired excitatory responses to CS presentation whereas another pool of neurons (CEL_{off}) within the same subnucleus displayed inhibitory responses. Reciprocal inhibitory interactions between both pools of neurons were also reported. In addition, CeA (Wilensky et al.,

2006) and more specifically its capsulateral portion (Ciocchi et al., 2010) seem to represent a parallel or complementary site for fear plasticity (see also Killcross et al., 1997) and CeM was found to be under the control of inhibitory CeL neurons (i.e. CEL_{off} neurons) (Cassell et al., 1999; Ciocchi et al., 2010; Huber et al., 2005; Sun et al., 1994; Veinante et al., 2003). In view of the above, it was suggested (Ciocchi et al., 2010) that glutamatergic thalamic inputs to CEL_{on} neurons carrying information on CS would inhibit CEL_{off} cells with the consequent increase in the anxiogenic output of the amygdala. Consistent with these results CEL_{off} neurons were found to fully overlap with a population of CeL neurons that express PKC-δ and most importantly with an enhancement of freezing behavior after its genetic silencing (Haubensak et al., 2010). These results were confirmed by Duvarci et al. (2011). However, since in their work the proportion of CEL_{off} cells 24 h after fear conditioning tripled with no change in the number of CEL_{on} cells the potentiation of an extrinsic inhibitory input, such as the dorsal cluster of the medial IPC cell group capable of inhibiting CEL_{off} cells was suggested as responsible for the inhibition of CEL_{off} neurons after CS presentation. An overview of the events leading to fear conditioning is depicted in Fig. 3.

7.2. *Role of IPC islands in fear extinction*

In view of the fact that fear extinction does not erase fearful CS–US associations but instead creates a new inhibitory association which competes with the original CS–US association its mechanism is not just the reversal of fear conditioning. On the other hand, given that increased activity in CeM neurons is responsible for conditioned freezing (Ciocchi et al., 2010) and other manifestations of anxiety (Davis and Whalen, 2001) the ultimate goal in fear extinction will be the long-lasting suppression of CeM activity.

Converging evidence points out to IL, a subregion of mPFC, and IPC islands as important substrates for fear extinction (for a review see: Herry et al., 2010; Paré et al., 2004; Quirk and Mueller, 2008; Sotres-Bayon and Quirk, 2010). Accordingly, IL lesions (Morgan et al., 1993; Quirk et al., 2000) or temporary inactivation of IL but not of the adjacent PL by local application of muscimol (Laurent and Westbrook, 2009) interferes with the expression of extinction. In line with this, the electrical stimulation of IL reduces freezing (Milad and Quirk, 2002). Particularly interesting in this regard is the finding that whereas microstimulation of IL favors the consolidation of extinction memory the microstimulation of the PL results in fear underlying the specificity of IL in the consolidation and expression of the extinction memory (Vidal-Gonzalez et al., 2006).

The association of IL activity and fear extinction is further supported by the findings that the presentation of a tone to fear conditioned rats induces high frequency bursting in IL neurons after extinction training (Burgos-Robles et al., 2009) and that animals which have not been trained for extinction when receive conditioned tones paired with a brief electrical shock applied to IL experience less freezing than non electrically stimulated rats (Milad and Quirk, 2002). Furthermore, in rats subjected to auditory fear conditioning and extinction, IL neurons fire to the tone upon extinction retrieval 24 h after extinction training but not before indicating that IL neurons participate in extinction consolidation and retrieval rather than its acquisition (Milad and Quirk, 2002). It is worth mentioning to this respect that the degree of IL neuronal activity has been positively correlated with extinction retrieval (Herry and Garcia, 2002; Milad and Quirk, 2002) indicating as suggested by Paré et al. (2004) that there exists a causal relationship between IL activation and extinction retrieval.

Electrophysiological studies by Quirk et al. (2003) recording extracellularly from brainstem-projecting-CeA neurons showed that stimulation of the mPFC, at the border of the IL and PL cortices, reduced the responsiveness of CeM output neurons to BLA outputs giving evidence that IL is functionally related with CeA and exerts a control on the activity of its output neurons. However, since the anatomical link between IL and CeM is rather poor the connection between both nuclei seems to be indirect and occurs through the intermediation of the medial IPC islands (Quirk et al., 2003) with which IL keeps strong connections (Freedman et al., 2000; McDonald et al., 1996; Vertes, 2004). In agreement with this, early gene expression studies carried out in normal rats (Knapska and Maren, 2009) and mice which had been rescued from impaired extinction with a Zn^{2+} deficient diet, show that the expression of both c-Fos and Zif268 in LA and BLA upon extinction retrieval is elevated (Hefner et al., 2008; Whittle et al., 2010). This change was found to correlate positively with increases in early gene expression within both IL and presumably medial (Hefner et al., 2008; Knapska and Maren, 2009; Whittle et al., 2010) but not lateral IPC islands (Hefner et al., 2008), and negatively with the expression of these genes in CeM.

Given that the above changes correlate with the degree of freezing behavior expressed by the animals upon extinction retrieval (Hefner et al., 2008; Knapska and Maren, 2009; Whittle et al., 2010) they agree with the notion (Quirk et al., 2003; see also Amano et al., 2010; Pape and Paré, 2010; Paré et al., 2004) that combined glutamatergic BLA (Royer et al., 1999) and IL inputs on ventral IPC neurons elicit feedforward inhibition on CeM output neurons thereby reducing the anxiogenic output of the amygdala (Fig. 3). In support of this view it has been shown that IL stimulation is followed by orthodromic responses in IPC neurons (Amir et al., 2011) and in line with the finding that dishinibition of IL neurons by the local injection of picrotoxin induces a large increase on c-fos expression within medial IPC neurons (Berretta et al., 2005) indicating a functional link between IL and medial IPC clusters. In addition it has been demonstrated that extinction training causes a potentiation of BLA inputs to medial IPC islands which is dependent of higher transmitter release probability and changes in postsynaptic $GABA_A$ receptors at IPC synapses (Amano et al., 2010). Interestingly, in the same study it was shown that this potentiation requires IL activity since it was blocked by inactivation of IL neurons with muscimol prior extinction training. Thus, since BLA inputs to medial IPC islands exhibit LTP (Royer and Paré, 2002) it may be possible that converging BLA and IL inputs on IPC neurons will produce enough depolarization to allow in this way the induction of extinction-related plasticity in medial IPC neurons. The role of IPC neurons in extinction has been elegantly studied by Likhtik et al. (2008) which taking advantage of the μ-opioid richness of these neurons have been able to selectively destroy them by the use of a complex formed by a μ-opioid ligand (dermorphin) coupled to a toxin (saporin) capable to be taken up by a receptor mediated

endocytosis process. The results of these experiments conclusively showed that IPC neurons are required for extinction retrieval and that the degree of freezing displayed by lesioned animals is correlated negatively with the number of surviving IPC neurons but not with the number of living CeA cells. In line with these results, the bilateral infusion of the neuropeptide S which increases glutamatergic transmission to IPC neurons facilitates extinction retrieval (Jüngling et al., 2008).

IPC islands, with the exception of a small population of large neurons located at their edges, are populated by GABAergic cells that keep inhibitory interactions among each other both within individual clusters and with IPC cells belonging to other islands giving rise to a "binary" system in which an active inhibitory neuron (1) by inhibiting an adjacent neuron (0) allows activity in the next neuron (1) of a chain of inhibitory neurons. Then, the question of how excitatory inputs in the presence of the inter-IPC inhibiton can sustain either fear conditioning or fear extinction becomes a difficult question to solve. However, experiments by Li et al. (2011) in a model which takes into account the short term plasticity described by Geracitano et al. (2007) and discussed above (see Section 5) and the bistable properties of IPC neurons (Royer et al., 2000b) discussed within the electrophysiological properties of IPC neurons (Section 3) showed that concurrent CS-related BLA and brief IL inputs can overcome the inter-IPC inhibition and elicit sustained activity in IPC neurons which according to their own connectivity may as discussed above induce fear conditioning or fear extinction.

In spite of the fact that the pharmacological inactivation of BL alone (Herry et al., 2008), BL and BM (Amano et al., 2011), and LA or BL (Sierra-Mercado et al., 2011) interferes with extinction the role of the BA nuclei in extinction and its interactions with IPC neurons in this phenomenon are still poorly understood. As indicated above and depicted in Fig. 3 BA nuclei receive a massive projection from LA (Krettek and Price, 1978; Pitkänen et al., 1997; Smith and Paré, 1994) and seem to participate in extinction consolidation and retrieval by eliciting feedforward inhibition on CeM output neurons (Royer et al., 1999) via activation of ventrally located medial IPC islands (Amir et al., 2011). A recent discovery of discrete populations of "fear" and "extinction" neurons has been made by Herry et al. (2008) in BM and confirmed by Amano et al. (2011) within BL which seems to work as a neural switch which allows to transit from a high to a low fear state and viceversa. This indicates that BA is not just a relay station for relaying CS related information to CeM output neurons but an integrative station which will provide us with a number of surprises in the years to come.

From the foregoing discussion, it is clear that fear extinction is also a complex phenomenon the mechanism of which involves, as suggested by Amano et al. (2011), not only LA- and IL-dependent feedforward inhibition of CeM output neurons mediated by medial IPC cells but also another phenomenon. Thus, rapid shifts in synaptic weight take place modulated by "fear" and "extinction" cells within the BA and perhaps also in other amygdaloid regions. Under these grounds it will be possible that behavioral treatments (i.e. Cognitive Behavioral Therapy) based on Wolpe's (1958) systematic desensitization therapy for the behavioral treatment of anxiety disorders in which the gradual exposure to the feared object or situation relives patients from their symptoms may involve the formation of new memories and take advantage of the extinction phenomenon discussed above.

7.3. *Role of medial IPC islands in unconditioned fear/anxiety*

Unconditioned fear/anxiety is a defensive response toward phylogenetically determined stimuli or environmental situations which put in risk an individual's integrity such as the exposure to open spaces devoid of thigmotactic cues, strongly illuminated places or the presence in their vicinity of specific depredators (for a review see, Rodgers, 1997). Information about innate fear/anxiety is wired within the brain and can be experimentally expressed in a number of unconditioned paradigms such as the Elevated Plus-Maze (Pellow et al., 1985), which exploits the natural tendency of rodents to avoid open spaces (Treit et al., 1993), the White and Dark Box test in which animals are exposed to highly environmental lit conditions (Bourin and Hascoët, 2003), the Shock-Probe Burying test that takes advantage of the innate drive of rodents to bury any potentially harmful object in its environment (De Boer and Koolhaas, 2003) or the cat's smell test (Zangrossi and File, 1992) which elicits defensive responses to the potential presence of a predator among other models.

In contrast to conditioned fear little is known about the mechanisms involved in the modulation of unconditioned (innate) fear/anxiety. However, since it has been reported that the intra-amygdaloid administration of a wide variety of neurotransmitter selective drugs modifies the behavior of animals in number of unconditioned tests of anxiety (reviewed in, Engin and Treit, 2008; Pérez de la Mora et al., 2010) and the activity of CeM output neurons is the common final pathway for the expression of fear/anxiety in both types of models, despite of differences in the processing and expression of conditioned vs unconditioned fear/anxiety (Blanchard and Blanchard, 1972; Corcoran and Quirk, 2007), there is agreement upon the participation of the amygdala in the modulation of unconditioned fear/anxiety (LeDoux, 2000). In agreement with this, it is known, since the seminal work of Kluver and Bucy (1939) that innate fear responses such as the aversion of monkeys for snakes, or the fear that these animals experience for individuals of upper hierarchical position within the colony that they belong is disturbed following bilateral lesions of the amygdala. Likewise, deficits in passive avoidance to an electrified probe have been found in rats having large electrolytic amygdala lesions when they were exposed to the Shock-Probe Burying test (Treit and Menard, 1997). In addition, more recently, in line with these early studies it was shown that lesions of specific amygdaloid nuclei also affect unconditioned fear behavior in rats. Specifically, it was found that lesions, which included CeA, MeA and cortical nuclei but spared BLA, blocked both flight behavior in an oval runaway task and defensive responses in a barrel test (Kemble et al., 1990). Interestingly, similar type of lesions impaired fear responses to a predator but had no effects on the responses to conditioned fear (Blanchard and Blanchard, 1972). In line with these results, the functional inactivation of CeA but not BLA by local injections of muscimol was found to impair the avoidance of rats to the open arms of the Elevated Plus-Maze (Moreira et al., 2007). Conversely, BLA excitotoxic lesions (Wallace and Rosen, 2001) or functional BLA

inactivation with muscimol (Ribeiro et al., 2011) impairs conditioned but not unconditioned fear responses. The results of all these studies tend to minimize the role of BLA activity in unconditioned fear/anxiety and underline the participation of the CeA and MeA in its modulation. However, results by Vazdarjanova et al. (2001) suggest that BLA may also have a role in unconditioned fear since local application of lidocain to BLA or its excitotoxic lesion impaired unconditioned freezing and avoidance behaviors in response to a ball of cat hair. On the other hand, results from our group (Pérez de la Mora et al., 2012) have shown that following the blockade of CeA dopamine D2 receptors anxiogenic effects were elicited in the Shock-Probe Burying test but not in the Elevated Plus-Maze which were interpreted as being produced as a consequence of a differential involvement of BLA and CeA in the behavior of animals in both paradigms. Interestingly, as suggested by the work of Blanchard and Blanchard (1972) and that of Kemble et al. (1990) the MeA seems to play an important role in the modulation of unconditioned fear/anxiety. In support of this, the exposure of rats to pieces of a collar previously worn by a cat was found to elicit an increased expression of c-fos within the MeA (Dielenberg et al., 2001). It was also reported by these authors that the functional inactivation of either MeA or BLA interferes with the freezing response to the predator odor trimethylthiazoline. In contrast, whereas the effects of MeA inactivation were found to occur immediately the interference attributable to BLA inactivation showed a 6 min delay suggesting that MeA is directly involved in freezing but that BLA has only a modulatory role on this response.

Like in conditioned fear IPC neurons seem also to play an important role in the modulation of unconditioned fear/anxiety as suggested by its connectivity (Fig. 2) which links them with both CeA- and MeA-mediated responses. This is also indicated, as will be discussed below, by the behavioral effects of intra-amygdaloid infusions of dopamine D1 receptor drugs in unconditioned tests of fear/anxiety.

7.4. *Role of other IPC insular groups in the modulation of anxiety*

In contrast to the medial IPC islands, the role of other IPC cell groups in the integration of fear/anxiety is less understood. Detailed and careful studies by Marowsky et al. (2005) have however paved the road to unveil the relationship of lateral IPC islands with other IPC insular groups and to understand their role in the amygdaloid modulation of anxiety. In this study, by recording from lateral IPC–BLA cell pairs and stimulating lateral IPC afferents within the external capsule Marowsky et al. (2005) found that IPC neurons receive excitatory inputs from putative glutamatergic mPFC afferents and convey feedforward inhibition into BLA projection neurons. Thus, stimulation of capsular fibers elicited excitatory postsynaptic potentials (EPSPs) and resulted in cell firing within lateral IPC islands. In turn, lateral IPC stimulation was found to elicit monosynaptic IPSCs in BLA projection neurons. Furthermore, since the amygdala is kept under a powerful cortical-related inhibitory control (Lang and Paré, 1998; Quirk et al., 2003; Rosenkranz and Grace, 2001, 2002) experiments were designed to assess the contribution of the lateral IPC islands to the total cortical inhibition. The results of such experiments showed that lateral IPC neurons contribute with 70% of the total cortical inhibition underlying the importance of the insular IPC system in the modulation of the output of the amygdala and its consequent participation in the brain integration of fear/anxiety. Furthermore, these results also suggest that lateral IPC neurons may play an important role in the maintenance of the basal levels of anxiety and that changes in their inhibitory tone upon BLA projection neurons will contribute to the intensity and duration of anxiety responses.

On the other hand, particularly interesting to the role of IPC islands in fear-conditioning and extinction is the discovery of Busti et al. (2011a) showing that whereas medial IPC islands are activated during fear expression the IM is selectively turned on during extinction training and retrieval. The mechanism underlying these effects is unknown. However, since the activity of IM neurons can be either enhanced by direct glutamatergic inputs from the MeA or decreased by inhibition from ventrally-located medial IPC islands (Busti et al., 2011a; Manko et al., 2011) their effects on either fear conditioning or extinction will depend on the balance between both types of inputs which in turn will be dependent on the nature of their respective afferents.

Less is even known about the role of the anterior IPC group of islands in the amygdaloid integration of fear and anxiety. However based on the dendritic and axonal patterns of their neurons as well as in their connectivity it can be surmised that they may influence the activity of extra-amygdaloid regions including widespread cortical regions through projections to the basal forebrain (Paré and Smith, 1994).

8. Neurotransmitter interactions with IPC islands

IPC islands differ in their chemical neuroanatomy (Table 2) but are homogeneously rich in dopamine D1 and μ-opioid-1 receptors. Thus, although in principle several neurotransmitter systems may influence their activity only the effects of the dopaminergic system and particularly those mediated by D1 receptors have been studied (see Pérez de la Mora et al., 2010 for a review).

8.1. *Electrophysiological studies*

Work from Marowsky et al. (2005) has shown that unlike local BLA interneurons which are stimulated by dopamine (see Kröner et al., 2005; Rosenkranz and Grace, 2002) IPC cells are inhibited by this neurotransmitter. It was disclosed by these authors that such inhibitory effects take place via a G-protein-mediated activation of inwardly rectifying K^+ channels (GIRK channels) which leads to a hyperpolarization of the IPC neurons associated with a decrease in its input resistance and an inhibition of its firing. Since dopamine is released within the amygdala under stressful conditions (Abercrombie et al., 1989; Inglis and Moghaddam, 1999) and following fear conditioning (Ribeiro de Oliveira et al., 2011; Yokoyama et al., 2005) these results are relevant since as pointed out by Pape (2005) an increase in D1 receptor activity will shift the synaptic balance from inhibition to excitation within the amygdala. In this way, dopamine by allowing activity at the level of BLA and CeA, which

represent the main input and output stations of the amygdala, will enable IPC islands to participate in the modulation of fear and anxiety.

8.2. *Behavioral studies*

A common approach to study the role of any particular brain region in behavior involves the microinfusion of drugs with selected properties within its anatomical limits and look at their behavioral outcome. The anatomical specificity of treatment is however strongly influenced by the diffusion rate of the drug from its site of injection and its relative distribution between its target and adjacent regions having similar types of receptors. Although the small size of IPC islands precludes its injection within the limits of any particular island, the anatomical specificity of the treatment takes advantage of the overwhelming D1 receptor richness of these islands in relation to adjacent amygdaloid regions. As a consequence of this, behavioral experiments aimed to study the participation of the dopaminergic system on the role of IPC islands in anxiety have relied on the diffusion of drugs to IPC islands from adjacent injection sites. Thus, since IPC islands surround BLA and in the case of medial IPC islands these cell clusters are also in vicinity to CeA dopamine drugs infused into BLA or CeA may reach by diffusion the medial IPC insular group or even the IM and exert their behavioral effects by binding to their dopamine D1 receptors. By the same reason, any D1 receptor agonist or antagonist infused into BLA may have effects on lateral IPC islands.

In support of the D1-receptor-mediated disinhibitory effects of dopamine on BLA and CeA activities and the ensuing increase in the anxiogenic output of the amygdala suggested in the experiments of Marowsky et al. (2005) the intra-amygdaloid D1 receptor activation or its blockade resulted in either anxiogenic or anxiolytic effects in conditioned tests of anxiety. Thus, the intra-amygdaloid administration of SCH23390 a selective D1 receptor antagonist (Hyttel, 1983) blocked the acquisition and expression of the fear-potentiated startle (Greba and Kokkinidis, 2000; Lamont and Kokkinidis, 1998). In agreement with these results Guarraci et al. (1999a, 1999b) reported that intra-amygdaloid administration of the same antagonist attenuated conditioned freezing during the retention testing. Finally, SCH23390 decreased second-order conditioning following its intra-amygdaloid infusion (Nader and LeDoux, 1999). In contrast, but consistent with the effects of the antagonists, the intra-amygdaloid administration of selective D1-receptor agonists (SKF82958 and 38393) facilitated freezing responses in the Conditioned Freezing test (Guarraci et al., 1999a, 1999b). Since these results suggest that D1 receptor activity has an anxiogenic role, and as discussed above, activity in IPC neurons is required for both fear conditioning and extinction it is rather intriguing why D1 receptor blockade by SCH23390 infused in the proximity of medial IPC islands was found to impair fear extinction (Hikind and Maroun, 2008).

It is worth mentioning, that in all the experiments discussed above cannulae were not aimed to any IPC insular group but instead either to BLA (Greba and Kokkinidis, 2000; Lamont and Kokkinidis, 1998) or CeA (Guarraci et al., 1999a,1999b). However, since D1 receptor density is by far higher in the IPC islands in comparison to CeA and BLA (Fig. 1) (Fuxe et al., 2003; Scibilia et al., 1992) we assume that the effects of these compounds on anxiety were mainly mediated by the blockade of D1 receptors located on IPC neurons.

The role of IPC islands on the modulation of unconditioned fear/anxiety has also been studied following the same experimental approach but using the White and Black Box test and the Elevated Plus-Maze for the evaluation of behavior. The results obtained so far support the role of IPC neurons in the modulation of unconditioned fear/anxiety. However, unlike the uniformity of effects observed in the conditioned models the effects of dopamine drugs on unconditioned fear/anxiety seem to differ between models and even within the same paradigm.

In agreement with the anxiogenic role of dopamine D1 receptor activity in conditioned fear, our studies (Pérez de la Mora et al., 2005) indicate that the administration of SCH23390 through cannulae aimed to the rostrolateral part of the IM, which contains a high density of dopamine D1 receptors, and to the medial IPC cell group elicited anxiolytic effects in the White and Black Box test. Thus, SCH23390 treatment reduced the natural fear of rats for the illuminated compartment of the White and Black Box and increased the latency to enter into its “safe” dark compartment (Fig. 4). Subsequent studies using the Elevated Plus-Maze showed that the intra-amygdaloid infusion of similar doses of SCH23390 through cannulae aimed mostly to BLA, but that may have allowed diffusion of the dopamine receptor antagonist into medial IPC clusters and elicited anxiolytic (Bananej et al., 2011), anxiogenic (Zarrindast et al., 2011) or no effects (Rezayoff et al., 2009). The reason for these discrepancies is at the present unknown but differences in the neural pathways activated by the anxiogenic stimuli evoked by each test within the amygdala and in the contribution of dopamine D1 receptors to their modulation in different amygdaloid nuclei (for a discussion on that see Pérez de la Mora et al., 2010, 2012) may be involved in the differential effects observed between paradigms. Differences within the same test are more difficult to explain. However, since as discussed above there exists a considerable heterogeneity among the medial IPC islands (Busti et al., 2011a; Geracitano et al., 2007) and medial IPC neurons seem to process stimuli in a spatial–temporal differentiated way (see Royer et al., 2000a) it may be possible that slight differences in the location of the cannulae from one experiment to another may have influenced different IPC clusters (dorsal vs ventral) and hence given different results.

9. Concluding remarks

The evidence reviewed here allows us to conclude that the IPC insular system is a distributed collection of small GABAergic neurons that in spite of its cumulative small size have a significant role on brain function, and particularly, on the amygdaloid modulation of fear/anxiety. IPC neurons are endowed with a unique set of properties which make them especially well suited to fulfill its role as members of an integrative neural system. Such properties, aside of their basic active and passive electrophysiological features include the presence of both a slowly deactivating voltage-dependent K^+ conductance which lets them to fire tonically upon suprathreshold stimulation, and a

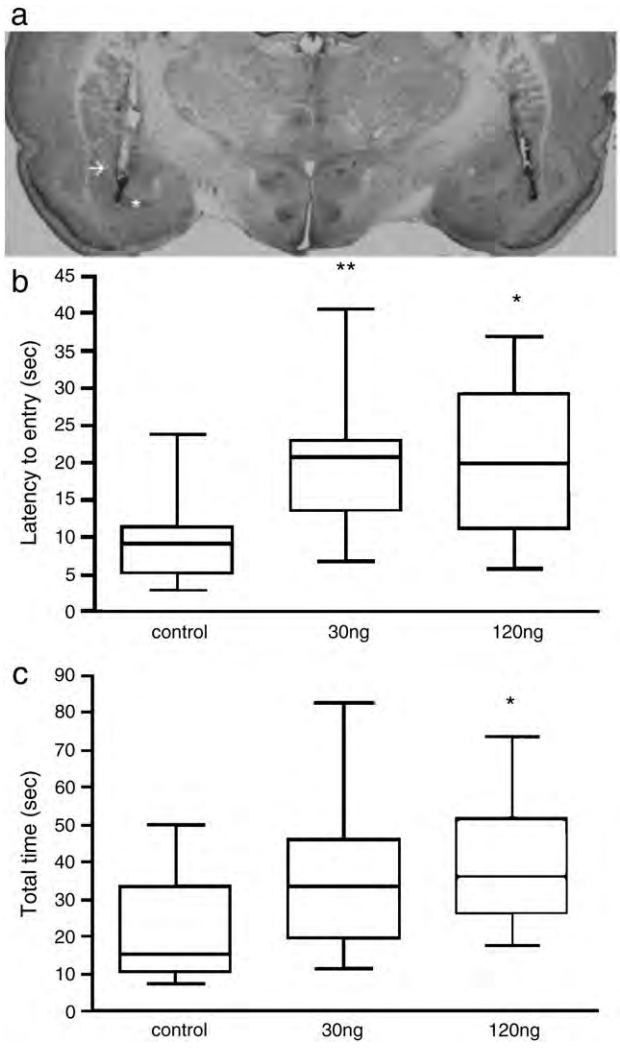

Fig. 4 – Effects of the bilateral intra-amygdaloid administration of SCH23390 on the behavior of rats in the White and Dark Box test. Blockade of dopamine D1 receptors with SCH23390 infused into a region populated by neurons belonging to the main intercalated island and the medial intercalated paracapsular islands (a) resulted in anxiolytic effects as both the latency (b) to enter into the dark compartment of the box and the time spent in its illuminated compartment (c) were increased. Results are expressed (in seconds) as medians with the respective interquartile range (boxes). The whiskers indicate the highest and lowest values within the sample. * $P<0.05$; ** $P<0.01$ against the control. Kruskal–Wallis test followed by Dunn's test comparing SCH23390-treated groups against the control. Control: n=12; SCH23390; 30 ng/side, n=16; SCH23390; 120 ng/side, n=10.
Reproduced from Pérez de la Mora et al. (2005) with permission from Elsevier.

special kind of short-term plasticity that allows the maintenance of total synaptic weight and reliable firing patterns within the IPC islands network. In addition, IPC neurons display a particular type of GIRK channel which allows them to be differentially modulated by certain neurotransmitters, i.e. dopamine, in relation to other types of interneurons within the amygdala. On the other hand, whereas the specific lesion of IPC islands was found to disrupt fear extinction the neuropeptide S-induced glutamate stimulation of these islands results in anxiogenic activity and facilitates the extinction of conditioned fear responses.

It can also be concluded that in spite of the apparent homogeneity of the IPC network there is a considerable heterogeneity among the different IPC groups of islands and even between neurons from the same cluster in terms of axonal and dendritic patterns, connectivity, plasticity and chemical neuroanatomy in such a way that different facets of the same phenomenon such as fear conditioning and extinction engage distinct IPC cell groups. In addition, given that intra- and extra-amygdaloid inputs have been found to reach different IPC islands, the existence of either reciprocal, or polarized inhibitory interactions between islands of the same or different IPC groups is particularly interesting since it provides the insular system with special computational capabilities.

Thus, the above properties combined with the strategical anatomical relationship of IPC islands with the main input (BLA) and output (CeA) stations of the amygdala make this system ideally suited to participate in the amygdaloid modulation of fear and anxiety.

It remains for the future by combining traditional tracing and conventional electrophysiological recording methods with the newly developed optogenetic and calcium imaging techniques to unveil the precise connectivity between IPC neurons. Particularly important in this regard will be to know whether the information flow between the IPC islands from a particular cell group (i.e. medial or lateral IPC islands) or between different IPC islands groups is in fact polarized or not. It will be interesting to know what is the relationship that a particular IPC insular system bears with the different inputs to the IPC insular system. It will be also important by combining this methodology with behavioral methods to further study whether separate IPC subsystems are involved in fear conditioning vs extinction, and whether the IPC insular system is involved in unconditioned fear/anxiety.

It will be equally important as a prelude to the design of novel pharmacological treatments to study by means of conditional and region specific knock-out/knock-in mice the role of locally important neurotransmitter receptors on different aspects of the anxiogenic behavior. Also it is important to gather information on the possible existence of separate IPC systems possessing a distinct chemical and wiring neuroanatomy, as well as to study the nature of the local neurotransmitter receptor–receptor interactions in the IPC islands and their associated plasticity.

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REFERENCES

Abercrombie, E.D., Keefe, K.A., DiFrischia, D.S., Zigmond, M.J., 1989. Differential effect of stress on in vivo dopamine release in striatum, nucleus accumbens, and medial frontal cortex. J. Neurochem. 52, 1655–1658.

Agnati, L.F., Fuxe, K., 2000. Volume transmission as a key feature of information handling in the central nervous system: possible new interpretative value of the Turing's B-Type machine. In: Agnati, L.F., Fuxe, K., Nocholson, C., Syková, E. (Eds.), Volume Transmission Revisited. Elsevier, Amsterdam, pp. 3–19.

Agnati, L.F., Guidolin, D., Guescini, M., Genedani, S., Fuxe, K., 2010. Understanding wiring and volume transmission. Brain Res. Rev. 64, 137–159.

Akimova, E., Lanzenberger, R., Kasper, S., 2009. The serotonin-1A receptor in anxiety disorders. Biol. Psychiatry 66, 627–635.

Amano, T., Unal, C.T., Paré, D., 2010. Synaptic correlates of fear extinction in the amygdala. Nat. Neurosci. 13, 489–494.

Amano, T., Duvarci, S., Popa, D., Paré, D., 2011. The fear circuit revisited: contributions of the basal amygdala nuclei to conditioned fear. J. Neurosci. 31, 15481–15489.

Amir, A., Amano, T., Paré, D., 2011. Physiological identification and infralimbic responsiveness of rat intercalated amygdala neurons. J. Neurophysiol. 105, 3054–3066.

Asan, E., 1993. Comparative single and double immunolabelling with antisera against catecholamine biosynthetic enzymes: criteria for the identification of dopaminergic, noradrenergic and adrenergic structures in selected rat brain areas. Histochemistry 99, 427–442.

Bananej, M., Karimi-Sori, A., Zarridast, M.R., Ahmadi, S., 2011. D1 and D2 dopaminergic systems in the rat basolateral amygdala are involved in anxiogenic-like effects induced by histamine. J. Psychopharmacol. 1–11.

Barbour, B., Häusser, M., 1997. Intersynaptic diffusion of neurotransmitter. Trends Neurosci. 20, 377–384.

Bauman, M.D., Amaral, D.G., 2005. The distribution of serotonergic fibers in the macaque monkey amygdala: an immunohistochemical study using antisera to 5-hydroxytryptamine. Neuroscience 136, 193–203.

Berkelbach van der Sprengel, H., 1926. Stria terminalis and amygdala in the brain of the opossum (*Didelphis virginiana*). J. Comp. Neurol. 42, 211–254.

Berretta, S., Pantazopoulos, H., Caldera, M., Pantazopoulos, P., Paré, D., 2005. Infralimbic cortex activation increases c-Fos expression in intercalated neurons of the amygdala. Neuroscience 132, 943–953.

Bissière, S., Humeau, Y., Lüthi, A., 2003. Dopamine gates LTP induction in lateral amygdala by suppressing feedforward inhibition. Nat. Neurosci. 6, 587–592.

Blair, H.T., Schaffe, G.F., Bauer, E.P., Rodrigues, S.M., Le Doux, J.E., 2001. Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. Learn. Mem. 8, 229–242.

Blanchard, D.C., Blanchard, R.J., 1972. Innate and conditioned reactions to threat in rats with amygdaloid lesions. J. Comp. Physiol. Psychol. 81, 281–290.

Bombardi, C., 2011. Distribution of 5-TH2A receptor immunoreactivity in the rat amygdaloid complex and colocalization with γ-aminobutyric acid. Brain Res. 1370, 112–128.

Bourin, M., Hascoët, M., 2003. The mouse light/dak test. Eur. J. Pharmacol. 473, 55–65.

Braak, H., Braak, E., Yilmazer, D., de Vos, R.A., Jansen, E.N., Bohl, J., Jellinger, K., 1994. Amygdala pathology in Parkinson's disease. Acta Neuropathol. 88, 493–500.

Brodal, A., 1947. The amygdaloid nucleus in the rat. J. Comp. Neurol. 87, 7–16.

Burgos-Robles, A., Vidal-Gonzalez, I., Quirk, G.J., 2009. Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. J. Neurosci. 29, 8474–8482.

Busti, D., Geracitano, R., Whittle, N., Dalezios, Y., Mánko, M., Kaufmann, W., Sätzler, K., Singeward, N., Capogna, M., Ferraguti, F., 2011a. Different fear states engage distinct networks within the intercalated cell clusters of the amygdala. J. Neurosci. 31, 51–5144.

Busti, D., Bienvenu, T., Micklem, B., Magill, P.J., Shigemoto, R., Capogna, M., Ferraguti, F., 2011b. Morphological characterization of large intercalated neurons provides novel insight on intrinsic networks of the amygdale. BMC Pharmacol. 11, A9.

Canteras, N.S., Swanson, L.W., 1992. Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: a PHAL anterograde tract-tracing study in the rat. J. Comp. Neurol. 324, 180–194.

Canteras, N.S., Resstel, L.B., Bertoglio, L.J., Carobrez, A.de P., Guimarães, F.S., 2010. Neuroanatomy of anxiety. Curr. Top. Behav. Neurosci. 2, 77–96.

Cassell, M.D., Gray, T.S., Kiss, J.Z., 1986. Neuronal architecture in the rat central nucleus of the amygdala. J. Comp. Neurol. 246, 478–499.

Cassell, M.D., Freedman, L.J., Shi, C., 1999. The intrinsic organization of the central extended amygdala. Ann. N. Y. Acad. Sci. 29, 217–241.

Charles, K.J., Evans, M.L., Robbins, M.J., Calver, A.R., Leslie, R.A., Pangalos, M.N., 2001. Comparative immunohistochemical localisation of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits in rat brain, spinal cord and dorsal root ganglion. Neuroscience 106, 447–467.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S.B.E., Letzkus, J.J., Vlachos, I., Ehrlich, I., Sprengel, R., Deisseroth, K., Stadler, M.B., Müller, C., Lüthi, A., 2010. Encoding of conditioned fear in central amygdala inhibitory circuits. Nature 468, 277–282.

Cools, R., Roberts, A.C., Robbins, T.W., 2008. Serotonergic regulation of emotional and behavioral control processes. Trends Cogn. Sci. 12, 31–40.

Corbit, L.H., Balleine, B.W., 2005. Double dissociation of basolateral and central amygdala lesions on the general and outcome-specific forms of pavlovian-instrumental transfer. J. Neurosci. 25, 962–970.

Corcoran, K.A., Quirk, G.J., 2007. Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. J. Neurosci. 27, 840–844.

Crosby, E.C., Humphrey, H., 1941. Studies of the vertebrate telencephalon. II. The nuclear patterns of the anterior olfactory nucleus, tuberculum olfactorium and the amygdaloid complex in adult man. J. Comp. Neurol. 74, 309–352.

Davis, M., 1990. Animal models of anxiety based on classical conditioning: the conditioned emotional response (CER) and the fear-potentiated startle effect. Pharmacol. Ther. 47, 147–165.

Davis, M., Whalen, P.J., 2001. The amygdala: vigilance and emotion. Mol. Psychiatry 6, 13–34.

Dawson, T.M., Barone, P., Sidhu, A., Wamsley, J.K., Chase, T.N., 1988. The D1 dopamine receptor in the rat brain: quantitative autoradiographic localization using an iodinated ligand. Neuroscience 26, 83–100.

De Boer, S.F., Koolhaas, J.M., 2003. Defensive burying in rodents: ethology, neurobiology and psychopharmacology. Eur. J. Pharmacol. 463, 145–161.

Delaney, A.J., Pankaj, S., 2001. Pathway-specific targeting of GABA receptor subtypes to somatic and dendritic synapses in the central amygdale. J. Neurophysiol. 86, 717–723.

Dielenberg, R.A., Hunt, G.E., McGregor, L.S., 2001. When a rat smell a cat: the distribution of fos immunoreactivity in rat brain following exposure to a predatory odor. Neuroscience 104, 1085–1097.

Duvarci, S., Popa, D., Paré, D., 2011. Central amygdala activity during fear conditioning. J. Neurosci. 31, 289–294.

Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., Luthi, A., 2009. Amygdala inhibitory circuits and the control of fear memory. Neuron 62, 757–771.

Elde, R., Arvidsson, U., Riedl, M., Vulchanova, L., Lee, J.H., Dado, R., Nakano, A., Chakrabarti, S., Zhang, X., Loh, H.H., Law, P.Y., Hökfelt, T., Wessendorf, M., 1995. Distribution of neuropeptide receptors. New views of peptidergic neurotransmission made possible by antibodies to opioid receptors. Ann. N. Y. Acad. Sci. 757, 390–404.

Engin, E., Treit, D., 2008. The effects of intra-cerebral drug infusions on animals' unconditioned fear reactions: a systematic review. Prog. Neuropsychopharmacol. Biol. Psychiatry 32, 1399–1419.

Everitt, B.J., Cardinal, R.N., Parkinson, J.A., Robbins, T.W., 2003. Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning. Ann. N. Y. Acad. Sci. 985, 233–250.

Fallon, J.H., Ciofi, P., 1992. Distribution of monoamines within the amygdala. In: Aggleton, J.P. (Ed.), The Amygdala: Neurobiological Aspects of Emotion, Memory and Mental Dysfuntion. Wiley-Liss, Inc., New York, pp. 97–114.

Fanselow, M.S., 1980. Conditioned and unconditional components of post-shock freezing. Pavlov J. Biol. Sci. 15, 177–182.

Ferraguti, F., Crepaldi, L., Nicoletti, F., 2008. Metabotropic glutamate 1 receptor: current concepts and perspectives. Pharmacol. Rev. 60, 536–581.

Flores-Gracia, C., Nuche-Bricaire, A., Crespo-Ramírez, M., Miledi, R., Fuxe, K., Pérez de la Mora, M., 2010. $GABA_A$ ρ receptor mechanism in the rat amygdale and its role in the modulation of fear and anxiety. Psychopharmacology 212, 475–484.

Fox, C.A., 1940. Certain basal telencephalic centers in the cat. J. Comp. Neurol. 72, 1–62.

Freedman, L.J., Insel, T.R., Smith, Y., 2000. Subcortical projections of area 25 (subgenual cortex) of the macaque monkey. J. Comp. Neurol. 421, 172–188.

Fuxe, K., 1965a. Evidence for the existence of monoamine neurons in the central nervous system. III. The monoamine nerve terminal. Z. Zellforsch. 65, 573–596.

Fuxe, K., 1965b. Evidence for the existence of monoamine neurons in the central nervous system. IV. Distribution of monoamine nerve terminals in the central nervous system. Acta Physiol. Scand. 64, 39–85.

Fuxe, K., Agnati, L.F., 1991. Transmission in the Brain: Novel Mechanisms of Neuronal Transmission. Raven Press, Ney York.

Fuxe, K., Jacobsen, K.X., Höistad, M., Tinner, B., Jansson, A., Staines, W.A., Agnati, L.F., 2003. The dopamine D1 receptor-rich main and paracapsular intercalated nerve cell groups of the rat amygdala: relationship to the dopamine innervation. Neuroscience 119, 733–746.

Fuxe, K., Dahlström, A., Jonsson, G., Marcellino, D., Guescini, M., Dam, M., Manger, P., Agnati, L., 2010. The discovery of central monoamine neurons gave volume transmission to the wired brain. Prog. Neurobiol. 90, 82–100.

Geracitano, R., Kauffman, W.A., Szabo, G., Ferraguti, F., Capogna, M., 2007. Synaptic heterogeneity between mouse paracapsular intercalated neurons of the amygdala. J. Physiol. 585, 117–134.

Gewirtz, J.C., Davis, M., 1997. Second-order fear conditioning prevented by blocking NMDA receptors in amygdala. Nature 388, 471–474.

Ghashghaei, H.T., Barbas, H., 2002. Pathways for emotion: interaction of prefrontal and anterior temporal pathways in the amygdala of rhesus monkey. Neuroscience 115, 1261–1279.

Goosens, K.A., Maren, S., 2003. Pretraining NMDA receptor blockade in the basolateral complex but not the central nucleus, of the amygdala prevents savings of conditional fear. Behav. Neurosci. 117, 738–750.

Goosens, K.A., Maren, S., 2004. NMDA receptors are essential for the acquisition, but not expression, of conditional fear and associative spike firing in the lateral amygdala. Eur. J. Neurosci. 20, 537–548.

Gray, T.S., 1989. Autonomic neuropeptide connections of the amygdala. In: Tache, Y., Morley, J.E., Brown, M.R. (Eds.), Neuropeptides and Stress. Springer-Verlag, New York, pp. 92–106.

Greba, Q., Kokkinidis, L., 2000. Peripheral and intra-amygdalar administration of the dopamine D1 receptor antagonist SCH23390 block fear-potentiated startle but not shock reactivity or the shock sensitization of acoustic startle. Behav. Neurosci. 114, 262–272.

Guarraci, F.A., Frohardt, R.J., Kapp, B.S., 1999a. Amygdaloid D1 dopamine receptor involvement in Pavlovian fear conditioning. Brain Res. 827, 28–40.

Guarraci, F.A., Frohardt, R.J., Young, S.L., Kapp, B.S., 1999b. A functional role for dopamine transmission in the amygdala during conditioned fear. Ann. N. Y. Acad. Sci. 877, 732–736.

Gurdijan, E.S., 1928. The corpus striatum of the rat, studies on the brain of the rat. No.3. J. Comp. Neurol. 45, 249–281.

Hall, E., 1972. The amygdale of the cat; a Golgi study. Z. Zellforsch. 134, 439–458.

Haubensak, W., Kunwar, P.S., Cai, H., Ciocchi, S., Wal, N.R., Ponnusamy, R., Biag, J., Dong, H.W., Deisseroth, K., Callaway, E.M., Fanselow, M.S., Lüthi, A., Anderson, D.J., 2010. Genetic dissection of an amygdala microcircuit that gates conditioned fear. Nature 468, 270–276.

Hefner, K., Whittle, N., Juhasz, J., Norcross, M., Karlsson, R.M., Saksida, L.M., Bussey, T.J., Singewald, N., Holmes, A., 2008. Impaired fear extinction learning and cortico-amygdala circuit abnormalities in a common genetic mouse strain. J. Neurosci. 28, 8074–8085.

Herry, C., Garcia, R., 2002. Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice. J. Neurosci. 22, 577–583.

Herry, C., Ciocchi, S., Senn, V., Demmou, L., Müller, C., Lüthi, A., 2008. Switching on and off fear by distinct neuronal circuits. Nature 454, 600–606.

Herry, C., Ferraguti, F., Singewald, N., Letzkus, J.J., Ehrlich, I., Luthi, A., 2010. Neuronal circuits of fear extinction. Eur. J. Neurosci. 31, 599–612.

Hikind, N., Maroun, M., 2008. Microinfusion of the D1 receptor antagonist, SCH23390 into the IL but not the BLA impairs consolidation of extinction of auditory fear conditioning. Neurobiol. Learn. Mem. 90, 217–222.

Hökfelt, T., Skirboll, L., Rehfeld, J.F., Goldstein, M., Markey, K., Dann, O., 1980. A subpopulation of mesencephalic dopamine neurons projecting to limbic areas contains a cholecystokinin-like peptide: evidence from immunohistochemistry combined with retrograde tracing. Neuroscience 5, 2093–2124.

Hökfelt, T., Herrera-Marschitz, M., Seroogy, K., Ju, G., Staines, W.A., Holets, V., Schalling, M., Ungerstedt, U., Post, C., Rehfeld, J.F., Frey, P., Fischer, J., Dockray, G., Hamaoka, T., Walsh, J.H., Goldstein, M., 1988. Immunocytochemical studies on cholecystokinin (CCK)-immunoreactive neurons in the rat using sequence specific antisera and with special reference to the caudate nucleus and primary sensory neurons. J. Chem. Neuroanat. 1, 11–51.

Holland, P.C., Hatfield, T., Gallagher, M., 2001. Rats with basolateral amygdala lesions show normal increases in conditioned stimulus processing but reduced conditioned potentiating of eating. Behav. Neurosci. 115, 945–950.

Hopkins, D.A., Holstege, G., 1978. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. Exp. Brain Res. 32, 529–547.

Hori, K., Tanaka, J., Nomura, M., 1993. Effects of discrimination learning on the rat amygdala dopamine release: a microdialysis study. Brain Res. 621, 296–300.

Huber, D., Veinante, P., Stoop, R., 2005. Vasopressin and oxytocin excite distinct neuronal populations in the central amygdale. Science 308, 245–248.

Humphrey, T., 1936. The telencephalon of the bat. I. The neocortical nuclear masses and certain pertinent fiber connections. J. Comp. Neurol. 65, 603–711.
Hyttel, J., 1983. SCH 23390 — the first selective dopamine D-1 antagonist. Eur. J. Pharmacol. 91, 153–154.
Inglis, F.M., Moghaddam, B., 1999. Dopaminergic innervations of the amygdale is highly responsive to stress. J. Neurochem. 72, 1088–1094.
Jacobsen, K.X., Höistand, M., Staines, W.A., Fuxe, K., 2006. The distribution of dopamine D1 receptor and μ-opioid receptor 1 receptor immunoreactivities in the amygdala and interstitial nucleus of the posterior limb of the anterior commissure: relationships to tyrosine hydroxylase and opioid peptide terminal systems. Neuroscience 141, 2007–2018.
Jahnsen, H., Llinás, R., 1984. Ionic basis for the electro-responsiveness and oscillatory properties of guinea-pig thalamic neurones in vitro. J. Physiol. 349, 227–247.
Jansson, A., Descarries, L., Cornea-Hébert, V., Riad, M., Vergé, D., Bancila, M., Agnati, L.F., Fuxe, K., 2002. Transmitter-receptor mismatches in central dopamine, serotonin and neuropeptide systems. Further evidence for volume transmission. In: Walz, W. (Ed.), The Neuronal Environment: Brain Homeostasis in Health and Disease. Humana Press Inc., Totowa, NJ, pp. 83–108.
Jüngling, K., Seidenbecher, T., Sosulina, L., Lesting, J., Sangha, S., Clark, S.D., Okamura, N., Duangdao, D.M., Xu, Y.L., Reinscheid, R.K., Pape, H.C., 2008. Neuropeptide S-mediated control of fear expression and extinction; role of intercalated GABAergic neurons in the amygdala. Neuron 59, 298–310.
Kamal, A.M., Tömböl, T., 1975. Golgi studies on the amygdaloid nuclei of the cat. J. Hirnforsch. 16, 175–201.
Kaoru, T., Liu, F.C., Ishida, M., Oishi, T., Hayashi, M., Kitagawa, M., Shimoda, K., Takahashi, H., 2010. Molecular characterization of the intercalated cell masses of the amygdala: implications for the relationship with the striatum. Neuroscience 166, 220–230.
Kemble, E.D., Blanchard, D.C., Blanchard, R.J., 1990. Effects of regional amygdaloid lesions on flight and defensive behaviors of wild black rats (*Rattus rattus*). Physiol. Behav. 48, 1–5.
Killcross, S., Robbins, T.W., Everitt, B.J., 1997. Different types of fear conditioned behavior mediated by separate nuclei within amygdala. Nature 388, 377–380.
Kishi, T., Sumori, T., Yokota, S., Yasui, Y., 2006. Topographical projections from the hippocampal formation to the amygdala: a combined anterograde and retrograde tracing study in the rat. J. Comp. Neurol. 496 (3), 349–368.
Kluver, H., Bucy, P.C., 1939. Preliminary analysis of functions of the temporal lobes in monkeys. Arch. Neurol. Psychiatry 42, 979–1000.
Knapska, E., Maren, S., 2009. Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. Learn. Mem. 16, 486–493.
Krettek, J.E., Price, J.L., 1978. Amygdaloid projections to subcortical structures within the basal forebrain and brain stem in the rat and cat. J. Comp. Neurol. 178, 225–254.
Kröner, S., Rosenkranz, J.A., Grace, A.A., Barrionuevo, G., 2005. Dopamine modulates excitability of basolateralamygdala neurons in vitro. J. Neurophysiol. 93, 1598–1610.
Lamont, E.W., Kokkinidis, L., 1998. Infusion of the dopamine D1 receptor antagonist SCH 23390 into the amygdala blocks fear expression in a potentiated startle paradigm. Brain Res. 795, 128–136.
Lang, E.J., Paré, D., 1998. Synaptic responsiveness of interneurons of the cat lateral amygdaloid nucleus. Neuroscience 83, 877–889.
Lantos, T.A., Palkovits, M., Rostène, W., Bérod, A., 1996. Neurotensin receptors in the human amygdaloid complex. Topographical and quantitative autoradiographic study. J. Chem. Neuroanat. 11, 209–217.
Lauer, E.W., 1945. The nuclear pattern and fiber connections of certain basal telencephalic centers in the macaque. J. Comp. Neurol. 82, 215–254.
Laurent, V., Westbrook, R.F., 2009. Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. Learn. Mem. 16, 520–529.
LeDoux, J.E., 2000. Emotion circuits in the brain. Annu. Rev. Neurosci. 23, 155–184.
LeDoux, J.E., 2007. The amygdala. Curr. Biol. 17, 868–874.
LeDoux, J.E., Ciccetti, P., Xagoraris, A., Romanski, L.M., 1990. The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. J. Neurosci. 10, 1062–1069.
Li, G., Amano, T., Paré, D., Nair, S.S., 2011. Impact of infralimbic inputs on intercalated amygdala neurons. A biophysical modeling study. Learn. Mem. 18, 226–240.
Likhtik, E., Popa, D., Apergis-Schoute, J., Fidacaro, G.A., Paré, D., 2008. Amygdala intercalated neurons are required for expression of fear extinction. Nature 454, 642–645.
Luiten, P.G., Ono, T., Nishijo, H., Fukuda, M., 1983. Differential input from the amygdaloid body to the ventromedial hypothalamic nucleus in the rat. Neurosci. Lett. 35, 253–258.
Manko, M., Geracitano, R., Capogna, M., 2011. Functional connectivity of the main intercalated nucleus of the mouse amygdala. J. Physiol. 589, 1911–1925.
Mansour, A., Watson Jr., S.J., 1995. Dopamine receptor expression in the central nervous system. In: Bloom, F.E., Kupfer, D.J. (Eds.), Psychopharmacology IV Generation of Progress. Raven Press, New York, pp. 207–219.
Marcellino, D., Kehr, J., Agnati, L.F., Fuxe, K., 2011. Increased affinity of dopamine for D_2 like versus D_1-like receptors. Relevance for volume transmission in interpreting PET findings. Synapse 66, 196–203.
Maren, S., Quirk, G.J., 2004. Neuronal signalling of fear memory. Nat. Rev. Neurosci. 5, 844–852.
Marowsky, A., Fritschy, J.M., Vogt, K.E., 2004. Functional mapping of GABA A receptor subtypes in the amygdala. Eur. J. Neurosci. 20, 1281–1289.
Marowsky, A., Yanagawa, Y., Obata, K., Vogt, K.E., 2005. A specialized subclass of interneurons mediates dopaminergic facilitation of amygdala function. Neuron 48, 1025–1037.
McDonald, A.J., 1998. Cortical pathways to the mammalian amygdala. Prog. Neurobiol. 55, 257–332.
McDonald, A.J., Augustine, J.R., 1993. Localization of GABA-like immunoreactivity in the monkey amygdala. Neuroscience 52, 281–294.
McDonald, A.J., Mascagni, F., 1996. Immunohistochemical localization of the β2 and β3 subunits of the $GABA_A$ receptor in the basolateral amygdala of the rat and monkey. Neuroscience 75, 407–419.
McDonald, A.J., Mascagni, F., 2004. Parvalbumin-containing interneurons in the basolateral amygdala express high levels of the α1 subunit of the $GABA_A$ Receptor. J. Comp. Neurol. 473, 137–146.
McDonald, A.J., Mascagni, F., Augustine, J.R., 1995. Neuropeptide Y and somatostatin-like immunoreactivity in neurons of the monkey amygdala. Neuroscience 66, 959–982.
McDonald, A.J., Mascagni, F., Guo, L., 1996. Projections from the medial and lateral prefrontal cortices to the amygdala: a *Phaseolus vulgaris* leucoagglutinin study in the rat. Neuroscience 71, 55–75.
McNally, G.P., Johansen, J.P., Blair, H.T., 2011. Placing prediction into the fear circuit. Trends Neurosci. 34, 283–292.
Micevych, P., Akesson, T., Elde, R., 1988. Distribution of cholecystokinin-immunoreactive cell bodies in the male and female rat: II. Bed nucleus of the strial terminalis and amygdala. J. Comp. Neurol. 269, 381–391.
Milad, M.R., Quirk, G.J., 2002. Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 420, 70–74.

Millhouse, O.E., 1986. The intercalated cells of the amygdala. J. Comp. Neurol. 247, 246–271.
Miserendino, M.J., Sananes, C.B., Melia, K.R., Davis, M., 1990. Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. Nature 345, 716–718.
Moga, M.M., Gray, T.S., 1985. Peptidergic efferents from the intercalated nuclei of the amygdala to the parabranchial nucleus in the rat. Neurosci. Lett. 61, 13–18.
Moreira, C.M., Masson, S., Carvalho, M.C., Brandao, M.L., 2007. Exploratory behaviour of rats in the elevated plus-maze is differentially sensitive to inactivation of the basolateral and central amygdaloid nuclei. Brain Res. Bull. 71, 466–474.
Morgan, M.A., Romanski, L.M., LeDoux, J.E., 1993. Extinction of emotional learning: contribution of medial prefrontal cortex. Neurosci. Lett. 163, 109–113.
Nader, K., LeDoux, J.E., 1999. Inhibition of the mesoamygdala dopaminergic pathway impairs the retrieval of conditioned fear associations. Behav. Neurosci. 113, 891–901.
Narayanan, R.T., Seidenbecher, T., Sangha, S., Stork, O., Pape, H.C., 2007. Theta resynchronization during reconsolidation of remote contextual fear memory. Neuroreport 18, 1107–1111.
Nitecka, L., Ben-Ari, Y., 1987. Distribution of GABA-like immunoreactivity in the rat amygdaloid complex. J. Comp. Neurol. 266, 45–55.
Nitecka, L., Frotscher, M., 1989. Organization and synaptic interconnections of GABAergic and cholinergic elements in the rat amygdaloid nuclei: single and double immunolabeling studies. J. Comp. Neurol. 279, 470–488.
O'Rourke, H., Fudge, J.L., 2006. Distribution of serotonin transporter labeled fibers in amygdaloid subregions. Biol. Psychiatry 60, 473–490.
Ohishi, H., Akazawa, C., Shigemoto, R., Nakanishi, M., Mizuno, N., 1995. Distribution of mRNAs for L-2-amino-4-phosphonobutyrate- sensitive metabotropic glutamate receptors, mGluR4 and mGluR7, in the rat brain. J. Comp. Neurol. 360, 555–570.
Pape, H.C., 2005. GABAergic neurons: gate masters of the amygdala, mastered by dopamine. Neuron 48, 877–879.
Pape, H.C., Paré, D., 2010. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol. Rev. 90, 419–463.
Pape, H.C., Paré, D., Driesang, R.B., 1998. Two types of intrinsic oscillations in neurons of the lateral and basolateral nuclei of the amygdala. J. Neurophysiol. 79, 205–216.
Pape, H.C., Narayanan, R.T., Smid, J., Stork, O., Seidenbecher, T., 2005. Theta activity in neurons and networks of the amygdala related to long-term fear memory. Hippocampus 15, 874–880.
Paré, D., Collins, D.R., 2000. Neuronal correlates of fear in the lateral amygdala: multiple extracellular recordings in conscious cats. J. Neurosci. 20, 2701–2710.
Paré, D., Smith, Y., 1993a. The intercalated cell masses project to the central and medial nuclei of the amygdala in cats. Neuroscience 57, 1077–1090.
Paré, D., Smith, Y., 1993b. Distribution of GABA immunoreactivity in the amygdaloid complex of the cat. Neuroscience 57, 1061–1076.
Paré, D., Smith, Y., 1994. GABAergic projection from the intercalated cell masses of the amygdala to the basal forebrain in cats. J. Comp. Neurol. 344, 33–49.
Paré, D., Dong, J., Gaudreau, H., 1995. Amygdalo-entorhinal relations and their reflection in the hippocampal formation: generation of sharp sleep potentials. J. Neurosci. 15, 2482–2503.
Paré, D., Quirk, G., LeDoux, J.E., 2004. New vistas on amygdala networks in conditioned fear. J. Neurophysiol. 92, 1–9.
Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press, London.
Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Meth. 14, 149–167.
Pérez de la Mora, M., Cárdenas-Cachón, L., Vázquez-García, M., Crespo-Ramírez, M., Jacobsen, K., Höistad, M., Agnati, L., Fuxe, K., 2005. Anxiolytic effects of intra-amygdaloid injection of the D1 antagonist SCH23390 in the rat. Neurosci. Lett. 377, 101–105.
Pérez de la Mora, M., Lara-García, D., Jacobsen, K.X., Vázquez-García, M., Crespo-Ramírez, M., Flores-Gracia, C., Escamilla-Marván, E., Fuxe, K., 2006. Anxiolytic-like effects of the selective metabotropic glutamate receptor 5 antagonist MPEP after its intra-amygdaloid microinjection in three different non-conditioned animal models of anxiety. Eur. J. Neurosci. 33, 2749–2759.
Pérez de la Mora, M., Hernández-Gómez, A.M., Arizmendi-García, Y., Jacobsen, K.X., Lara-García, D., Flores-Gracia, C., Crespo-Ramírez, M., Gallegos-Cari, A., Nuche-Bricaire, A., Fuxe, K., 2007. Role of the amygdaloid cholecystokinin (CCK) 7 gastrin-2 receptors and terminal networks in the modulation of anxiety in the rat. Eur. J. Neurosci. 26, 3614–3630.
Pérez de la Mora, M., Jacobsen, K.X., Crespo-Ramírez, M., Flores-Gracia, C., Fuxe, K., 2008. Wiring and volume transmission in rat amygdala. Implications for fear and anxiety. Neurochem. Res. 33, 1618–1633.
Pérez de la Mora, M.P., Gallegos-Cari, A., Arizmendi-Garcia, Y., Marcellino, D., Fuxe, K., 2010. Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: structural and functional analysis. Prog. Neurobiol. 90, 198–216.
Pérez de la Mora, M., Gallegos-Cari, A., Crespo-Ramírez, M., Marcellino, D., Hansson, A.C., Fuxe, K., 2012. Distribution of dopamine D(2)-like receptors in the rat amygdala and their role in the modulation of unconditioned fear and anxiety. Neuroscience 201, 252–266.
Pinard, C.R., Muller, J.F., Mascagni, F., McDonald, A.J., 2008. Dopaminergic innervation of interneurons of the rat basolateral amygdala. Neuroscience 157, 850–863.
Pinto, A., Sesack, S.R., 2008. Ultrastructural analysis of prefrontal cortical inputs to the rat amygdala: spatial relationships to presumed dopamine axons and D1 and D2 receptors. Brain Struct. Funct. 213, 159–175.
Pitkänen, A., Amaral, D.G., 1994. The distribution of GABAergic cells, fibers, and terminals in the monkey amygdaloid complex: an immunohistochemical and in situ hybridization study. J. Neurosci. 14, 2200–2224.
Pitkänen, A., Amaral, D.G., 1998. Organization of the intrinsic connections of the monkey amygdaloid complex: projections originating in the lateral nucleus. J. Comp. Neurol. 398, 431–458.
Pitkänen, A., Stefanacci, L., Farb, C.R., Go, G.G., LeDoux, J.E., Amaral, D.G., 1995. Intrinsic connections of the rat amygdaloid complex: projections originating in the lateral nucleus. J. Comp. Neurol. 356, 288–310.
Pitkänen, A., Savander, V., LeDoux, J.E., 1997. Organization of intra-amygdaloid circuitries in the rat: emerging tramework for understanding functions of the amygdala. Trends Neurosci. 20, 517–523.
Pouille, F., Scanziani, M., 2001. Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. Science 293, 1159–1163.
Poulin, J.F., Chevalier, B., Laforest, S., Drolet, G., 2006. Enkephalinergic afferents of the centromedial amygdala in the rat. J. Comp. Neurol. 496, 859–876.
Poulin, J.F., Castonguay-Lebel, Z., Laforest, S., Drolet, G., 2008. Enkephalin co-expression with classical neurotransmitters in the amygdaloid complex of the rat. J. Comp. Neurol. 906, 543–551.
Pu, S., Zhuang, H., Lu, Z., Wu, X., Han, J., 1994. Cholecystokinin gene expression in rat amygdaloid neurons: normal distribution an effect of morphine tolerance. Brain Res. Mol. Brain Res. 21, 183–189.
Quirk, G.J., Mueller, D., 2008. Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33, 56–72.

Quirk, G.J., Repa, C., LeDoux, J.E., 1995. Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. Neuron 15, 1029–1039.

Quirk, J.G., Russo, G.K., Barron, J.L., Lebron, K., 2000. The role of ventromedial prefrontal cortex in the recovery of extinguished fear. J. Neurosci. 20, 6225–6231.

Quirk, G.J., Likhtik, E., Pelletier, J.G., Paré, D., 2003. Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. J. Neurosci. 23, 8800–8807.

Rezayoff, A., Hosseini, S.S., Zarrindast, M.R., 2009. Effects of morphine on rat behaviour in the elevated plus maze: the role of central amygdala dopamine receptors. Behav. Brain Res. 202, 171–178.

Ribeiro de Oliveira, A., Reimer, A.E., Antunes de Macedo, C.E., de Carvalho, M.C., de Souza Silva, M.A., Brandao, M.L., 2011. Contidioned fear is modulated by D_2 receptor pathway connecting the ventral tegmental area and basolateral amygdala. Neurobiol. Learn. Mem. 95, 37–45.

Ribeiro, A.M., Barbosa, F.F., Munguba, H., Costa, M.S.M.O., Cavalcante, J.S., Silva, R.H., 2011. Basolateral amygdala inactivation impairs learned (but not innate) fear response in rats. Neurobiol. Learn. Mem. 95, 433–440.

Rice, M.E., Cragg, S.J., 2008. Dopamine spillover after quantal release: rethinking dopamine transmission in the nigrostriatal pathway. Brain Res. Rev. 58, 303–313.

Rodgers, R.J., 1997. Animal models of 'anxiety': where next? Behav. Pharmacol. 8, 477–496.

Rodrigues, S.M., Schafe, G.E., LeDoux, J.E., 2004. Molecular mechanisms underlying emotional learning and memory in the lateral amygdala. Neuron 44, 75–91.

Romanski, L.M., LeDoux, J.E., 1993. Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. J. Neurosci. 12, 4501–4509.

Roozendaal, B., McEwen, B.S., Chattarji, S., 2009. Stress, memory and the amygdala. Nat. Rev. Neurosci. 10, 423–433.

Rosenkranz, J.A., Grace, A.A., 2001. Dopamine attenuates prefrontal cortical suppression of sensory inputs to the basolateral amygdala of rats. J. Neurosci. 21, 4090–4103.

Rosenkranz, J.A., Grace, A.A., 2002. Cellular mechanisms of infralimbic and prelimbic prefrontal cortical inhibition and dopaminergic modulation of basolateral amygdala neurons in vivo. J. Neurosci. 22, 324–337.

Royer, S., Paré, D., 2002. Bidirectional synaptic plasticity in intercalated amygdala neurons and the extinction of conditioned fear resposes. Neuroscience 115, 455–462.

Royer, S., Paré, D., 2003. Conservation of total synaptic weight through balanced synaptic depression and potentiation. Nature 422, 518–522.

Royer, S., Martina, M., Paré, D., 1999. An inhibitory interface gates impulse traffic between the input and output stations of the amygdala. J. Neurosci. 19, 10575–10583.

Royer, S., Martina, M., Paré, D., 2000a. Polarized synaptic interactions between intercalated neurons of the amygdala. J. Neurophysiol. 83, 3509–3518.

Royer, S., Martina, M., Paré, D., 2000b. Bistable behavior of inhibitory neurons controlling impulse traffic through the amygdala: role of a slowly deinactivating K+ current. J. Neurosci. 20, 9034–9039.

Samson, R.D., Paré, D., 2005. Activity-dependent synaptic plasticity in the central nucleus of the amygdala. J. Neurosci. 25, 1847–1855.

Scibilia, R.J., Lachowicz, J.E., Kilts, C.D., 1992. Topographic nonoverlapping distribution of D1 and D2 dopamine receptors in the amygdaloid nuclear complex of the rat brain. Synapse 11, 146–154.

Seidenbecher, T., Laxmi, T.R., Stork, O., Pape, H.C., 2003. Amygdalar and hippocampal theta rhythm synchronization during fear memory retrieval. Science 301, 846–850.

Sierra-Mercado, D., Padilla-Coreano, N., Quirk, G.J., 2011. Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology 36, 529–538.

Sims, K.S., Williams, R.S., 1990. The human amygdaloid complex: a cytologic and histochemical atlas using nissl, myelin, acetylcholinesterase and nicotinamide adenine dinucleotide phosphate diaphorase staining. Neuroscience 36, 449–472.

Smith, Y., Paré, D., 1994. Intra-amygdaloid projections of the lateral nucleus in the cat: PHA-L anterograde labeling combined with postembedding GABA and glutamate immunocytochemistry. J. Comp. Neurol. 342, 232–248.

Sorvari, H., Soininin, H., Pitkänen, A., 1996. Calbindin-D28K-immunoreactive cells and fibres in the human amygdaloid complex. Neuroscience 75, 421–443.

Sotres-Bayon, F., Quirk, G.J., 2010. Prefrontal control of fear: more than just extinction. Curr. Opin. Neurobiol. 20, 231–235.

Spiegel, E., 1919. Dic Kerne im Vorderhim der Sauger. Arb. Neurol. Inst. Wien Univ. 22, 418–497.

Sun, N., Yi, H., Cassell, M.D., 1994. Evidence for a GABAergic interface between cortical afferents and brainstem projection neurons in the rat central extended amygdala. J. Comp. Neurol. 340, 43–64.

Swanson, L.W., Petrovich, G.D., 1998. What is the amygdala? Trends Neurosci. 21, 323–331.

Tömböl, T., Szafrańska-Kosmal, A., 1972. A golgi study of the amygdaloid complex in the cat. Acta Neurobiol. Exp. (Wars) 32, 835–848.

Treit, D., Menard, J., 1997. Dissociations among the anxiolytic effects of septal, hippocampal, and amygdaloid lesions. Behav. Neurosci. 111, 653–658.

Treit, D., Robinson, A., Rotzinger, S., Pesold, C., 1993. Anxiolytic effects of serotonergic interventions in the shock-probe burying test and the elevated plus-maze test. Behav. Brain Res. 54, 23–34.

Vazdarjanova, A., Cahill, L., McGaugh, J.L., 2001. Disrupting basolateral amygdala function impairs unconditioned freezing and avoidance in rats. Eur. J. Neurosci. 14, 709–718.

Veeinig, J.G., Swanson, L.W., Sawchenko, P.E., 1984. The organization of projections from the central nucleus of the amygdala to brain stem-sites involved in central autonomic regulation: a combined retrograde transport-immunohistochemical study. Brain Res. 303, 337–357.

Veinante, P., Stoeckel, M.E., Lasbennes, F., Freund-Mercier, M.J., 2003. c-Fos and peptide immunoreactivities in the central extended amygdala of morphine-dependent rats after naloxone-precipitated withdrawal. Eur. J. Neurosci. 18, 1295–1305.

Vertes, R.P., 2004. Differential projections of the infralimbic and prelimbic cortex in the rat. Synapse 51, 32–58.

Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S.L., Quirk, G.J., 2006. Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. Learn. Mem. 13, 728–733.

Völsch, M., 1910. Zurvergleichenden anatomie des Mandelkerns und seiner Nachbargebilde. II. Teil. Arch. Mikrosk. Anat. Entwgesch. 76, 373–523.

Waclaw, R.R., Ehrman, L.A., Pierani, A., Campbell, K., 2010. Developmental origin of the neuronal subtypes that comprise the amygdalar fear circuit in the mouse. J. Neurosci. 30, 6944–6953.

Wallace, K.J., Rosen, J.B., 2001. Neurotoxic lesions of the lateral nucleus of the amygdala decrease conditioned fear but not unconditioned fear of a predator odor: comparison with electrolytic lesions. J. Neurosci. 21, 3619–3627.

Weiner, D.M., Levey, A.I., Sunahara, R.K., Niznik, H.B., O'Dowd, B.F., Seeman, P., Brann, M.R., 1991. D1 and D2 dopamine receptor mRNA in rat brain. Proc. Natl. Acad. Sci. U. S. A. 88, 1859–1863.

Whittle, N., Hauschild, M., Lubec, G., Holmes, A., Singewald, N., 2010. Rescue of impaired fear extinction and normalization of cortico-amygdala circuit dysfunction in a genetic mouse model by dietary zinc restriction. J. Neurosci. 30, 13586–13596.

Wilensky, A.E., Schafe, G.E., Kristensen, M.P., LeDoux, J.E., 2006. Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. J. Neurosci. 26, 12387–12396.

Wolpe, J., 1958. Psychotherapy by Reciprocal Inhibition. Stanford University Press, Stanford, California.

Yokoyama, M., Suzuki, E., Sato, T., Maruta, S., Watanabe, S., Miyaoka, H., 2005. Amygdalic levels of dopamine and serotonin rise upon exposure to conditioned fear stress without elevation of glutamate. Neurosci. Lett. 379, 37–41.

Young, A.M.J., Rees, K.R., 1998. Dopamine release in the amygdaloid complex of the rat, studied by brain microdialysis. Neurosci. Lett. 249, 49–52.

Zangrossi Jr., H., File, S.E., 1992. Behavioral consequences in animal tests of anxiety and exploration of exposure to cat odor. Brain Res. Bull. 29, 381–388.

Zarrindast, M.R., Mahboobi, S., Sadat-Shirazi, M.S., Ahmadi, S., 2011. Anxiolytic-like effect induced by the cannabinoid CB1 receptor agonist, arachydonilcyclopropylamide (ACPA), in the rat amygdala is mediated through the D1 and D2 dopaminergic systems. J. Psychopharmacol. 25, 131–140.

Zezula, J., Cortés, R., Probst, A., Palacios, J.M., 1988. Benzodiazepine receptor sites in the human brain: autoradiographic mapping. Neuroscience 25, 771–795.

Zhang, J.H., Sato, M., Tohyama, M., 2004. Region-specific expression of the mRNAs encoding β subunits (β1, β2, and β3) of $GABA_A$, receptor in the rat brain. J. Comp. Neurol. 303, 637–657.

Zoli, M., Jansson, A., Syková, E., Agnati, L.F., Fuxe, K., 1999. Volume transmission in the CNS and its relevance for neuropsychopharmacology. TIPS 20, 142–150.