Rol de las Hipocretinas en el área preóptica medial de ratas lactantes en la regulación del sueño y el comportamiento maternal

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RESUMEN

Durante el período posparto, en la hembra ocurren cambios neuroendócrinos que permiten el desarrollo del comportamiento maternal, necesario para el adecuado cuidado y desarrollo de las crías. Este es un comportamiento altamente demandante, que requiere, entre otros, una adaptación del sueño y vigilia de la madre. En particular, el área preóptica medial (APOm) es un área clave no solo en el control del comportamiento maternal, sino en la regulación de los estados de vigilia y sueño, así como de un proceso fisiológico básico como la temperatura corporal.

Las hipocretinas (HCRT) son dos neuropéptidos moduladores, la HCRT-1 y HCRT-2, sintetizados por neuronas de la región postero-lateral del hipotálamo que proyectan a amplias regiones del sistema nervioso central. Las HCRT han sido implicadas en la generación y mantenimiento de la vigilia, así como en la regulación de comportamientos motivados. Sin embargo, no se conoce el rol de las HCRT en ratas lactantes, especialmente a nivel del APOm. Por lo tanto, el objetivo de esta tesis es determinar el rol del sistema hipocretinérgico en el APOm en la regulación del comportamiento maternal y el sueño de ratas lactantes. Para ello, evaluamos estas variables luego de la microinyección de HCRT-1 y de antagonistas durante el posparto. A su vez, evaluamos, bajo anestesia con uretano, los efectos de HCRT-1 sobre la actividad neuronal del APOm de ratas lactantes y sus diferencias respecto a ratas vírgenes.

Los principales resultados de esta tesis indican que las HCRT en el APOm promueven los comportamientos maternales activos junto con un incremento de la vigilia, y con un aumento leve pero significativo de la temperatura corporal. Por otro lado, el bloqueo del efecto de la HCRT endógena promueve el sueño y el amamantamiento de forma simultánea sin afectar la temperatura corporal. Por otra parte, observamos que el efecto de las HCRT fue heterogéneo, facilitando o reduciendo la frecuencia de descarga en distintas neuronas, sin encontrar diferentes respuestas entre animales lactantes o vírgenes.

Concluimos que, en ratas lactantes, las HCRT a nivel del APOm promueven en forma integrada la generación de la vigilia y de comportamientos maternales activos mientras que la disminución de la acción de las HCRT endógenas aumentan el sueño y el amamantamiento.

INTRODUCCIÓN

1. Comportamiento maternal

El comportamiento maternal existe en una gran variedad de especies, aunque está más desarrollado en aves y mamíferos (Clutton-Brock, 1991). Este comportamiento es clave para la supervivencia de las crías, ya que las provee de calor, refugio, limpieza, defensa y orientación, además de nutrientes alimenticios (Stern, 1989). En los mamíferos, dado que la principal fuente de alimento de las crías es la leche, y es la hembra la que amamanta, la madre suele ser la principal responsable de los cuidados parentales. La maternidad está asociada a una serie de adaptaciones comportamentales, controladas por mecanismos neuroendócrinos, que permiten a la hembra cuidar adecuadamente a su descendencia (Numan, 2006). El inicio del comportamiento maternal está facilitado por los cambios hormonales que suceden hacia el final de la gestación y en el parto (Bridges, 1984). Una vez establecido, este comportamiento es regulado principalmente por los estímulos de las crías en desarrollo (Rosenblatt, 1980).

Las respuestas maternas se pueden agrupar en dos categorías: las dirigidas hacia la cría y las indirectamente relacionadas con la cría. En roedores, las respuestas dirigidas a las crías incluyen comportamientos tales como acarreo de las crías al nido, re-arreglos en el nido, lamidos corporales y anogenitales de las crías, así como adopción de una postura típica para el amamantamiento. También hay respuestas indirectas que consisten en la construcción y arreglos del nido, y cambios afectivos que se visualizan en caso de defensa de las crías, por ejemplo, un aumento de la agresión hacia intrusos en el nido y una reducción del miedo y ansiedad experimental (Stern, 1996). A su vez, los comportamientos dirigidos hacia las crías pueden catalogarse como comportamientos maternales de tipo activo, que implican movimientos dirigidos a las crías, mientras que el amamantamiento podría considerarse un comportamiento pasivo, desde el punto de vista de la actividad motora (Terkel et al., 1979). Durante el amamantamiento suele haber una inhibición de todos los comportamientos activos y, en algunas especies como la rata, la adopción típica de una postura de tipo arqueada sobre las crías (Stern & Lonstein, 2001). Dicha postura de amamantamiento es provocada por los estímulos somatosensoriales de las crías en la zona ventral de la hembra (Stern & Johnson, 1990). A su vez, el estímulo de succión de las crías provoca la secreción de prolactina y oxitocina que promueven la síntesis y la eyección de leche (Stern & Lonstein, 2001).

2. Ciclo sueño-vigilia

En todas las especies de animales estudiadas se reconocen los estados comportamentales de sueño y vigilia, con una ritmicidad circadiana. Durante la vigilia ocurren los

comportamientos de interacción con el ambiente. El sueño, por el contrario, puede definirse como un estado de inmovilidad de rápida reversibilidad, caracterizado por una respuesta reducida a los estímulos del ambiente (Siegel, 2005). A su vez, en mamíferos y aves se pueden distinguir dos estados diferentes de sueño: el sueño REM (de la sigla en inglés: “rapid eye movements”) y el sueño lento o no REM (NREM).

Los cambios comportamentales de los estados de sueño y vigilia son acompañados por correlatos electrofisiológicos que los definen y permiten diferenciarlos con precisión. En este sentido, la polisomnografía es una herramienta clásica que consiste en el registro del electroencefalograma (EEG) junto con otras variables fisiológicas y es sumamente utilizada para el diagnóstico de sueño y vigilia, tanto a nivel clínico como en investigación.

Específicamente, durante la vigilia se reconoce un estado de activación en la corteza cerebral, con ondas de alta frecuencia y baja amplitud en el EEG, determinado por la "desincronización" de la actividad de las neuronas talámicas y corticales. Desde la vigilia se ingresa al sueño NREM, en el que se pueden reconocer distintas fases asociadas a la profundidad del sueño. El correlato electrofisiológico del sueño NREM consiste en ondas de baja frecuencia y alta amplitud en el EEG, generadas por la “sincronización” de las neuronas talámicas y corticales. Desde el sueño NREM se ingresa al sueño REM periódicamente y, a pesar de que el sueño es profundo, el correlato electrofisiológico de este estado es de activación cortical, similar a la vigilia. Sin embargo, la característica atonía muscular presente en el sueño REM permite diferenciarlo de la vigilia (Siegel, 2005).

3. Sueño durante el posparto

Estudios previos de nuestro grupo de trabajo han mostrado que las ratas madres coordinan su propio ciclo de sueño-vigilia con el cuidado materno de las crías durante el posparto. Específicamente, se demostró que las madres permanecen la mayor parte del tiempo en sueño NREM mientras amamantan en la postura arqueada baja, que es la postura de amamantamiento más frecuente (Benedetto et al., 2017b). A su vez, la fragmentación del sueño, es decir períodos de sueño interrumpidos por breves despertares, es mayor durante los episodios de amamantamiento en comparación a cuando las madres duermen lejos de sus crías, pero la profundidad del sueño (medida a través de la amplitud de las ondas lentas) no varía entre estos comportamientos (Benedetto et al., 2017b). El sueño y el amamantamiento en ratas han sido asociados desde hace varias décadas, ya que se ha descrito que el sueño NREM previo es un requisito para la eyección láctea, y si las madres están privadas de sueño ésta no ocurriría (Lincoln et al., 1980; Voloschin & Tramezzani, 1979). Sin embargo, un trabajo reciente de nuestro grupo

indica que, si bien el sueño y el amamantamiento están asociados desde el punto de vista comportamental, el sueño previo no es un requerimiento para la eyección láctea (Peña et al., 2022).

4. Área preóptica medial (APOm)

El APOm es parte del área preóptica (APO) localizada en el hipotálamo rostral. Esta es un área heterogénea que integra diversos comportamientos y variables fisiológicas vitales para la supervivencia del individuo y de la especie, entre ellos el sueño, el comportamiento maternal y la regulación de la temperatura corporal (Kumar, 2004; Numan, 1974; Szymusiak et al., 1998).

El APOm está compuesta por poblaciones neuronales de diversos fenotipos, entre ellas GABAérgicas, glutamatérgicas, nitrérgicas y neuropeptidérgicas (Melander et al., 1986; Ottersen & Storm-Mathisen, 1984; Skofitsch & Jacobowitz, 1985). Sin embargo, la gran mayoría de las neuronas del APOm son GABAérgicas. Estas neuronas se activan durante el sueño (Szymusiak et al., 1998; Takahashi et al., 2009), y durante el comportamiento maternal (Fleming et al., 1994b; Lonstein et al., 1998b; Tsuneoka et al., 2013).

Durante la transición a la maternidad, y durante el período posparto, suceden varios cambios anatómicos y fisiológicos en el APOm. Se han descrito modificaciones morfológicas en las neuronas tanto en la complejidad de las espinas dendríticas (Parent et al., 2017; Shams et al., 2012), en la densidad de receptores de varios neurotransmisores (Akbari et al., 2013; Bosch & Neumann, 2008; Dobolyi, 2009; Driessen et al., 2014; Gammie, 2005), e incluso en el fenotipo neuronal. Por ejemplo, la expresión del neuropéptido Hormona Concentradora de Melanina (MCH) en neuronas de la APOm ocurre solamente durante el período posparto (Rondini et al., 2010).

4.1. Rol del APOm en el comportamiento maternal

Como se mencionó previamente, los cambios hormonales que ocurren al final de la gestación y el parto facilitan el desencadenamiento y mantenimiento del comportamiento maternal en el posparto, modificando la actividad de circuitos cerebrales que regulan esta conducta. El APOm es un centro regulador clave de estos circuitos maternales (Bridges et al., 1990; Numan, 2006; Stolzenberg et al., 2019). Lesiones del APOm, así como su inactivación farmacológica reversible, interrumpen varios componentes de este comportamiento sin afectar otros como la alimentación (Arrati et al., 2006; Numan, 1974; Numan et al., 1988; Pereira & Morrell, 2009). A su vez, utilizando la proteína Fos como índice de actividad neuronal, se ha observado que la actividad neuronal en esta área está elevada cuando la madre despliega conductas maternales activas, y se reduce cuando es separada de sus crías (Fleming et al., 1994a; Lonstein et al., 1998a). Estudios recientes muestran que la activación quimio-genética y opto-genética de grupos específicos de

neuronas del APOm, ya sean GABAérgicas (Dimen et al., 2021), galaninérgicas (Wu et al., 2014), o que expresan receptor de estrógenos (Fang et al., 2018), inducen o aumentan las respuestas maternas en ratones.

El comportamiento maternal varía durante el período posparto, probablemente en respuesta a los cambios en las demandas de las crías. Se ha postulado que modificaciones en el APOm subyacen al menos parte de estos cambios. Específicamente, se ha reportado que el APOm cambia su rol de facilitar el comportamiento maternal en el posparto temprano (primera semana), a inhibirlo en la segunda semana posparto de la rata (Pereira & Morrell, 2009). En concordancia, la administración de MCH en el APOm disminuye los comportamientos maternales activos durante la primera semana (Benedetto et al., 2014), mientras que no causa ningún efecto durante la segunda semana posparto (Benedetto et al., 2018).

Si bien el rol del APOm en el control del comportamiento maternal ha sido ampliamente abordado desde distintos enfoques, hasta el momento no se ha estudiado las características electrofisiológicas básicas de las neuronas del APOm, ni su actividad eléctrica durante el posparto, ni si esta es diferente en relación a animales vírgenes.

4.2. Rol del APOm en el sueño

Existe una amplia evidencia experimental, con distintos abordajes metodológicos, que indica que la APO promueve la generación y el mantenimiento del sueño. Por ejemplo, lesiones en esta área interrumpen el sueño, y la actividad neuronal aumenta durante el sueño NREM, observado tanto con la proteína Fos (Gong et al., 2004; Lu et al., 2000; Sherin et al., 1996), como con registros de unidades neuronales (Alam et al., 1997; Koyama & Hayaishi, 1994). Estas neuronas con actividad relacionada al sueño se han registrado tanto en el APOm como en regiones más laterales, y están entremezcladas con neuronas activas durante la vigilia (Alam et al., 1997; Alam et al., 1995; Szymusiak et al., 1998; Takahashi et al., 2009). Se ha mostrado un rol causal entre la activación farmacológica de estas neuronas activas durante el sueño y la inducción de sueño (Zhang et al., 2015). Estas neuronas somnogénicas han sido identificadas principalmente como GABAérgicas y galaninérgicas, y se ha mostrado que proyectan hacia distintas regiones de los sistemas activadores como el hipotálamo postero-lateral, núcleo tuberomamilar del hipotálamo, locus coeruleus y núcleo dorsal del rafe (Chung et al., 2017; Steininger et al., 2001; Vanini & Torterolo, 2021). A pesar de esta evidencia, el mecanismo mediante el cual el cerebro realiza la transición entre el sueño y la vigilia permanece solo parcialmente comprendido. Actualmente, el modelo más aceptado es el conocido como "flip-flop", en el cual las interacciones inhibitorias

recíprocas entre los grupos de neuronas que promueven el sueño y la vigilia, subyacen la transición y alternancia entre estos estados comportamentales (Saper et al., 2001).

4.3. APOm en el control integrado del sueño y la termorregulación

El APOm juega un papel clave en la termorregulación (Srividya et al., 2006). Se ha sugerido una interrelación entre la regulación de la temperatura corporal y del sueño, y que el APOm integraría la regulación térmica con el estado comportamental (Kumar, 2004; Harding et al., 2018). Neuronas del APOm, que aumentan su frecuencia de descarga durante el sueño NREM ("NREM sleep-on"), también son sensibles al calor, ya que aumentan su descarga en respuesta a aumentos locales de la temperatura (Alam et al., 1997; Alam et al., 1995). Además, el calentamiento local del APO promueve el sueño y aumenta la amplitud de las ondas lentas del EEG durante el sueño NREM (McGinty et al., 1994; Roberts & Robinson, 1969). Asimismo, recientemente se ha identificado que la activación de un grupo de neuronas nitrérgicas/glutamatérgicas en el núcleo preóptico mediano (MnPO)/APOm (Harding et al., 2018), así como galaninérgicas del APO ventrolateral (Kroeger et al., 2018), inducen tanto sueño NREM como hipotermia. Sin embargo, no se conoce cómo es esta interrelación durante el posparto.

4.4. APOm en el control del sueño y el comportamiento materno

Los cambios en la anatomía y función de las redes neurales durante el posparto podrían alterar el sueño de la madre en función de los nuevos requerimientos fisiológicos. En este sentido, en trabajos recientes exploramos cómo diferentes neurotransmisores a nivel el APOm de ratas lactantes modifican el sueño y el comportamiento maternal. La microinyección en el APOm del antagonista del receptor de dopamina D2 Raclopride reduce el sueño REM sin afectar el sueño NREM, mientras que reduce la latencia al inicio del amamantamiento (Benedetto et al., 2017a). A su vez, el antagonista del receptor GABA-A bicuculina, aumenta los comportamientos maternales activos, sin afectar el sueño de las madres (Benedetto et al., 2021). En conjunto, estos resultados sugieren que distintos sistemas de neurotransmisión podrían estar desempeñando un rol específico en la regulación tanto del sueño como del comportamiento materno durante el posparto.

5. Sistema Hipocretinérgico

Desde su descubrimiento en el año 1998, el sistema hipocretinérgico (HCRTérgico) se ha convertido en uno de los sistemas reguladores más estudiados (Jacobson et al., 2022). Las hipocretinas (HCRT) consisten de dos neuropéptidos excitadores (HCRT-1 y HCRT-2, también conocidos como orexina A y orexina B, respectivamente), sintetizados por un grupo de neuronas localizados exclusivamente en el hipotálamo postero-lateral, cuyas proyecciones y receptores

están ampliamente distribuidos en el sistema nervioso central (Peyron et al., 1998; Sakurai et al., 1998; Taheri & Bloom, 2001; Trivedi et al., 1998). Las HCRT se unen con diferente afinidad a dos receptores metabotrópicos denominados tipo 1 (HCRT-R1) y tipo 2 (HCRT-R2). Ambos receptores se expresan en el APOm (Marcus et al., 2001; Trivedi et al., 1998).

5.1. HCRT en la regulación de la vigilia

Una extensa evidencia indica que las HCRT juegan un rol clave en el inicio y mantenimiento de la vigilia (Adamantidis et al., 2007; McGregor et al., 2011; Sakurai, 2005; Torterolo et al., 2001; Torterolo et al., 2003; Torterolo et al., 2011). A través del uso de la proteína Fos como índice de actividad neuronal, Torterolo y colaboradores demostraron que las neuronas HCRTérgicas se activan sólo si existe actividad motora, por lo que su función primaria sería facilitar el aumento de la vigilancia que acompaña a la actividad motora, principalmente durante los comportamientos dirigidos a un objetivo o recompensa (Torterolo et al., 2001; Torterolo et al., 2003). A su vez, las neuronas HCRTérgicas disminuyen su actividad durante el sueño NREM y muestran descargas fásicas durante el sueño REM (Lee et al., 2005; Mileykovskiy et al., 2005; Torterolo et al., 2001). Además, mediante la estimulación optogenética de las neuronas HCRTérgicas, se ha demostrado su rol causal en la estabilidad de la vigilia y en el aumento de la probabilidad de transición hacia esta desde el sueño NREM o sueño REM (Adamantidis et al., 2007). La administración de HCRT a nivel del APO en ratas macho, tanto en la región lateral (Methippara et al., 2000), como medial (España et al., 2001), provoca un aumento del tiempo en vigilia. Sin embargo, debido a los cambios funcionales que experimenta el APOm durante el posparto, los efectos sobre la vigilia y el sueño de la perfusión de HCRT dentro del APOm podrían diferir durante dicho período.

5.2. HCRT en la termorregulación

Se ha mostrado que la administración de HCRT-1 en el tercer ventrículo aumenta la temperatura corporal (Yoshimichi et al., 2001 1684), mientras que las inyecciones sistémicas del antagonista del receptor dual de orexina (DORA) o del antagonista específico del HCRT-R1 (SB-334867) reducen la temperatura corporal (Martin et al., 2019 1685; Rusyniak et al., 2011 1687). Dada la asociación entre el sueño y la termorregulación, las HCRT podrían influir en la temperatura corporal a través del estado de vigilancia. Hasta el momento, se desconoce el rol de las HCRT sobre la temperatura corporal actuando específicamente en el APOm.

5.3. HCRT en la regulación del comportamiento maternal

El sistema HCRTérgico ha sido propuesto como promotor de varios comportamientos motivados, incluyendo la alimentación, la actividad exploratoria, la búsqueda de drogas, el comportamiento sexual y el comportamiento maternal (Boutrel et al., 2010; D'Anna & Gammie, 2006; Harris et al., 2005; Muschamp et al., 2007). Durante el posparto, mediante la expresión de Fos, se ha reportado un aumento en la actividad de las neuronas HCRTérgicas en ratones hembras lactantes comparado con vírgenes (Espana et al., 2004). A su vez, la administración intracerebroventricular de HCRT-1 en ratones modula el comportamiento maternal (D'Anna & Gammie, 2006). De manera interesante, el sistema HCRTérgico forma parte de los cambios anátomo-funcionales que experimenta la hembra durante el período posparto. Particularmente, los niveles de la proteína precursora de las HCRT, la prepro-HCRT, y el ARNm del receptor HCRT-R1 en el hipotálamo de hembras lactantes están aumentados (Wang et al., 2003). Sin embargo, si bien las HCRT se han involucrado en la modulación del comportamiento maternal, hasta el momento se desconocía si este efecto es mediado por el APOm.

5.4. Efecto de las HCRT sobre las neuronas del APOm

Hasta el momento, el efecto de las HCRT en la actividad de las neuronas del APOm no ha sido estudiado. En registros *in vitro* de otras áreas del APO como el MnPO, la HCRT-1 aumenta la excitabilidad neuronal (Kolaj et al., 2008). Sin embargo, también mediante registros *in vitro* en neuronas del núcleo ventrolateral preóptico (VLPO), presumiblemente promotoras de sueño, Eggermann et al. (2001) reportaron ausencia de efecto de las HCRT en su actividad. Dado que estos trabajos fueron realizados en ratas macho, se desconoce el efecto de las HCRT en neuronas del APO en animales lactantes.

JUSTIFICACIÓN E HIPÓTESIS DE TRABAJO

1) Dada la importancia del sistema HCRTérgico en los comportamientos motivados, así como los cambios de este sistema durante la lactancia, planteamos la hipótesis de que las HCRT en el APOm promueven el comportamiento maternal activo en ratas lactantes. Además, dado que se han propuesto modificaciones en el rol del APOm durante el período posparto, hipotetizamos que los efectos de las HCRT pueden ser diferentes en la primera y segunda semana posparto

2) Dado el rol del sistema HCRTérgico como promotor de la vigilia y que las ratas madre principalmente duermen mientras amamantan, planteamos la hipótesis que un aumento de la actividad del sistema HCRTérgico en el APOm promueve la vigilia y el comportamiento maternal activo, mientras que su disminución promueve el sueño y el amamantamiento.

3) En relación a la actividad de las neuronas del APOm, debido a los cambios anátomo-funcionales que sufre esta área durante el posparto, hipotetizamos que la actividad basal de las neuronas es diferente en hembras posparto en comparación a las vírgenes. Además, dado que en esta zona se encuentran receptores para HCRT, asociadas al control de distintos comportamientos, la administración yuxtacelular de HCRT modula la actividad individual de las neuronas del APOm.

OBJETIVOS

Objetivo general

Determinar el rol del sistema hipocretinérgico en el APOm en la regulación del comportamiento maternal y el sueño de ratas lactantes.

Objetivos específicos

1. Determinar cómo las HCRT, actuando en el APOm, modulan el comportamiento maternal de ratas lactantes durante la primera y segunda semana posparto (capítulo I).

2. Determinar los efectos de las HCRT a nivel del APOm, simultáneamente sobre el sueño y la vigilia, el comportamiento maternal y la temperatura corporal en ratas lactantes en la primera semana posparto (capítulo II).

3. Evaluar los efectos de las HCRT sobre la actividad neuronal del APOm de ratas posparto y sus diferencias respecto a hembras vírgenes ciclantes (capítulo III).

3.1. Realizar la caracterización electrofisiológica de las neuronas del APOm en ratas posparto y vírgenes ciclantes.

3.2. Describir la relación de la actividad de las neuronas del APOm con el estado del electroencefalograma.

3.3. Evaluar los efectos de las HCRT en la actividad de las neuronas del APOm en ratas posparto y vírgenes ciclantes.

ESTRATEGIA DE INVESTIGACIÓN Y ORGANIZACIÓN DE LA TESIS

Las secciones correspondientes a metodología, resultados y discusión están organizadas en tres capítulos. A su vez, previamente se describen en forma breve las principales metodologías empleadas. Específicamente, en cada capítulo se exponen los artículos publicados en relación al objetivo específico abordado, precedidos por un breve resumen. En el primero abordamos el rol de las HCRT en el APOm en el control del comportamiento maternal en la primera y segunda

semana posparto, mediante la microinyección de HCRT-1 y su antagonista en esta área. En el segundo, estudiamos cómo las HCRT en el APOm regulan el comportamiento maternal integrándolo con sus efectos en el sueño-vigilia y la temperatura corporal en ratas lactantes durante la primera semana posparto, mediante registros polisomnográficos y de videos en simultáneo luego de las microinyecciones de HCRT-1 y el antagonista. Y en el tercero, describimos la actividad electrofisiológica basal de las neuronas del APOm en ratas lactantes, en comparación a la de hembras vírgenes, y evaluamos el efecto de la administración yuxtacelular de HCRT-1 en la actividad individual de estas neuronas. Al final de la tesis, se realizan comentarios adicionales a la discusión de los trabajos y una síntesis de las principales conclusiones y perspectivas.

METODOLOGÍA DE ESTUDIO

A continuación, se comentan las principales características de cada técnica utilizada. En cada artículo se encontrará una descripción detallada de los métodos utilizados, así como de los diseños y protocolos experimentales

Todos los experimentos se realizaron en el Laboratorio de Neurobiología del Sueño, del Departamento de Fisiología, Facultad de Medicina, Universidad de la República (UdelaR).

Animales

Se utilizaron ratas hembras primíparas (*Rattus norvegicus*) de la cepa Wistar de aproximadamente 3 meses de edad y crías de 5-8 y 14-15 días de vida. Las hembras gestantes fueron alojadas en cajas individuales 2 a 3 días antes del parto y hasta la finalización de los experimentos. Los animales fueron obtenidos de la Unidad de Reactivos para Biomodelos de Experimentación (URBE). Los protocolos experimentales nº 070153-000304-13 y 070153-000550-18 fueron aprobados por la Comisión Honoraria de Experimentación Animal (CHEA).

Cirugía estereotáxica

Para los experimentos de los capítulos I y II se realizaron cirugías estereotáxicas bajo anestesia para el implante de cánulas bilaterales en el APOm. Además, se colocaron electrodos sobre la corteza cerebral y los músculos de la nuca para el registro polisomnográfico en los experimentos del capítulo II.

Polisomnografía

Para el estudio del ciclo sueño-vigilia se utilizaron los registros del EEG y electromiograma (EMG). Los registros polisomnográficos se realizaron durante 4 horas luego de las microinyecciones de HCRT o su antagonista en el APOm. Además del diagnóstico del sueño y vigilia, se realizó un análisis más detallado del EEG comparando las potencias (el cuadrado de la amplitud) para las distintas frecuencias registradas.

Registro del comportamiento maternal

Para el estudio de este comportamiento se utilizaron dos estrategias. En el primer trabajo (cap. I), se realizó una prueba de comportamiento maternal de 30 min mediante observación directa, en la que se registró el número de los distintos comportamientos, así como la latencia y duración de las distintas posturas. En el segundo trabajo (cap. II), se analizó el comportamiento materno a partir de los videos adquiridos durante las 4 horas siguientes a la administración del fármaco, mediante la clasificación en tres estados comportamentales.

Otras pruebas de comportamiento

En los experimentos del cap. I, con el fin de descartar alteraciones motoras por la microinyección de HCRT, se realizó una prueba de actividad locomotora inmediatamente después de la de comportamiento maternal. Seguido a esta prueba, para determinar si los efectos de las HCRT en el comportamiento maternal se debían a cambios afectivos, se utilizó el laberinto elevado en cruz como modelo de ansiedad.

Registros de unidades neuronales

Para los registros electrofisiológicos, los animales se anestesiaron con uretano, se fijaron a un aparato estereotáxico y se realizó una perforación en el cráneo en el lugar de registro en el APOm. Se utilizaron como microelectrodos micropipetas de vidrio dobles fabricadas en el laboratorio, una de ellas rellena de solución conductora y otra con HCRT-1 para su administración yuxtacelular mediante pulsos de presión controlados. Además, se colocaron electrodos para el registro del EEG junto con el registro de unidades. Este experimento lo realizamos bajo anestesia con uretano, ya que con este anestésico ocurren alternancias espontáneas y rítmicas entre dos patrones electroencefalográficos diferentes, que se asemejan a los de sueño-vigilia (Clement et al., 2008).

Análisis histológico

Luego de finalizados todos los experimentos, los cerebros fueron procesados, seccionados en cortes frontales seriados y observados en lupa o microscopio óptico para la verificación del sitio de microinyección (cap. I y II), así como la localización de los microelectrodos (cap. III) en el APOm.

CAPÍTULO I. El Sistema Hipocretinérgico en el APOm modula el comportamiento maternal de ratas lactantes

En este primer trabajo, determinamos, por primera vez, cómo afecta la modulación por las HCRT del APOm al comportamiento maternal en ratas lactantes. Si bien los antecedentes bibliográficos indican que estos neuropéptidos participan en el control de este comportamiento, hasta ahora se desconocía si lo hacían específicamente a través de esta zona neural. Para explorar esta idea, administramos HCRT-1 y el antagonista de su receptor mediante microinyecciones en el APOm y posteriormente estudiamos el comportamiento materno en la primera y segunda semana posparto. Inmediatamente después de evaluar el comportamiento maternal, realizamos pruebas de actividad locomotora y de ansiedad experimental. En este trabajo pudimos concluir que las HCRT son capaces de modular el comportamiento maternal a través del APOm. Específicamente, mostramos que la HCRT-1 promueve los comportamientos maternos de tipo activo, mientras que su bloqueo los disminuye, a la vez que promueve los pasivos. Observamos un efecto mayor en la segunda semana posparto con la administración exógena de HCRT-1, mientras que el antagonista tuvo mayor efecto en la primera semana, sugiriendo que los niveles endógenos de HCRT-1 podrían ser mayores en la primera semana respecto a la segunda. Además, no se registraron cambios en la actividad locomotora ni en la ansiedad experimental de las madres, mostrando que el efecto de las HCRT en el APOm es específico del comportamiento maternal.

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Hypocretinergic system in the medial preoptic area promotes maternal behavior in lactating rats

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ABSTRACT

Hypocretin-1 and 2 (HCRT-1 and HCRT-2, respectively) are neuropeptides synthesized by neurons located in the postero-lateral hypothalamus, whose projections are widely distributed throughout the brain. The hypocretinergic (HCRTergic) system has been associated with the generation and maintenance of wakefulness, as well as with the promotion of motivated behaviors. In lactating rats, intra-cerebroventricular HCRT-1 administration stimulates maternal behavior, whilst lactation *per se* increases the expression of HCRT type 1 receptor (HCRT-R1). Due to the fact that HCRTergic receptors are expressed in the medial preoptic area (mPOA), a region critically involved in maternal behavior, we hypothesize that HCRT-1 promotes maternal behavior acting on this region. In order to evaluate this hypothesis, we assessed the maternal behavior of lactating rats following microinjections of HCRT-1 (10 or 100 μM) and the selective HCRT-R1 antagonist SB-334867 (250 μM) into the mPOA, during the first and second postpartum weeks. While intra-mPOA microinjections of HCRT-1 (100 μM) increased corporal pup licking during the second postpartum week, the blockade of HCRT-R1 significantly decreased active components of maternal behavior, such as retrievals, corporal and ano-genital lickings, and increased the time spent in nursing postures in both postpartum periods. We conclude that HCRTergic system in the mPOA may stimulate maternal behavior, suggesting that endogenous HCRT-1 is necessary for the natural display of this behavior.

1. Introduction

The hypocretinergic (HCRTergic) system has been associated with the promotion of motivated behaviors, including exploratory activity as well as food, sexual and drug seeking behaviors [6,9,19,23,25,42,44,45]. The hypocretins (HCRT) consist of two neuropeptides: HCRT-1 and HCRT-2, also known as orexin A and orexin B respectively. These neuropeptides are synthesized by neurons located in the postero-lateral hypothalamus whose projections are widely distributed throughout the brain [22,32,38,43] HCRT exert their biological functions through two metabotropic receptors: the HCRT type 1 (HCRT-R1) and type 2 receptors (HCRT-R2) that differ in their affinity to HCRT-1 [33].

Several experimental studies indicate that the HCRTergic system plays a role during the lactating period. In this regard, the number of HCRTergic neurons expressing Fos-immunoreactivity is larger in lactating female mice versus virgin ones [14]. Furthermore, prepro-HCRT mRNA and HCRT-R1 mRNA levels in the entire hypothalamus are significantly higher on day 1 of lactation than during late pregnancy and late lactation [48], suggesting that HCRT may be involved in the regulation of certain aspects of maternal behavior in the early stages of lactation. Despite this evidence, only one study has directly examined the effect of HCRT on maternal behavior; D'Anna and Gammie showed that intracerebroventricular (i.c.v.) injections of HCRT-1 increase the number of lickings and groomings of pups, and the number of nursing bouts at intermediate doses in lactating mice [10]. However, the specific areas that mediate this effect remain unknown. Interestingly, HCRT receptors, HCRT-R1 in particular, are expressed in the medial preoptic area (mPOA) [21,46], a critical region involved in the onset and maintenance of maternal behavior [26,28,36].

The mPOA is a key neural site where the hormones of pregnancy and parturition act to synchronize maternal responsiveness [7,27]. In addition, it has been suggested that several neuromodulators, including oxytocin, melanin-concentrating hormone (MCH), amylin and neurotensin mediate the mPOA adjustments,

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underlying the postpartum expression of maternal behavior [3,8,11,18,37,47]. Based on these findings and taking the importance of HCRT on the motivational aspects of behavior into account, as well as its relation with lactation, we hypothesized that HCRT promote maternal behavior acting in the mPOA. In order to evaluate this hypothesis, we assessed the maternal behavior of lactating rats following microinjections of HCRT-1 and the selective HCRT-R1 antagonist SB-334867 into the mPOA during the first and second postpartum weeks.

2. Material and methods

2.1. Animals and housing

Fifty-one primiparous Wistar female rats (240–310 g) and pups were used in this study. The experimental procedures were in strict accordance with the "Guide for the care and use of laboratory animals" (8th edition. National Academy Press, Washington D. C., 2011) and approved by the Institutional Animal Care Committee. All efforts were made in order to minimize the number of animals and their suffering. We used the same experimental protocol as Benedetto et al. [3]. Briefly, animals were housed in a temperature-controlled room under a 12-h light/dark cycle, with *ad libitum* access to food and water. Two days before giving birth, pregnant females were housed individually. On postpartum day 1 (PPD1, birth = day 0), litters were culled to four female and four male pups per mother.

2.2. Stereotaxic surgery

On the morning of PPD3 or PPD11, females were anesthetized with a mixture of ketamine/xylazine/acepromazine maleate (80/2.8/2.0 mg/kg i.p.). Female rats were bilaterally implanted with 22-gauge stainless steel guide cannulae (Plastic One, Roanoke, VA) aimed 2 mm dorsal to the mPOA: AP −0.5 mm (from Bregma); ML ± 0.5 mm (from midline); DV −6.5 mm (from skull) according to Paxinos and Watson [29]. In addition, three stainless steel screws were implanted into the skull as anchors and, together with the guide cannulae, they were secured to the skull with dental cement.

Immediately after surgery, each mother was reunited with her pups in the home cage. All females remained healthy throughout the experiment, exhibiting typical maternal behaviors.

2.3. Experimental design

Animals were randomly assigned to one of the following six independent groups according to drug dosage and the postpartum stage: (1) 10 μM HCRT-1 in the first postpartum week (1stWK) (n = 6), (2) 100 μM HCRT-1 in 1stWK (n = 8), (3) 10 μM HCRT-1 in the second postpartum week (2ndWK) (n = 7), (4) 100 μM HCRT-1 in 2ndWK (n = 8), (5) 250 μM SB-334867 in 1stWK (n = 8) and (6) 250 μM SB-334867 in 2ndWK (n = 8). In every group, each female was microinjected with vehicle and drug on two different days (PPD 6 and 7: 1stWK or PPD 14 and 15: 2ndWK) in a counterbalanced order.

2.4. Drugs

HCRT-1 (Human, mouse, rat; Phoenix Pharmaceuticals Inc., Belmont, CA) was diluted in a sterile saline solution to obtain a final concentration of 10 and 100 μM (the dose was adjusted according to Ref. [49]). Aliquots for these doses were prepared in advance, frozen at −20 °C, and thawed immediately before use. SB-334867, a HCRT-R1 antagonist [35] (provided by Glaxo-Smith-Kline, Essex, UK), was diluted in dimethyl sulfoxide (DMSO) 2% to obtain a final concentration of 250 μM (the dose was adjusted according to Ref. [50]).

2.5. Microinjection procedure

Females were bilaterally microinjected with 0.3 μl of either HCRT-1, SB-334867 or the same volume of vehicle into the mPOA over a period of 3 min, with the injection cannulae (28 gauge; Plastic One, Roanoke, VA) extending 2 mm beyond the tip of the guide cannulae, with a constant-rate infusion pump (Harvard apparatus, USA). The administration cannulae were left in place for an additional minute to allow for the diffusion of the drug. A similar microinjection volume was used in previous studies of the group [3,20].

2.6. Behavioral testing

All behavioral tests were performed during the light phase of the light/dark cycle, between 09:00 and 11:00 AM.

2.6.1. Maternal behavior testing

Following the microinjections procedure, the females were returned to their home cages. 10 min later, the pups were scattered in the mothers' home cages opposite to the nest. Maternal behaviors such as retrievals of the pups to the nest, mouthings, corporal lickings, ano-genital lickings and nest building were counted over a 30 min period. The latencies to retrieve the first pup and to group the entire litter into the nest were also measured. In addition, the latencies and durations of hovering over the pups and nursing postures were recorded. The number of eating, drinking and self-grooming behaviors was also annotated [31].

2.6.2. Locomotor activity

In order to evaluate any non-specific motor disturbance induced by HCRT-1 or SB-334867 administration, the locomotor activity was assessed immediately after the maternal behavioral test. Due to the fact that this test can be conducted only once per animal, it was carried out on independent groups of animals. The following groups were employed: 100 μM HCRT-1-treatment day in the 1stWK (from group 2) and 2ndWK (from group 4), SB-334867-treatment day in the 2ndWK (from group 6) and vehicle-treatment day in the 1stWK (from group 1) and in the 2stWK (from group 3). The number of line-crosses and rearings were measured over a 5-min session in a cage measuring 60 × 40 × 40 cm, that was divided into six equal quadrants (adapted from Ref. [16]).

2.6.3. Elevated plus-maze test

The elevated plus maze (EPM) test was performed after the locomotor activity test in the same animals. The EPM apparatus consists of an elevated (50 cm above the floor) plus-shaped maze with four arms (50 cm long × 10 cm wide), two of which are enclosed by walls 40 cm in height (closed arms), while the other arms lack walls (open arms) [30]. The cumulative time spent in the open arms and the number of open arm entries were recorded over a 5-min session. The data is expressed as the percentage of time spent in the open arms and the number of open arm entries.

2.7. Histological verification of injection sites

At the end of the experiment the animals were euthanized with an overdose of ketamine/xylazine, perfused with 4% paraformaldehyde, and their brains were removed for histological processing. Thereafter, the brains were cut in 100 μm coronal sections with a vibratome. The location of mPOA microinjection sites was verified according to Paxinos and Watson [29].

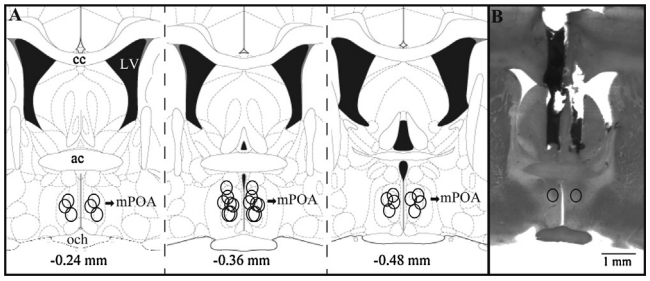

Fig. 1. Microinjections sites. (A) Schematic representations of coronal sections at the level of mPOA. Circles indicate the microinjection sites of the HCRT-1 10 and 100 μM in the 1stWK (groups 1 and 2, n = 14); bottom numbers indicate distance from Bregma. Plates were taken from the atlas of Paxinos and Watson [29]. ac, anterior commisure; cc, corpus callosum; LV, lateral ventricle; och, optic chiasm. (B) Representative topographic microphotography showing cannula track, circles indicate the microinjection sites.

2.8. Statistics

All behavioral data is presented as median (semi-interquartile ranges [SIQR]). As the data did not follow a normal distribution (Kolmogorov–Smirnov test, $p < 0.05$), non-parametric tests were utilized [34]. Mann–Whitney U tests were used for comparisons of independent groups and Wilcoxon matched-pair signed-ranks tests were used for intra group comparisons. A $p < 0.05$ was used in all analyses to discard the null hypothesis.

3. Results

3.1. Sites of injection

All microinjections of animals included in the study were located within the mPOA between −0.12 and −0.48 mm from Bregma, based on the examination of the cannulae tracks in Section 2.7 [29]. In six animals the guide cannulae was located outside the mPOA and because these animals belong to four different experimental groups, they were excluded from the data analysis. Therefore, a total of 45 animals were included in the study. Fig. 1 shows the microinjection sites of the HCRT-1 10 and 100 μM in the 1stWK (groups 1 and 2, n = 14). The location of the microinjection sites had similar distribution in the other groups.

3.2. Effect of HCRT-1 on maternal behavior

The low dose of HCRT-1 (10 μM) did not modify maternal behavior in any postpartum periods compared with the respective control group (Fig. 2). The high dose of HCRT-1 (100 μM) significantly increased corporal pup licking during the 2ndWK ($T_8 = 2.37$, $p = 0.018$) while it did not affect any maternal behavior in the 1stWK (Fig. 2). Moreover, compared with the control groups, latencies and durations of huddling/nursing behaviors did not differ after microinjections of both doses of HCRT-1 either on the 1stWK and 2ndWK (Table 1).

The HCRT-1 (100 μM) increased self-grooming on the 2ndWK (saline = 7.5 ± 5.8, HCRT-1 = 24.5 ± 3.38; $T_8 = 2.24$, $p = 0.025$). Other non-maternal behaviors such as drinking and eating were not affected by any of the HCRT-1 doses used (data not shown).

3.3. Effect of HCRT-R1 antagonist SB-334867 on maternal behavior

Microinjections of 250 μM SB-334867 into the mPOA significantly decreased the active components of maternal behavior both in the 1stWK and 2ndWK compared with vehicle microinjections (Fig. 3). Specifically, SB-334867 decreased the number of corporal

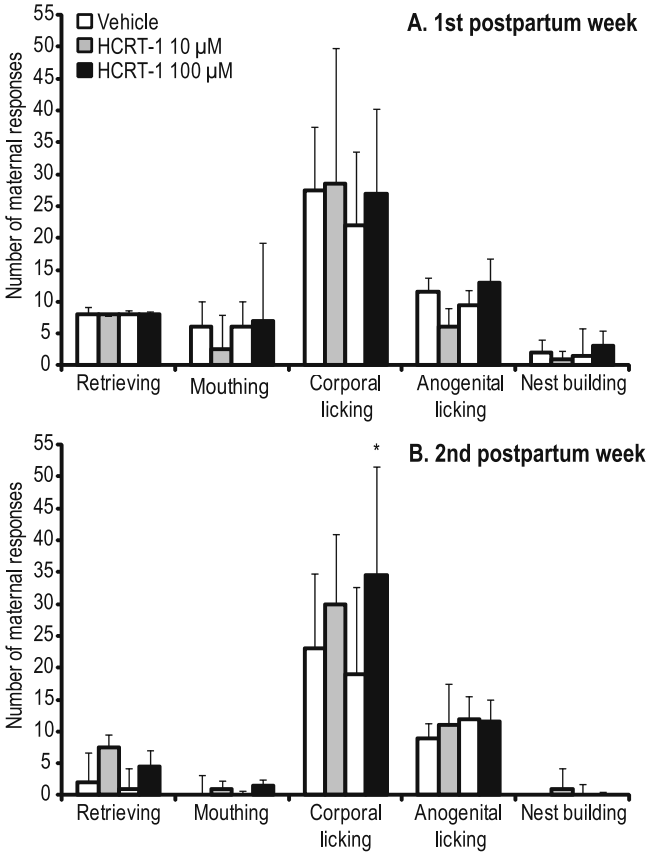

Fig. 2. Effect of HCRT-1 microinjections into the mPOA on active components of maternal behavior. The chart shows the number of different active maternal responses on first (A) and second (B) postpartum week following bilateral administration of either saline or HCRT-1 (10 and 100 μM/side) over a 30 min maternal test. Within-group comparisons were analyzed with Wilcoxon matched-pair signed-ranks test; significant differences are indicated by asterisks (*$p < 0.05$).

($T_8 = 2.2$, $p = 0.028$) and ano-genital lickings ($T_8 = 2.5$, $p = 0.012$) on 1stWK, as well as the number of retrievals ($T_8 = 2.2$, $p = 0.028$) and corporal lickings ($T_8 = 2.5$, $p = 0.012$) on the 2ndWK compared with the respective control groups. Moreover, the latency to retrieve the first pup was higher following the administration of SB-334867 compared with the vehicle in the 2ndWK ($T_8 = 2.02$, $p = 0.043$; Table 2). In addition, SB-334867 decreased the time spent in hover over both during the 1stWK and 2ndWK ($T_8 = 2.52$; $p = 0.012$ and $T_8 = 2.52$; $p = 0.012$), and it increased the time in nursing postures in the 1stWK ($T_8 = 1.96$; $p = 0.049$). The time spent in nursing postures tended to be higher in the 2ndWK ($T_8 = 1.82$; $p = 0.069$).

SB-334867 also decreased self-grooming behaviors (vehicle = 12 ± 3.8; SB-334867 = 5.5 ± 2.5; $T_8 = 2.17$, $p = 0.030$) but only during the 1stWK. SB-334867 did not affect drinking or eating behaviors (data not shown).

3.4. Anxiety-like behavior and locomotor activity

No differences were detected in the time spent or the number of entries into the open arms of the EPM, after intra-mPOA HCRT-1 or SB-334867 administration (Table 3). Moreover, no significant differences were found in the locomotor parameters among HCRT-1 100 μM, SB-334867 250 μM and vehicle-treated groups (Table 3).

4. Discussion

The present study shows that while intra-mPOA microinjections of HCRT-1 produced minor effects on maternal behavior, the

Table 1
Latencies and durations of maternal behaviors on first or second postpartum week, following either HCRT-1 (10 and 100 μM/side) or saline treatment.

	1st postpartum week				2nd postpartum week			
	Saline	HCRT 10 μM	Saline	HCRT 100 μM	Saline	HCRT 10 μM	Saline	HCRT 100 μM
Active behaviors								
Latency (s)								
First retrieval	7.5 ± 2.75	3.5 ± 5.5	7.0 ± 10.8	10.0 ± 5.0	19.0 ± 448.2	56.0 ± 449.0	48.0 ± 890.4	24.5 ± 247.5
Reunion litter	67.0 ± 31.1	83.5 ± 70.6	76.0 ± 20.8	71.0 ± 26.0	1800.0 ± 440.0	135.0 ± 871.3	1800.0 ± 0.0	1800.0 ± 213.3
Huddling and nursing behaviors								
Latency (s)								
Hover over	151.0 ± 15.0	329.0 ± 138.0	185.0 ± 51.3	232.0 ± 73.3	303.0 ± 271.8	231.0 ± 409.2	1298.5 ± 738.0	1525.0 ± 694.2
Nursing postures	1107.0 ± 661.5	731.0 ± 223.5	851.0 ± 140.0	898.0 ± 295.5	835.0 ± 396.3	631.0 ± 277.2	745.5 ± 317.0	721.5 ± 297.0
Duration (s)								
Hover over	719.0 ± 193.0	495.0 ± 190.0	564.0 ± 55.0	961.0 ± 166.5	449.0 ± 135.8	500.0 ± 95.7	333.5 ± 179.0	221.5 ± 32.1
Nursing postures	646.0 ± 501.0	1133.0 ± 287.5	874.0 ± 146.5	563.0 ± 102.8	1042.0 ± 253.1	930.0 ± 219.0	937.0 ± 215.6	644.0 ± 315.8

Data are expressed as median ± SIQR. Within-group comparisons were analyzed with the Wilcoxon match pairs signed-ranks test; there were not significant differences between groups.

Table 2
Latencies and durations of maternal behaviors on first or second postpartum week, following either HCRT-R1 antagonist SB-334867 or vehicle treatment.

	1st postpartum week		2nd postpartum week	
	Vehicle	SB-334867	Vehicle	SB-334867
Active behaviors				
Latency (s)				
First retrieval	5.5 ± 7.4	4.5 ± 6.7	4.0 ± 7.2	39.5 ± 899.6*
Reunion litter	252.0 ± 510.2	42.0 ± 885.5	137.0 ± 872.7	1800.0 ± 221.7
Huddling and nursing behaviors				
Latency (s)				
Hover over	164.0 ± 151.5	741.0 ± 842.6	384.5 ± 112.5	144.0 ± 523.6
Nursing postures	473.0 ± 291.8	385.0 ± 63.2	666.5 ± 132.9	379.0 ± 94.8
Duration (s)				
Hover over	484.0 ± 163.5	293.0 ± 110.9*	515.0 ± 86.8	307.0 ± 38.5*
Nursing postures	1056.5 ± 281.5	1409.0 ± 136.9*	883.5 ± 145.6	1196.5 ± 276.9

Data are expressed as median ± SIQR. Within-group comparisons were analyzed with the Wilcoxon match pairs signed-ranks test. Significant differences are indicated by asterisks.
* $p < 0.05$.

Table 3
Elevated plus maze test and locomotor activity. Effect of bilateral microinjections of HCRT-1 (100 μM) and SB-334867 (250 μM) into the mPOA on behavior during the elevated plus maze test and locomotor activity.

	1st postpartum week		2nd postpartum week		2nd postpartum week	
	Vehicle	HCRT	Vehicle	HCRT	Vehicle	SB-334867
Locomotor activity (counts/5 min)						
Crossings	45.5 ± 14.9	29.0 ± 15.3	46.0 ± 14.0	36.0 ± 14.5	39.5 ± 12.1	52.5 ± 1.5
Rearings	19.0 ± 4.3	15.0 ± 7.3	23.0 ± 6.5	18.0 ± 2.5	23.0 ± 4.9	27.0 ± 4.6
Elevated plus maze						
%Entries in open arms	40.0 ± 19.6	52.9 ± 13.6	54.5 ± 10.5	38.9 ± 7.8	50.6 ± 17.6	28.2 ± 5.1
%Time spent in open arms	17.0 ± 16.4	44.3 ± 25.9	20.3 ± 9.3	23.3 ± 2.7	18.3 ± 9.4	6.8 ± 1.9

All data are expressed as median ± SIQR. Group comparisons were analyzed with the Mann–Whitney U test. There were not significant differences between groups.

blockade of HCRT-R1 reduced the active maternal behavior whilst increasing the passive maternal components. These data suggest that by modulating the activity of mPOA neurons, the HCRTergic system regulates the expression of maternal behavior.

In this report we utilized the same microinjection procedure as in previous studies of our group [3,20], and similar volumes and concentration of the drugs have been utilized before [49,50]. Although the microinjection sites were localized within the mPOA, we cannot completely rule out that at least part of the effects of the drugs may be caused by a diffusion to surrounding areas (see Ref. [20] for further discussion about technical issues).

HCRT-1 microinjection into the mPOA increased pup-licking while SB-334867 decreased pup-licking and pup retrieval components of maternal behavior. These results are consistent with previous evidence that shows an increase in the number of lickings and groomings after i.c.v. microinjections of HCRT-1, while systemic administration of SB-334867 tends to impair this type of behavior in lactating mice [10]. In this study we extend the knowledge of the effects of HCRT-1 on maternal behavior, showing the intracerebral site of action. Interestingly we observed mostly an opposite but larger effect of the antagonist compared to the agonist. The SB-334867 is a non-peptide HCRT-R1 antagonist that was described by Smart et al. in Chinese hamster ovary cells that express HCRT receptors [35]. Although we cannot entirely discard a non-selective effect of the antagonist, we believe that the differences in the power of the effects could be due to a ceiling effect of the HCRT-1; *i.e.*, increasing the HCRT-1 levels exogenously only slightly increases an already active maternal behavior.

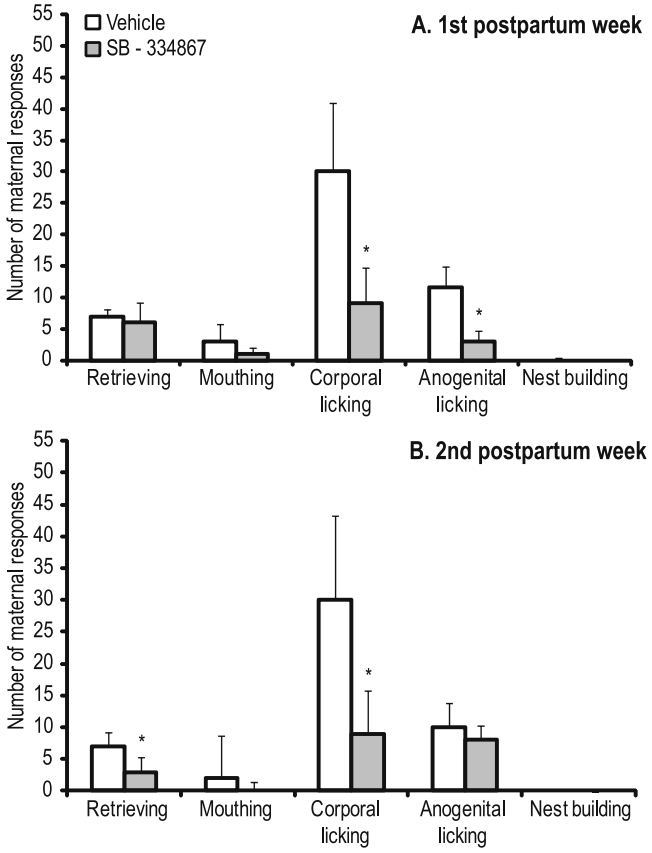

Fig. 3. Effect of HCRT-R1 antagonist SB-334867 microinjections into the mPOA on active components of maternal behavior. The chart shows the number of different active maternal responses on first (A) and second (B) postpartum week following bilateral administration of vehicle or SB-334867 (250 μM/side) over a 30 min maternal test. Within-group comparisons were analyzed with Wilcoxon matched-pair signed-ranks test; significant differences are indicated by asterisks (*$p < 0.05$).

Based on Wang et al., that showed that prepro-HCRT and HCRT-R1 mRNA levels are higher on postpartum day 1 than in late lactation, we expected to find some differences in the effects of HCRTergic agents between the two postpartum periods under study. However, we did not find major differences, except that HCRT-1 increased pup-licking only in the 2ndWK and SB-334867 decreased retrieval behavior only in the 2ndWK. One possible explanation is that we did not study postpartum day 1, when the HCRT-R1 mRNA expression is higher.

The total amount of the time spent in nursing, a passive behavior, significantly increased, while the time spent in hover over decreased after the blockade of endogenous HCRT-1 in the mPOA. This fact could be related to a sleep-promoting effect of the HCRT-R1 antagonist. This is in accordance with extensive evidence showing that HCRT promote wakefulness [9] and that the preoptic area is a sleep-promoting area [2,4,39]. Specifically, infusion of HCRT-1 into the mPOA elicits a greater increase in wakefulness than its administration into other structures [13]. However, the HCRTergic system is not involved in the maintenance of wakefulness *per se*, but is implicated when the animals are performing goal-directed behaviors [42]. Interestingly, studies in humans suggest that HCRT promote arousal associated with positive emotions and social activity rather than arousal in general [5]. In this regard, our results suggest that the HCRTergic system is involved in maternal behavior, a highly motivated behavior that heightens arousal.

After local administration of HCRT-1, self-grooming increased while the microinjection of SB-334867 provoked the opposite effect. These results are consistent with previous findings in males which show that the administration i.c.v. and intra-mPOA of HCRT-1 elicits an increase in self-grooming activity [12,13] and the HCRT cells exhibit a high discharge rate during this self-grooming behavior [24]. Although self-grooming could be related to anxiety [15], neither HCRT-1 nor the HCRT-R1 antagonist affected anxiety-like behavior or locomotor activity in this study. Although in a recent review Flores et al. concluded that a dysregulation of the HCRTergic system contributes to pathologies associated with generalized anxiety and/or impaired fear processing [17], it is unlikely that the mPOA plays a critical role in these processes.

A large amount of data shows a reciprocal, anatomical and functional relationship between the HCRTergic activity and the MCHergic neuronal system [40,41]. Basically, both systems play opposite roles in the regulation of motivated behaviors, as well as in several physiological processes, such as the sleep-wake cycle, energy expenditure and glucose metabolism [1,41]. It has also been suggested that both neuropeptides have complementary roles in motivated behavior, such as feeding, where HCRT promote food-seeking, while MCH is involved in ongoing food intake [1]. In this sense, we showed that the microinjection of MCH into the mPOA decreased the expression of active maternal behaviors in postpartum female rats [3]. Although nursing time did not increase, this could be due to the long latency to the reunion of the pups. This evidence together with the present results, suggests that these neuropeptides would act in coordination within the mPOA to modulate the expression of maternal behavior; HCRT promote active maternal behavior and inhibit nursing, while MCH produces the opposite effects.

In conclusion, these results suggest that maternal behavior, like other motivated behaviors, is stimulated by the HCRTergic system. In addition, endogenous levels of HCRT-1 in the mPOA are necessary for appropriate maternal behavior in lactating rats. Future studies should aim to elucidate if the HCRTergic projections toward the mPOA regulate simultaneouslyboth maternal behavior and sleep.

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CAPÍTULO II. Rol de las hipocretinas en el APOm en la regulación integrada del sueño, comportamiento maternal y temperatura corporal de ratas lactantes.

Dados los resultados obtenidos en el capítulo anterior y teniendo en cuenta la participación de las HCRT en la regulación de la vigilia, nos preguntamos si esta modulación en el comportamiento maternal es simultánea con cambios en el sueño-vigilia. Si bien es conocido el rol promotor de vigilia por las HCRT, hasta el momento no se conocía si estas modulaban el APOm durante el período posparto en ratas lactantes. En este sentido, hipotetizamos que la facilitación del amamantamiento por el bloqueo de la HCRT-1 endógena en el APOm se asocia a un efecto promotor de sueño. Además, dado que el APOm es un área importante en el control de la temperatura corporal, y ésta se ha asociado tanto al sueño como también a algunos aspectos del comportamiento maternal, evaluamos si las HCRT en el APOm producen cambios en la temperatura corporal de ratas lactantes. A través de este trabajo, mostramos que las HCRT en el APOm aumentan la vigilia junto con un leve pero significativo aumento en la temperatura corporal, sin afectar el comportamiento materno. A su vez, el bloqueo de la HCRT endógena aumenta el amamantamiento y el sueño simultáneamente, apoyando nuestra hipótesis. Estos cambios no fueron acompañados por variaciones en la actividad del EEG, evaluado por las potencias de las distintas frecuencias registradas. Estos resultados muestran, por primera vez, los efectos integrados de modular el APOm en el comportamiento maternal, el sueño y la temperatura corporal.

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Role of Hypocretin in the Medial Preoptic Area in the Regulation of Sleep, Maternal Behavior and Body Temperature of Lactating Rats

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Abstract—Hypocretins (HCRT), also known as orexins, includes two neuroexcitatory peptides, HCRT-1 and HCRT-2 (orexin A y B, respectively), synthesized by neurons located in the postero-lateral hypothalamus, whose projections and receptors are widely distributed throughout the brain, including the medial preoptic area (mPOA). HCRT have been associated with a wide range of physiological functions including sleep-wake cycle, maternal behavior and body temperature, all regulated by the mPOA. Previously, we showed that HCRT in the mPOA facilitates certain active maternal behaviors, while the blockade of HCRT-R1 increases the time spent in nursing. As mother rats mainly sleep while they nurse, we hypothesize that HCRT in the mPOA of lactating rats reduce sleep and nursing, while intra-mPOA administration of a dual orexin receptor antagonist (DORA) would cause the opposite effect. Therefore, the aim of this study was to determine the role of HCRT within the mPOA, in the regulation and integration of the sleep-wake cycle, maternal behavior and body temperature of lactating rats. For that purpose, we assessed the sleep-wake states, maternal behavior and body temperature of lactating rats following microinjections of HCRT-1 (100 and 200 μM) and DORA (5 mM) into the mPOA. As expected, our data show that HCRT-1 in mPOA promote wakefulness and a slightly increase in body temperature, whereas DORA increases both NREM and REM sleep together with an increment of nursing and milk ejection. Taken together, our results strongly suggest that the endogenous reduction of HCRT within the mPOA contribute to the promotion of sleep, milk ejection and nursing behavior in lactating rats.

Key words: hypocretin, orexin, hypothalamus, mPOA, sleep, nursing.

INTRODUCTION

Hypocretins (HCRT) or orexins, are two neuroexcitatory peptides (HCRT-1 and HCRT-2 or orexin A and B, respectively) that bind to two metabotropic receptors: HCRT-R1 and HCRT-R2. This neuropeptide system originates in neurons located in the postero-lateral hypothalamus that have a widespread distribution along the brain (Peyron et al., 1998; Sakurai et al., 1998; Trivedi et al., 1998; Taheri and Bloom, 2001). It is of particular interest to this study that HCRT receptors, particularly HCRT-R1, is found in the mPOA (Trivedi et al., 1998; Marcus et al., 2001), a critical region for the regulation of several physiological functions, including sleep and wakefulness, body temperature (Kumar, 2004), as well as the maternal care of the pups (Numan, 1974; Numan and Stolzenberg, 2009; Stolzenberg and Numan, 2011).

The hypocretinergic (HCRTergic) system has been associated with several physiological functions including the maintenance of wakefulness, the promotion of several motivated behaviors, such as maternal behavior, and the control of body temperature (Yoshimichi et al., 2001; Harris et al., 2005; D'Anna and Gammie, 2006; Muschamp et al., 2007; Boutrel et al., 2010; McGregor et al., 2011; Torterolo et al., 2011; Rivas et al., 2016). Besides, the sleep disorder called narcolepsy, caused by the degeneration of the hypocretinergic neurons, is characterized by sleep attacks and cataplexy as well as the disruption of thermoregulation (van der Heide et al., 2016; Harding et al., 2019).

There is a great experimental evidence showing that the mPOA regulates the wake-sleep cycle. Specifically, lesions of the mPOA produced a reduction in non-REM (NREM) and REM sleep (John and Kumar, 1998; Lu et al., 2000; Srividya et al., 2006). In addition, the administration of glutamate (Kaushik et al., 2011) or adenosine (Mendelson, 2000) into the mPOA, as well as the activation of GABAergic and galaninergic neurons in this area promote NREM sleep (Chung et al., 2017; Harding

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et al., 2018; Kroeger et al., 2018; Vanini et al., 2020). Recently, it has been reported that the chemogenetic activation of a group of glutamatergic neurons within the mPOA and surroundings areas increases wakefulness, and decreases both NREM and REM sleep (Vanini et al., 2020; Mondino et al., 2021), suggesting that mPOA contains not only sleep-inducing neurons, but also wake-promoting neurons.

Among the several changes that the mother rat experiences during the postpartum period (Numan and Insel, 2003), sleep pattern undergoes through several adjustments. For instance, the mother rat is partially sleep deprived (Rocha and Hoshino, 2009; Sivadas et al., 2016; Toth et al., 2020), sleep is fragmented and delta power band is increased during NREM sleep (Sivadas, 2016). In addition, during the first postpartum week they mostly sleep while they nurse, reducing sleep time during the second postpartum week (Benedetto et al., 2017b), probably as an adaptation of its own sleep physiology to the development of their pups. Not only sleep physiology is altered, but also it has been suggested that the thermoregulatory abilities of mother rats are reduced during lactation (Jans and Leon, 1983; Knecht et al., 1980; Eliason and Fewell, 1997). Additionally, there is a great body of evidence showing that the mPOA physiology is modified along the postpartum period (Fleming and Korsmit, 1996; Numan, 2006; Pereira and Morrell, 2009; Rondini et al., 2010; Uriarte et al., 2020). These changes may not only prepare the lactating female to the maternal care of the pups, but also alter the functionality of different neuronal networks that allow the orchestration of maternal behavior with its own physiology. In recent reports, we explored how different neurotransmitters modifies sleep and maternal behavior acting through the mPOA of the lactating rat. Particularly, the local delivery of raclopride, a dopamine D2 receptor antagonist, into the mPOA reduces REM sleep, and its transitional stage from NREM sleep, while NREM sleep is not affected (Benedetto et al., 2017a). On the contrary, the $GABA_A$ antagonist bicuculline has no effect on sleep but increases active maternal care of the pups (Benedetto et al., 2021). Although it has been shown that the administration of HCRT-1 into the mPOA promotes wakefulness and reduces both NREM and REM sleep in male rats (Espana et al., 2001), the effect in lactating rat is unknown.

The mPOA also plays an important role in thermoregulation (Srividya et al., 2006). In fact, not only the neural mechanisms that regulates both sleep and body temperature seem to coexist anatomically within the mPOA, but are also functionally linked (Kumar, 2004; Harding et al., 2018; Cerri and Amici, 2021). Regarding HCRT, the administration of HCRT-1 into the third ventricle increases body temperature (Yoshimichi et al., 2001), while is reduced by HCRT blockade (Rusyniak et al., 2011; Martin et al., 2019). However, the effect of intra-mPOA administration of HCRT is still unknown.

The mPOA is a crucial area where hormones and several neuromodulators regulate the maternal care of the pups (Benedetto et al., 2014; Rivas et al., 2016; Stolzenberg et al., 2019). In this regard, we have previously shown that administration of HCRT into the mPOA facilitates certain active maternal behaviors, while the blockade of HCRT-1 decreases active components of maternal behavior and promotes nursing (Rivas et al., 2016). Since mother rats mainly sleep while they nurse (Benedetto et al., 2017b), the increased time spent in nursing after HCRT blockade within the mPOA (Rivas et al., 2016) could be related to a sleep-promoting effect. Therefore, we hypothesize that HCRT administration into the mPOA of lactating rats reduces sleep and nursing, while the blockade of endogenous HCRT has the opposite effect. To assess this concept, this study aims to determine the effect of microinjections of HCRT-1 and the dual orexin receptor antagonist (DORA) into the mPOA on sleep and wakefulness, maternal behavior and body temperature in lactating rats.

EXPERIMENTAL PROCEDURES

Animals and housing

A total of 23 primiparous Wistar female rats (250 g) and their pups were utilized in this study. The experimental procedures were approved by the Institutional Animal Care Committee (expedient N° 070153-000304) and based in "Guide for the care and use of laboratory animals" (8th edition. National Academy Press, Washington D. C., 2011). Conditions and experimental protocols were similar as in previous studies (Benedetto et al., 2014; Benedetto et al., 2017a; Benedetto et al., 2017b). Animals were maintained in a temperature-controlled (22 ± 1 °C) room, with a 12-h light/dark cycle (lights on at 6:00 a.m.) and free access to food and water. Two days before parturition, pregnant females were separated in individual cages. On postpartum day 1 (PPD1, birth = day 0), litters were culled to four female and four male pups per mother.

Stereotaxic surgery

On PPD1, females were implanted for polysomnographic recordings and intracerebral microinjections under deep anesthesia (ketamine/xylazine/acepromazine maleate; 80/2.8/2.0 mg/kg i.p. respectively). For that purpose, a 22-gauge stainless steel bilateral guide cannulae (Plastic One, Roanoke, VA) was implanted 2 mm above the target area (mPOA) with the following coordinates: AP −0.5 mm (distance from Bregma); ML ± 0.5 mm (from midline); DV −6.5 mm (from skull) (Paxinos and Watson, 2005). Four EEG electrodes were also implanted (prefrontal cortex: AP = +3.0, ML = 2.0; parietal cortex: AP = −4.0, ML = 3.0; occipital cortex: AP = −7.0, ML = 3.0); an electrode in the cerebellum was also implanted as a reference electrode (AP = −11.0, ML = 0.0). In addition, a bipolar electromyogram (EMG) electrode was located in the dorsal neck muscle. Thereafter the electrodes were soldered to a connector; all components and two additional stainless-steel screws were anchored to the skull using dental acrylic

At the end of the stereotaxic surgery, the lower dorsal part of the flank was shaved and a 1.5–2 cm long incision was made on the skin. The temperature data logger

iButton (Thermochron, model DS2422), was implanted subcutaneously inside a silicone-coat capsule; thereafter, the skin was sutured. The iButton was implanted in the dorsal part of the animal to avoid interference with the mammary glands and suckling of the pups.

Finally, sterile saline (0.9%; 10 ml/kg, s.c.) and a single dose of analgesic (ketoprofen, 5 mg/kg, s.c.) were administrated (Benedetto et al., 2021). Also, topic antibiotic was applied into both surgery injuries (Benedetto et al., 2021).

Experimental design

Experiments were accomplished in the first postpartum week (PPD4-8) during the light phase. Animals were randomly assigned to one of the following independent groups: HCRT or DORA group. For the HCRT group, each animal received a total of three microinjections: HCRT-1 100 μM ($HCRT_{100}$), HCRT-1 200 μM ($HCRT_{200}$) and sterile saline as vehicle. For DORA group, each animal received two microinjections: DORA 5 mM and dimethyl sulfoxide (DMSO) as vehicle. All microinjections were administrated in a counterbalanced design; no experiments were performed the day after the microinjections.

Drugs

HCRT-1 (BACHEM, Bubendorf, Switzerland) was diluted in sterile saline to obtain a final concentration of 100 and 200 μM. Aliquots for these doses were prepared in advance, frozen at −20 °C and thawed immediately before use. DORA TCS-1102 (Sigma–Aldrich, St Louis, USA) was diluted in DMSO 10% to obtain a final concentration of 5 mM. Similar doses were used in previous studies (Hsiao et al., 2012; Korim et al., 2014; Rivas et al., 2016).

Microinjection procedure

Bilateral microinjections into the mPOA of either HCRT-1, DORA, or the same volume (0.2 μl) of its correspondent vehicle were carried out using injection cannulae 2 mm longer than the guide cannulae (28 gauge; Plastic One, Roanoke, VA). The injection cannulae were connected to an infusion pump with a constant rate of 0.1 μl/min (Harvard apparatus, USA), and were left in the target area for one extra minute to allow the correct diffusion of the drug.

Experimental sessions

During each experimental day, pups were removed from the maternal cage for three hours at 9 a.m. and placed under a heat lamp. Fifteen minutes before the completion of maternal separation, microinjection procedure was performed. Thereafter, the mother rat was returned to her home cage and connected to the recording system. Then, the entire litter was weighed and at the completion of the separation period the pups were scattered in the mothers' home cage, opposite to the nest. Subsequently, polysomnographic recording and videotaping of the maternal behavior were initiated for four hours. After each recording session, the mother rat was disconnected from the recording device and the entire litter was weighed again.

Sleep recording

Bioelectric signals were amplified (×1000), filtered (0.1–500 Hz), sampled (1024 Hz, 16 bits) and stored in a PC for further analysis using the Spike 2 software. The behavioral states of light sleep (LS), slow wave sleep (SWS), REM sleep and wakefulness (W) were established in 5-s epochs with standard criteria (Benedetto et al., 2013; Benedetto et al., 2017a; Benedetto et al., 2017b). In addition, the intermediate stage (IS, transition from NREM to REM sleep) was diagnosed (sleep spindles combined with theta activity) (Gottesmann, 1992). Total time spent in W, LS, SWS, NREM sleep (LS + SWS), IS and REM sleep over the total recording time and each hour individually were evaluated. Besides, sleep latencies (first episodes ≥ 20 s from the beginning of the recordings), number, and duration of episodes for each state were analyzed.

EEG spectral power analysis

EEG analysis of power (1–45 Hz) was conducted during the 4 hours after delivery of vehicle or drugs into mPOA. Spectrograms (time–frequency representation of the EEG signal) were examined in Spike 2. To further analyze the spectral characteristics, we studied the average power spectrum from the prefrontal and parietal cortices, employing the pwelch function in MATLAB. We employed 1 s sliding windows with 0.5 s overlap, and a frequency resolution of 1 Hz (parameters: window = 1024, noverlap = [], fs = 1024, nfft = 1024). We normalized the spectra, dividing the power at each frequency by the total power. We only considered the frequency bands up to 45-Hz, to avoid the contamination from the line noise (50 Hz) and its harmonics.

Maternal behavior

Maternal behavior was analyzed from digital videos and classified into three major categories: hovering over the pups (dam over the pups while actively engaged in any activity), nursing (low and high kyphosis and supine postures), and away from the pups. Maternal states were staged in 5-s epochs and analyzed for the 4-h-recording session; an analysis of each hour individually was also performed.

In addition, specific active maternal behaviors were measured: the number of retrievals of the pups into the nest and the latency to group the entire litter. Besides, we analyzed the latency to the first nursing bout ≥2 min, the number of milk ejections through the stretching behavior of the pups (Lincoln et al., 1973; Voloschin and Tramezzani, 1979), and the percentage of the litter weight gain as an indirect measurement of the amount of ejected milk (Lincoln et al., 1973; Peña et al., 2020; Stern, 1991).

Temperature recording

The body temperature was automatically recorded by the iButtons. The onset of measurement was set to initiate 3 days post-surgery for a 5-day period, taking temperature readings every 3 minutes. The temperature resolution of the iButtons was 0.0625 °C. After the experiments were completed and the animals were euthanized, the iButtons were removed and data acquired were downloaded to a PC.

Histological verification of microinjection sites

At the conclusion of all experiment sessions, postpartum rats were euthanized with an overdose of ketamine/xylazine and perfused with 0.9% sterile saline followed by 4% formaldehyde at room temperature. Thereafter, the brains were removed and cut in 100 μm coronal sections using a vibratome for histological processing. Thereafter, the location of the microinjection sites were corroborated (Paxinos and Watson, 2005).

Statistics

All values are presented as mean ± S.E.M (standard error). Data were previously tested for normality by Kolmogorov-Smirnov test and variance homogeneity by the Levene test. Comparisons of sleep and maternal parameters among HCRT-1 groups were evaluated by one-way repeated measures (ANOVA) followed by Tukey *post hoc* test, while paired Student *t*-test was used to compare DORA groups. Since the data did not fit a normal distribution, differences in EEG power among HCRT-1 groups were evaluated utilizing the non-parametric Friedman test using the Bonferroni correction for multiple comparisons and Wilcoxon signed-rank test for the DORA group. Pearson linear correlation was used to study the correlation between the total time in SWS and nursing, the total time in SWS and body temperature, and total time in nursing and body temperature. SWS was used for correlations since it is the longest sleep state while they nurse (Benedetto et al., 2017b). The criterion used to discard the null hypotheses was $p < 0.05$.

RESULTS

Sites of injection

As depicted in Fig. 1, all microinjections included in the study were located within the mPOA between −0.12 and −0.60 mm from Bregma, based on the examination of the cannulae tracks in the histological sections (Paxinos and Watson, 2005). The location of the cannulae track was situated out of the borders of the mPOA in six animals, so they were excluded from the data analysis. Hence, 16 animals were included in the analysis.

Effect of HCRT-1 on sleep and waking states

Representative hypnograms and spectrograms from 0 to 30 Hz are shown for the HCRT group in Fig. 2A. Sleep and waking parameters means are shown in Table 1 and Fig. 3. Compared to vehicle, the total time spent in W significantly increased after the delivery of both $HCRT_{100}$ and $HCRT_{200}$. Furthermore, $HCRT_{200}$ significantly decreased the total time spent in SWS, and tended to reduce the duration of the SWS episodes.

Different REM sleep parameters were modified after HCRT microinjections. Specifically, the total time spent in REM was significantly reduced following $HCRT_{200}$ microinjections. Also, the time spent in REM during the second hour was different among groups ($F_{2,16} = 11.11$, $p \leq 0.01$). Specifically, it was significantly reduced after local delivery of $HCRT_{100}$ ($p \leq 0.02$) and $HCRT_{200}$ ($p \leq 0.01$) compared to vehicle (see Fig. 3). Also, the number of REM episodes decreased and the latency to REM sleep increased after $HCRT_{200}$ when compared to control values (Table 1).

As shown in Table 1 and Fig. 3, no other sleep parameter differed among groups.

Effect of DORA on sleep and waking states

Representative hypnograms and spectrograms of the DORA groups are shown in Fig. 2B. The time spent in W was significantly reduced after microinjection of DORA compared to vehicle treatment, during the total recording time and the fourth recording hour ($t_7 = 2.75$, $p \leq 0.02$; see Table 2 and Fig. 4). In addition, there was a significant reduction in the duration of W episodes (Table 2). DORA local delivery also increased the time spent in SWS during the total recording and the fourth recording hour ($t_7 = 2.62$, $p \leq 0.03$; Fig. 4). Besides, after DORA microinjection REM sleep was increased during the total time and the fourth recording hour ($t_7 = 3.12$, $p \leq 0.01$; Fig. 4). Sleep parameters of LS and IS were not affected by DORA microinjection (Table 2 and Fig. 4).

EEG spectral analysis following administration of HCRT-1 and DORA

The mean EEG power spectrum during wakefulness and sleep following either HCRT or DORA administration into the mPOA are exhibit in Fig. 5. The EEG power did not differ from control in any frequency studied, neither in prefrontal or parietal cortex, during wakefulness or sleep. We also extended the analysis of the frequency bands up to 200 Hz, but no differences were found (data not shown).

Effect of HCRT-1 on maternal behavior

Table 3 and Fig. 6 show the results of HCRT-1 microinjection into mPOA on maternal behavior parameters. Only the litter weight gain decreased following $HCRT_{100}$ microinjection. HCRT-1 did not produce any additional significant changes in the maternal behaviors analyzed.

Effect of DORA on maternal behavior

Compared with vehicle administration, the local delivery of DORA into the mPOA increased the time that females spent nursing their pups. This augmentation was observed in the total recording time (Table 4 and

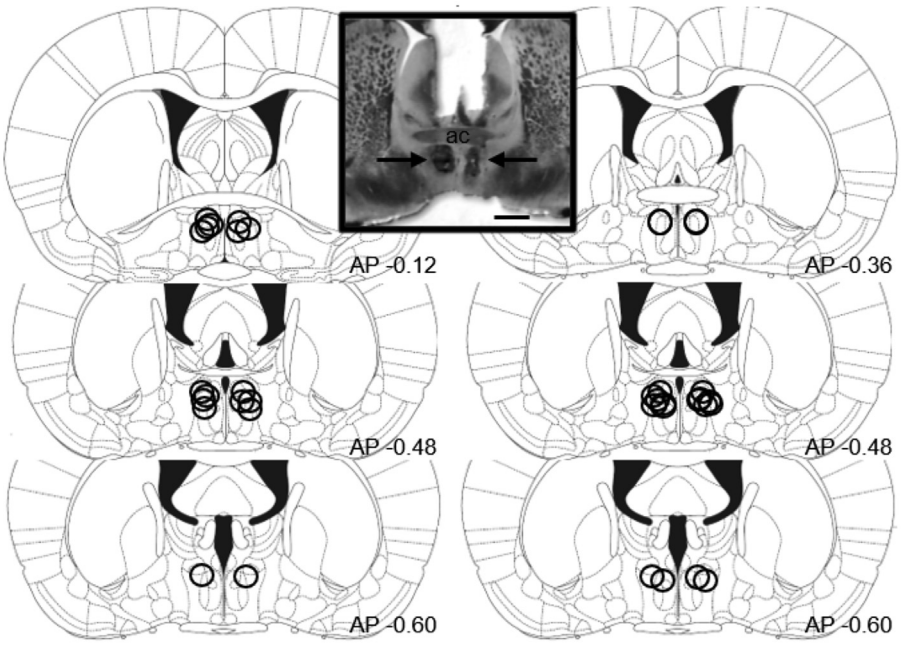


Fig. 1. Microinjection locations. Representative coronal sections plates taken from (Paxinos and Watson, 2005) at the level of mPOA are shown, where black circles indicate the microinjection sites of HCRT-1 (left) and DORA (right); distance from Bregma is indicated with bottom numbers. A representative photography of a microinjection site (arrows) is shown in the center of the Figure. ac: anterior commissure. Calibration bar: 1 mm.

Fig. 6) as well as during the fourth recording hour (t_6 = 2.40, $p \leq 0.03$; Fig. 6). In accordance, the number of milk ejections increased after the microinjection of DORA compared to vehicle, and litter weight gain tended to increase (Table 4). Furthermore, DORA local delivery decreased the time that dams spent away from the pups during the total recording (t_6 = 4.27, $p \leq 0.01$; Fig. 6), as well as during the fourth recording hour (t_6 = 4.81, $p \leq 0.01$; Fig. 6). There were no additional significant changes in other maternal behavior parameters analyzed (Table 4).

Effect of HCRT-1 and DORA on body temperature

The body temperature differed among HCRT-1 groups. Specifically, it increased after the microinjection of $HCRT_{200}$ during the first ($F_{2,\ 14}$ = 9.27, $p \leq 0.01$;

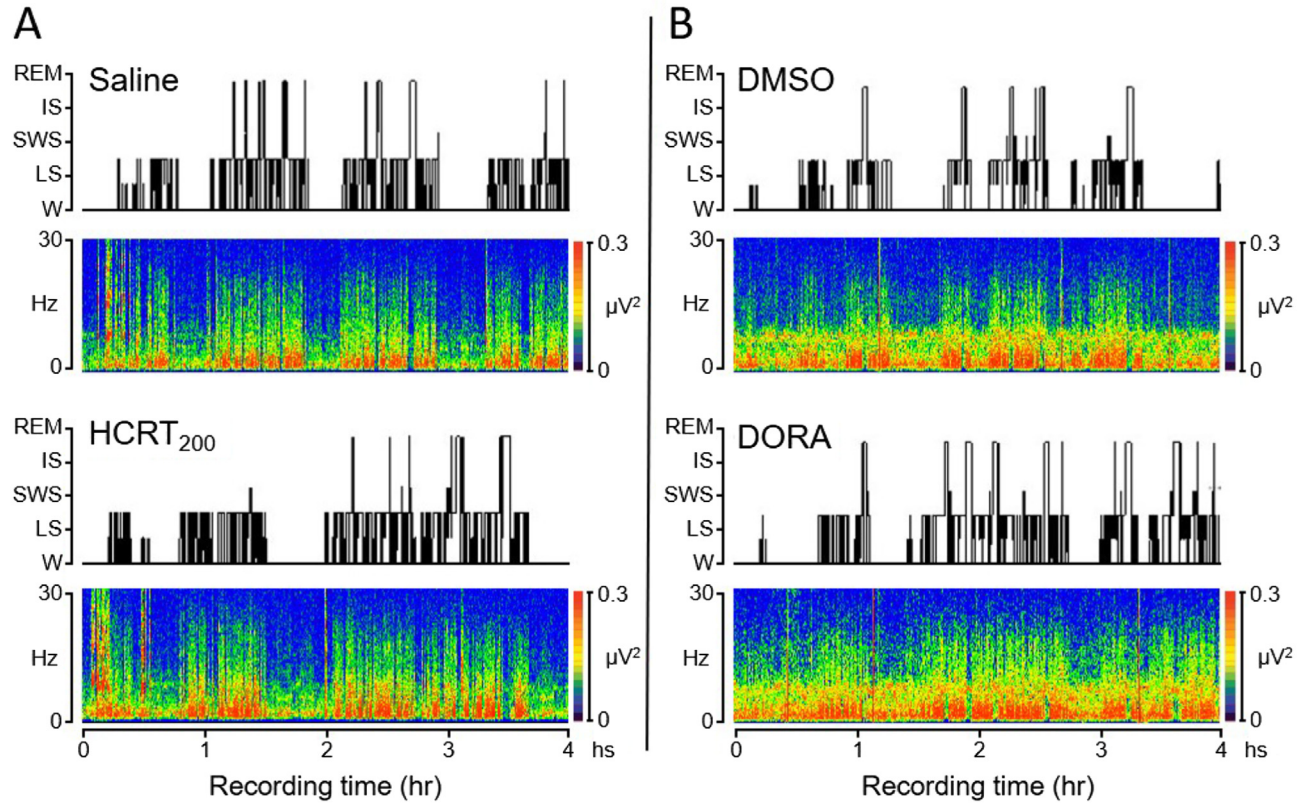


Fig. 2. Representative hypnograms and spectrograms after HCRT-1 and DORA microinjections into the mPOA. Spectrograms (1–30 Hz) from the prefrontal electroencephalogram and hypnograms after saline and $HCRT_{200}$ **(A)**, and DMSO and DORA **(B)** local administration are shown. W, wakefulness; LS, light sleep; SWS, slow wave sleep; IS, intermediate stage and REM, rapid eyes movements sleep.

Table 1. Effects of HCRT-1 administration into mPOA on sleep parameters during total recording time

	Vehicle	$HCRT_{100}$	$HCRT_{200}$	ANOVA		Tukey
				F	*p*	*p*
Wakefulness						
Total duration (min)	105.29 ± 8.01	117.43 ± 6.06*	120.74 ± 6.15*	7.64	0.005	0.001 0.003
Number of episodes	133.44 ± 8.32	145.33 ± 8.74	155.56 ± 12.40	1.51	0.250	
Episodes duration (min)	0.81 ± 0.09	0.83 ± 0.08	0.80 ± 0.08	0.09	0.911	
Light Sleep						
Total duration (min)	32.19 ± 2.62	32.00 ± 2.55	34.04 ± 2.94	0.31	0.737	
Number of episodes	186.44 ± 12.64	197.44 ± 14.87	209.56 ± 19.34	1.28	0.305	
Episodes duration (min)	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.93	0.414	
Slow Wave Sleep						
Total duration (min)	83.17 ± 4.78	73.20 ± 5.28	73.03 ± 4.69*	4.41	0.030	0.049
Number of episodes	133.78 ± 9.61	139.11 ± 11.22	143.78 ± 13.33	0.63	0.544	
Episodes duration (min)	0.63 ± 0.03	0.54 ± 0.06	0.52 ± 0.05	3.91	0.041	0.051
Intermediate stage						
Total duration (min)	5.22 ± 1.34	6.39 ± 1.15	4.66 ± 1.29	0.85	0.445	
Number of episodes	16.33 ± 2.99	19.33 ± 3.02	12.67 ± 3.40	2.87	0.086	
Episodes duration (min)	0.30 ± 0.05	0.32 ± 0.05	0.31 ± 0.06	0.09	0.915	
REM Sleep						
Total duration (min)	14.13 ± 2.09	10.98 ± 1.71	7.54 ± 1.54*	10.04	0.001	0.001
Number of episodes	12.44 ± 2.00	9.89 ± 1.48	6.33 ± 1.73*	8.67	0.003	0.002
Episodes duration (min)	1.23 ± 0.23	1.18 ± 0.20	1.21 ± 0.31	0.04	0.964	
Latency NREM (min)	13.13 ± 2.29	13.25 ± 4.10	18.90 ± 3.82	1.81	0.195	
Latency REM (min)	81.41 ± 11.88	91.90 ± 11.05	136.94 ± 19.90*	7.26	0.006	0.007

Data is presented as mean ± standard error ($n = 9$), * indicates significant difference compared to vehicle using one-way repeated measures ANOVA followed by Tukey test.

Tukey: $HCRT_{200}$ vs. saline $p \leq 0.03$) and second hour ($F_{2, 14} = 6.83$, $p \leq 0.01$; Tukey: $HCRT_{200}$ vs. saline $p \leq 0.02$) compared to control values (Fig. 7).

DORA microinjection into the mPOA did not cause significant changes in the body temperature of mother rats, neither in the total recording time nor during each hour analyzed separately (Fig. 7).

Correlations between sleep, nursing and body temperature after HCRT-1 and DORA

The total time in SWS and body temperature showed a significant positive correlation ($r = 0.84$, $p \leq 0.02$) after the microinjection of saline into the mPOA, while the time in SWS and nursing ($r = 0.55$, $p = 0.13$), and the time in nursing and body temperature ($r = 0.27$, $p = 0.56$) were not correlated. No correlation between these variables was detected after the administration of $HCRT_{200}$, DMSO or DORA into mPOA (Fig. 8).

DISCUSSION

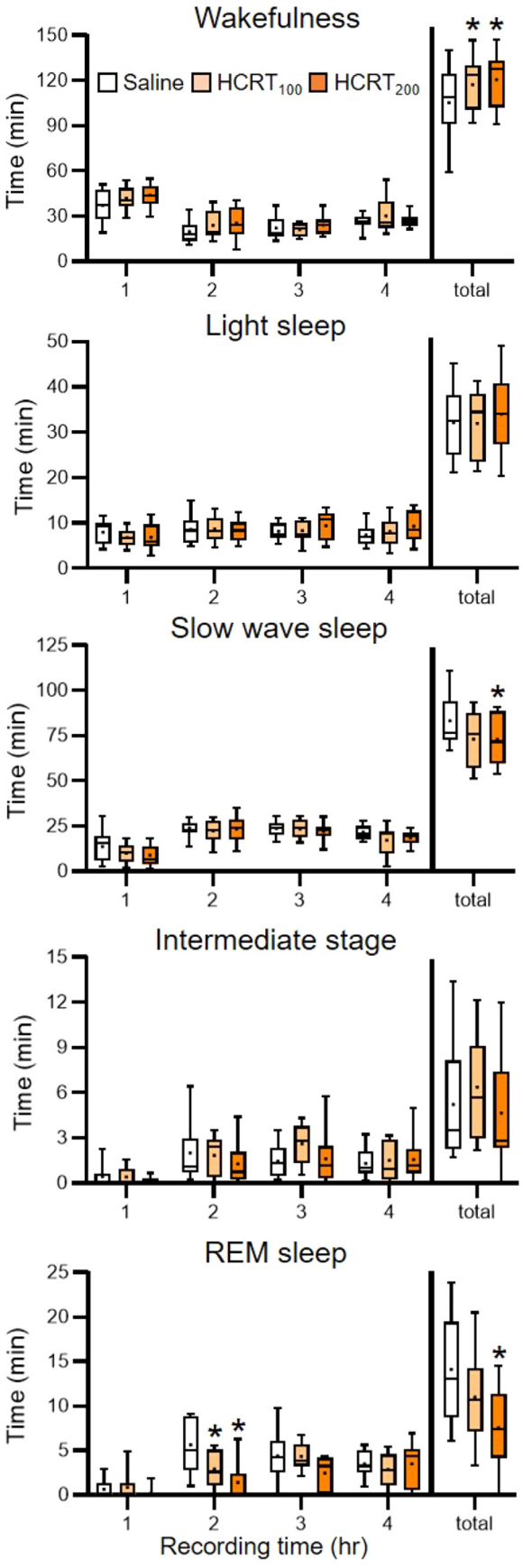
This study shows that the local perfusion of HCRT-1 into the mPOA of lactating rats increased total recording time spent awake and decreased the time in both SWS and REM sleep, while the dual receptor antagonist of HCRT-receptors (DORA) had the opposite effect. Together with the enhancement of sleep, the local administration of DORA increased the time spent in nursing the pups and the number of milk ejections, suggesting that sleep and nursing, can be promoted together. In addition, while HCRT-1 microinjections increased body temperature, this parameter was not affected by DORA treatment.

Technical considerations

We performed intracerebral microinjection of 200 nl of either HCRT-1 or DORA, similarly to previous studies (Lagos et al., 2009; Lagos et al., 2011; Benedetto et al., 2013; Benedetto et al., 2014; Rivas et al., 2016; Benedetto et al., 2017a). Although the same amount of methylene blue diffuses approximately 500 μm in the CNS (Lohman et al., 2005), we cannot discard that the drugs could have potentially affected adjacent areas, as the lateral preoptic area, which is also a somnogenic area. Furthermore, the microinjection sites in this study were mainly located in the dorsal zone of the mPOA. Due to the fact that the mPOA consists of heterogenous areas with different neuronal phenotypes and projections (Simerly et al., 1986; Simerly and Swanson, 1988), we cannot discard that the current findings may be different if microinjections had been directed towards the central or ventral area of the mPOA.

HCRT-1 microinjections into the mPOA modifies sleep but not EEG activity

The fact that HCRT-1 infusion into the mPOA of lactating mother rats reduced sleep is consistent with previous evidence in male rats showing that microinjections of HCRT-1 in the preoptic area (POA) promotes W, in



spite of the fact that the target of the injection was more lateral than in the present study (Methippara et al., 2000; Espana et al., 2001). Taken together, we can hypothesize that the function of HCRT-1 on sleep is widespread along the POA and not restricted to the medial zone of the mPOA, and these findings are consistent both in males and mother rats.

In accordance with HCRT-1 effects, our results show that local infusion of DORA decreased the total time spent awake while increased the time in SWS and REM sleep. These data strongly suggest that there is a tonic endogenous release of HCRT in mPOA sleeping circuits. It is important to note that the DORA Suvorexant has been approved in 2014 as a hypnotic drug by the Food Drug Administration (FDA) (Yang, 2014). Nevertheless, to the best of our knowledge, its effect during the postpartum period has not been studied before, neither in humans nor non-human animals.

The mPOA is a heterogeneous region composed of cells that release several neurotransmitters, such as GABA, glutamate, dopamine, and several neuropeptides (Simerly et al., 1986; Tsuneoka et al., 2013). Although most of the neurons whose activity have been related to sleep are GABAergic (Lonstein and De Vries, 2000; Tsuneoka et al., 2013; Fang et al., 2018), it has been recently shown that a subgroup of glutamatergic neurons within the mPOA promotes NREM sleep together with body cooling (Harding et al., 2018). In addition to somnogenic POA neurons, it has been recently reported the presence of glutamatergic neurons within the mPOA, whose chemogenetic activation increase wakefulness and decrease both NREM and REM sleep (Vanini et al., 2020; Mondino et al., 2021). Regarding the mechanisms underlying the effects of HCRT in POA neurons, (Eggermann et al., 2001) have studied the effects of HCRT-1 perfusion in neurons of the ventrolateral preoptic area (VLPO) in rat brain slices. Specifically, they show that HCRT have no effects on the GABA sleep-promoting neurons of the VLPO, whereas they have a robust and direct excitatory effect on the cholinergic neurons of the adjacent basal forebrain, and this effect is dependent on the activation of the HCRT-R2. Moreover, in the median preoptic nucleus (MnPO) neurons, through patch-clamp recording in rat brain slices, (Kolaj et al., 2008) have shown that HCRT applications induce a direct postsynaptic depolarization and excitation of glutamatergic currents but have no influence on GABAergic currents. Although sleep active and sleep promoting neurons of the POA are present mainly in the VLPO and MnPO (Sherin et al., 1996; Szymusiak et al., 1998; Gong et al., 2004; Kroeger et al., 2018; Vanini et al., 2020), somnogenic neurons are also present in mPOA

Fig. 3. Effect of HCRT-1 microinjection into mPOA on sleep and wakefulness. Box plot show the median (line) and the 25th and 75th quartiles, "whisker" show the 5th and 95th percentiles, and dots show the mean time spent in each state, during each hour individually and during the total recording time. Group differences were determined using one-way repeated measures ANOVA and Tukey was used as post *hoc* analysis; significant differences compared to control values are indicated with asterisks (*).

Table 2. Effects of DORA administration into mPOA on sleep parameters during total recording time

	DMSO	DORA	*t*	*p*
Wakefulness				
Total duration (min)	131.61 ± 5.63	115.88 ± 3.08*	3.10	0.017
Number of episodes	109.25 ± 12.19	127.88 ± 10.30	2.16	0.068
Episodes duration (min)	1.32 ± 0.18	0.95 ± 0.08*	2.61	0.035
Light Sleep				
Total duration (min)	27.75 ± 3.80	28.54 ± 3.06	0.34	0.743
Number of episodes	159.75 ± 16.23	171.63 ± 13.12	0.10	0.351
Episodes duration (min)	0.17 ± 0.01	0.16 ± 0.01	0.86	0.416
Slow Wave Sleep				
Total duration (min)	67.67 ± 4.64	78.26 ± 3.16*	3.68	0.008
Number of episodes	110.25 ± 7.01	120.00 ± 5.18	1.22	0.262
Episodes duration (min)	0.62 ± 0.04	0.66 ± 0.03	0.78	0.461
Intermediate stage				
Total duration (min)	4.83 ± 1.53	5.38 ± 1.08	0.63	0.550
Number of episodes	15.00 ± 3.24	15.62 ± 4.11	0.22	0.822
Episodes duration (min)	0.31 ± 0.03	0.38 ± 0.04	1.69	0.135
REM Sleep				
Total duration (min)	8.14 ± 1.22	11.94 ± 1.19*	2.57	0.037
Number of episodes	7.75 ± 1.61	8.87 ± 2.18	0.59	0.575
Episodes duration (min)	1.35 ± 0.30	1.71 ± 0.25	1.33	0.225
Latency REM (min)	86.43 ± 15.53	65.33 ± 7.38	1.95	0.091
Latency NREM (min)	14.40 ± 3.86	9.73 ± 0.99	1.18	0.276

Data is presented as mean ± standard error ($n = 8$), * indicates significant difference compared to vehicle using the Student paired *t*-test.

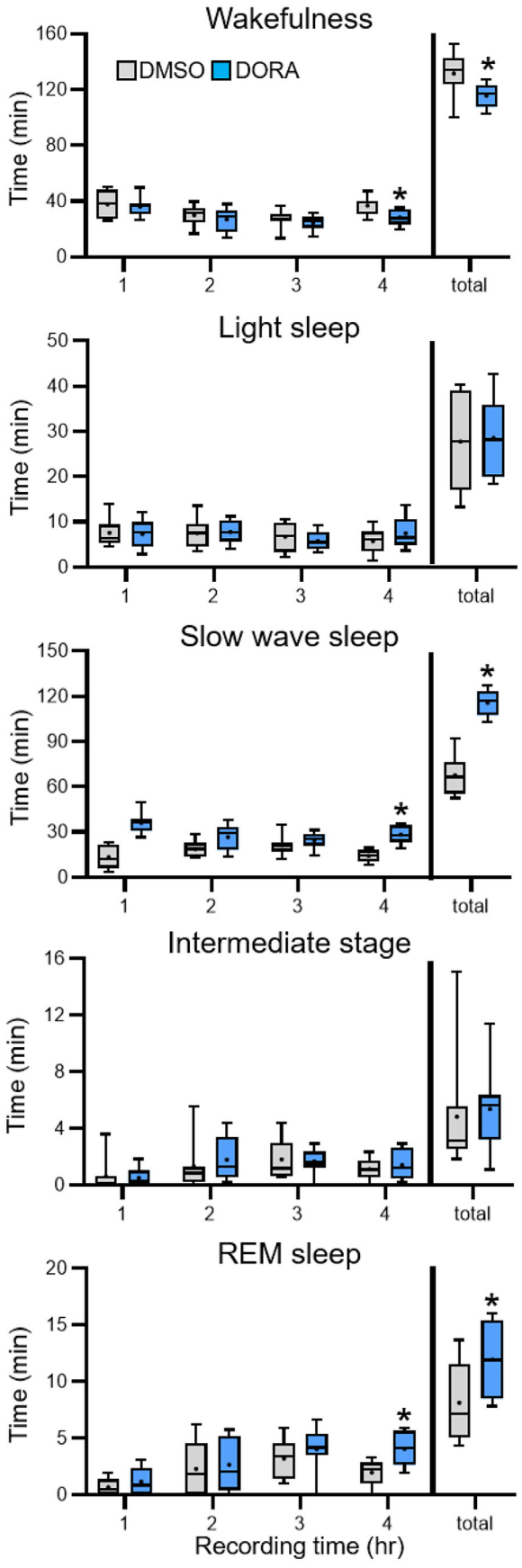
(Chung et al., 2017; Harding et al., 2018). However, there are no previous reports exploring the effect of HCRT in mPOA neurons. Although the present study does not provide information about the specific neurons that were affected by HCRT-1 to produce the observed effects, given previous evidence we can speculate that HCRT-1 local administration into the mPOA could be acting directly into glutamatergic rather than in GABAergic neurons.

Even though HCRT-1 and DORA in mPOA affected sleep-wake times, these changes were not accompanied by EEG power modifications in any frequency or behavioral stage studied. This is in accordance with (Hungs and Mignot, 2001), who showed that HCRT neuron ablation in mice did not affected the EEG power spectrum in any state. However, intracerebroventricular HCRT-1 administration in male rats decreased delta and alpha power, and increased theta and beta power (Toth et al., 2012); while a low dose of HCRT-1 (140 pmol) only decreased delta power but had no changes in theta-potency (Magdaleno-Madrigal et al., 2019). Collectively, these data suggest that HCRT-1 effects on EEG power are dependent on other brain areas rather than the mPOA. Interestingly, in humans, EEG power spectra in non-REM sleep was not affected by the hypnotic DORA SB-649868 (Bettica et al., 2012), while Suvorexant has restricted consequences on power spectral density at clinically effective doses, suggesting that the antagonism of the HCRT may have improvements in sleep without great modifications in the EEG profile (Ma et al., 2014).

HCRT in the mPOA modifies maternal behavior

Our results show that the blockade of endogenous HCRT intra-mPOA by DORA increased the time that mother rats spent nursing and the number of milk ejections. The increase in nursing occurred at the expense of a decrease in the time that dams spent away from the pups, without affecting the time in hovering over the pups, indicating that DORA increased the time that mothers spent in contact with the pups. This is in accordance with (Grieb et al., 2018), who showed that the endogenous levels of HCRT-1 within the mPOA are negatively correlated with the frequency of contact with the litter and kyphosis postures. However, the time hovering over the pups is negatively correlated with the high levels of HCRT-1. These results together suggest that natural variations in endogenous HCRT levels within the mPOA may lead to maternal behavior differences among individuals.

In contrast, most maternal behaviors analyzed in the present report were undisturbed by the administration of HCRT-1 into mPOA. $HCRT_{100}$ reduced litter weight gain, but without affecting nursing time. This is consistent with our previous study, in which latencies and durations of nursing behaviors were not different after microinjections of HCRT-1 10 and 100 μM, measured in a 30-minute maternal test (Rivas et al., 2016). Together, this lack of effect of HCRT-1 administration on maternal behaviors suggests that the endogenous tonic levels of HCRT in mPOA might be already high. Hence, HCRT-1 administered exogenously would have



no additional effect in the modulation of maternal behavior.

Recently, Diniz et al. (2018) showed the presence of a greater number of HCRT-immunoreactive neurons in lactating dams compared to that of virgin females, and this number decreases from PPD15 to PPD21 in response to regular suckling stimulus of pups, suggesting a role of HCRTergic system during the lactation period (Diniz et al., 2018). However, the specific role that HCRT play in maternal behavior remains to be elucidated.

HCRT, mPOA and thermoregulation

The higher dose of HCRT-1 ($HCRT_{200}$) increased body temperature during the first and second hour after its microinjection into mPOA. This is in accordance with the elevated body temperature after intracerebroventricular infusion of HCRT-1 in rats, with a peak at about three hours (Yoshimichi et al., 2001). Regarding the POA, only one study has administered HCRT-1 (1 mM) into lateral preoptic area of male rats, but brain temperature was not affected (Methippara et al., 2000). These data, together with the present results, suggest that the HCRT modulates body temperature acting specifically within the mPOA rather than in other POA areas, supporting the large body of evidence that establishes the mPOA as a key center of body temperature regulation (Boulant, 2000; Morrison, 2016; Harding et al., 2018). However, blocking endogenous HCRT action by the administration of DORA had no effect on body temperature of the mothers. This latter result could suggest that endogenous HCRT may not be playing a main role in maintaining basal temperature throughout the mPOA circuit in lactating dams, which could reflect the changes in the mPOA circuits during motherhood and lactation. In this sense, it is important to note that functional differences in mPOA networks have been reported between male and postpartum female rats (Raisman and Field, 1971; Gorski et al., 1978; Bleier et al., 1982; Brown et al., 1988; Ottem et al., 2004).

In the present report, the increase in maternal body temperature provoked by $HCRT_{200}$ was not accompanied by changes in the time spent in nursing. Although (Leon et al., 1978) suggest that nursing bouts are restricted by an increase in maternal temperature, and that the rate of temperature progression determines the length of each episode, there is evidence that shows that hyperthermic mothers nurse their litter normally, after morphine combined with naloxone administration, suggesting that nursing episodes are not limited by the mother's temperature in rats (Stern and Azzara, 2002). In this sense, we did not find a significant correlation

Fig. 4. Effect of DORA microinjection into mPOA on sleep and wakefulness. Box plot show the median (line) and the 25th and 75th quartiles, "whisker" show the 5th and 95th percentiles, and dots show the mean time spent in each behavioral state after local administration of DSMO (10%, vehicle) and DORA (5 mM) during each hour and the total recording time. Group differences were determined using paired Student test; * indicates significant differences compared to control values.

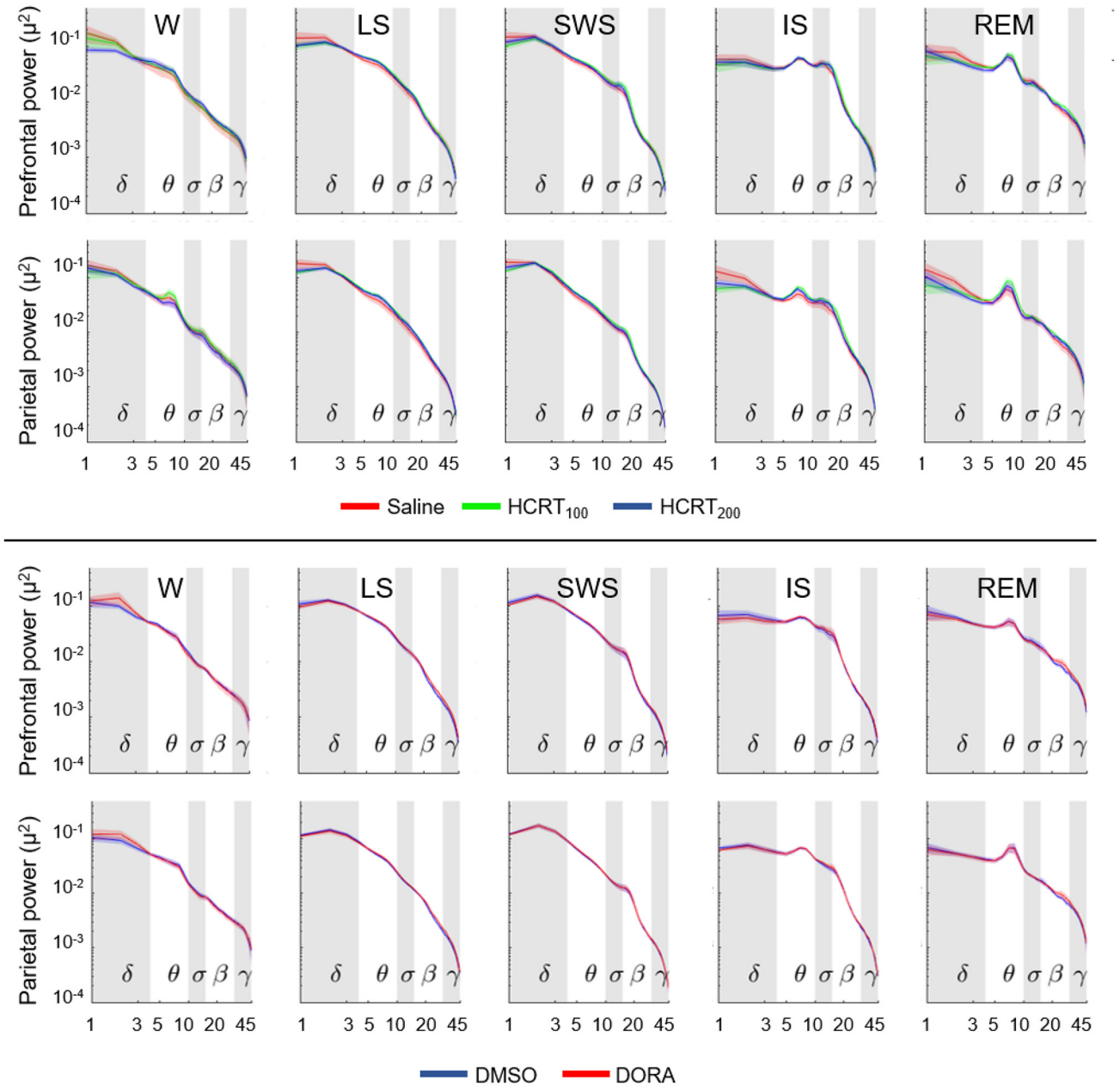


Fig. 5. EEG power of each behavioral state during the total recording time after HCRT-1 and DORA microinjections into mPOA. Graphs show spectral power changes in the prefrontal and parietal cortex during wakefulness and sleep for frequencies between 1 and 30 Hz for HCRT-1 (top) and DORA (bottom) groups. Traces represent mean values (thin, dark lines) ± SEM (shaded area above and below the mean). Frequency ranges are highlighted by varying horizontal-colored columns in the background of the graphs (Delta [1–4 Hz], Theta [4–10 Hz], Sigma [10–15 Hz], Beta [15–30 Hz] and Gamma [30–45 Hz]. For HCRT-1 group, Friedman test for multiple comparisons was employed for statistical comparison of spectral power in each frequency. Wilcoxon signed-rank test was used for DORA group. W, wakefulness; LS, light sleep; SWS, slow wave sleep; IS, intermediate stage and REM, rapid eyes movements sleep.

between body temperature and nursing behavior after control or drug treatments. However, the neural mechanisms that control the regulation of body temperature in lactating dams and suckling could be associated. In this regard, there is evidence that the tuberoinfundibular peptide of 39 residues (TIP39), the ligand of the parathyroid hormone receptor 2 receptor (PTH2R), participates in the regulation of maternal motivation, in the release of prolactin during lactation (Cservenak et al., 2010; Cservenak et al., 2013), and also in the raise of body temperature during this period (Gellen et al., 2017). Moreover, TIP39 neurons that increase their activity in response to the suckling, project to the mPOA (Cservenak et al., 2013). It could be hypothesized that the increase in body temperature by HCRT-1 could be associated with the interaction of the projections of the HCRT system with TIP-39 neurons within the mPOA. However, this hypothesis remains untested.

Table 3. Effects of HCRT-1 administration into mPOA on maternal behavior parameters during total recording time

	Vehicle	$HCRT_{100}$	$HCRT_{200}$	ANOVA *F*	Tukey *p*	*p*
*Latency to (*min*)*:Reunion litter	8.50 ± 4.11	14.20 ± 5.00	8.75 ± 4.05	0.62	0.549	
Nursing	8.69 ± 1.29	12.10 ± 3.92	8.74 ± 1.40	0.54	0.593	
*Duration (*min*):*						
Nursing total	171.52 ± 6.99	162.46 ± 7.61	154.43 ± 12.37	1.70	0.214	
Nursing Episodes	12.44 ± 1.25	10.50 ± 0.78	11.29 ± 1.61	0.78	0.476	
Number of:						
Nursing episodes	15.11 ± 1.31	15.78 ± 0.70	15.56 ± 1.58	0.07	0.928	
Milk ejections	21 ± 2.09	19.67 ± 2.06	16.67 ± 1.29	2.41	0.121	
Litter weight gain (%)	7.08 ± 0.72	4.77 ± 0.49*	5.21 ± 0.57	4.16	0.035	0.038

Data is presented as mean ± SEM (n = 9), * indicates significant difference compared to vehicle using one-way repeated measures ANOVA followed by Tukey test.

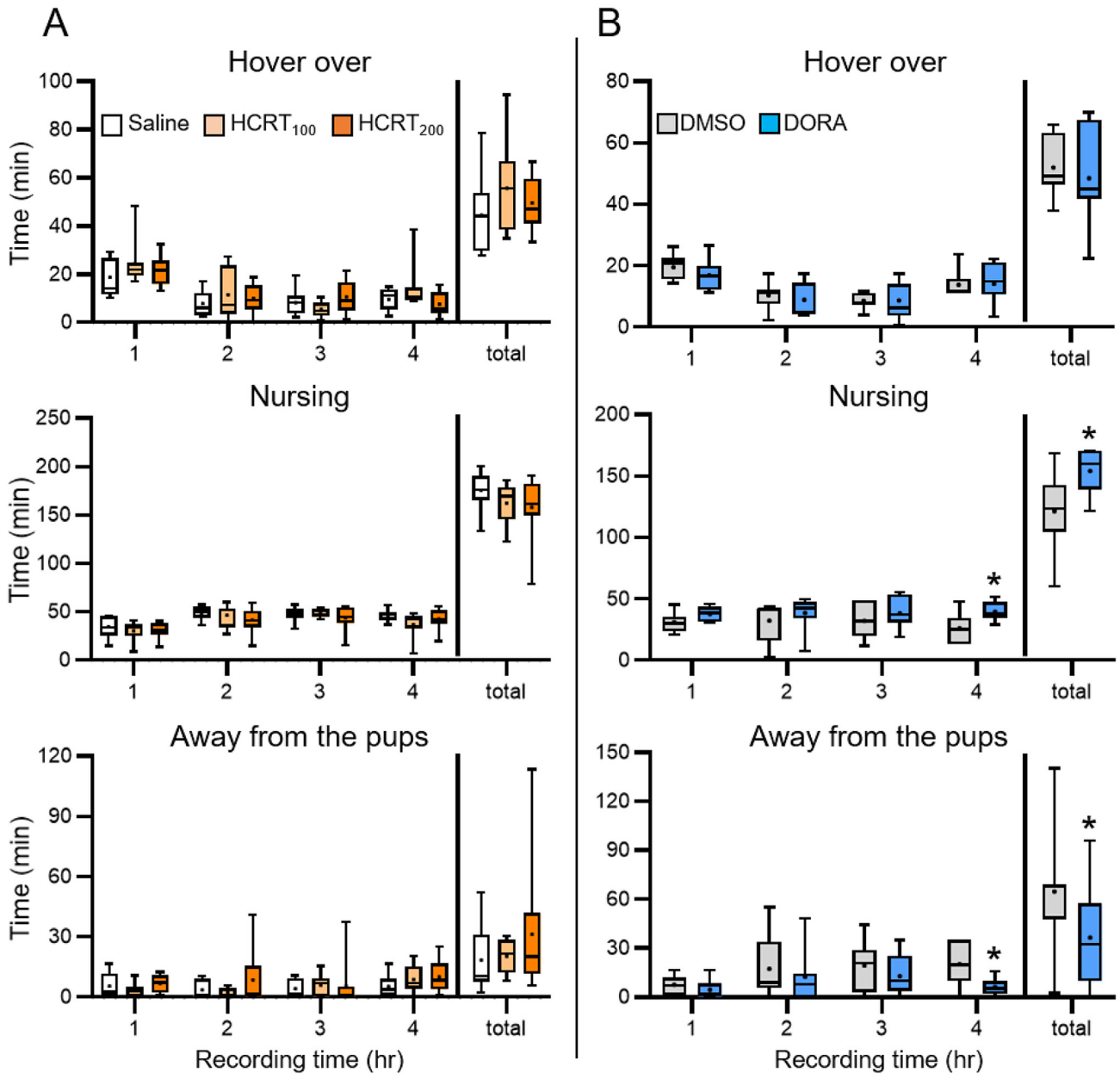


Fig. 6. Effect of HCRT-1 and DORA microinjection into mPOA on maternal behaviors. Box plot show the median (line) and the 25th and 75th quartiles, "whisker" show the 5th and 95th percentiles, and dots show the mean time spent in hover over, nursing, and away from the pups after local administration of saline, $HCRT_{100}$ and $HCRT_{200}$ **(A)**, and DMSO and DORA **(B)**, during each hour individually and the total recording time. Group differences were established by one-way repeated measures ANOVA and Tukey was used as *post hoc* analysis in HCRT-1 groups, and Student paired test in DORA group; significant differences compared to control values are indicated with asterisks (*).

Table 4. Effects of microinjection of DORA administration into mPOA on maternal behavior parameters during 4-hour sessions

	DMSO	DORA	*t*	*p*
Latency to (min)*:*				
Reunion litter	1.56 ± 0.09	9.75 ± 5.24	1.55	0.172
Nursing	7.71 ± 1.46	6.12 ± 0.72	0.87	0.418
Duration (min)*:*				
Nursing total	121.38 ± 12.67	154.15 ± 6.85*	4.07	0.007
Nursing Episodes	10. 01 ± 0.78	12.67 ± 2.05	0.10	0.356
Number of:				
Nursing episodes	12.14 ± 0.86	14 ± 2.12	1,30	0,239
Milk ejections	17 ± 2.31	27.14 ± 3.39*	4.33	0.005
Litter weight gain (%)	4.50 ± 0.57	5.32 ± 0.50	2.09	0.071

Data is presented as mean ± standard error (n = 7), * indicates significant difference between groups using Student paired *t*-test.

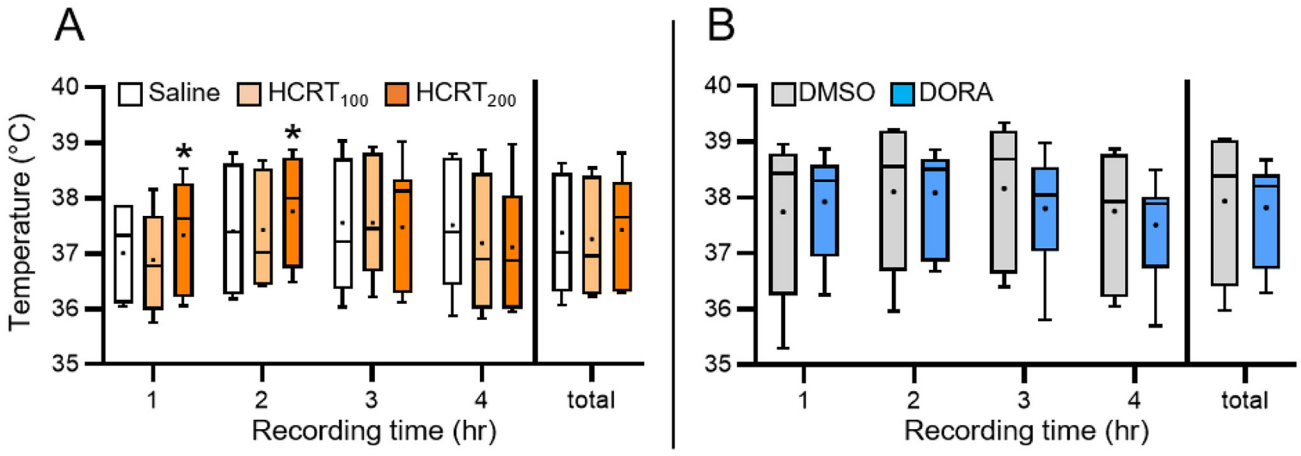


Fig. 7. Effect of HCRT-1 and DORA microinjection into mPOA on body temperature. Box plot show the median (line) and the 25th and 75th quartiles, "whisker" show the 5th and 95th percentiles, and dots show the average body temperature after local administration of saline, $HCRT_{100}$ and $HCRT_{200}$ **(A)**, and DMSO and DORA **(B)**, during each hour and in the total recording time. Group differences were established by one-way repeated measures ANOVA and Tukey was used as the post *hoc* analysis in HCRT-1 groups, and Student paired test in DORA group. Significant differences compared to control values are indicated with asterisks (*).

Correlation between sleep, nursing and body temperature and the effect of HCRT-1 and DORA

Although body temperature was positively correlated with SWS after saline treatment, this correlation was not observed after the injection with $HCRT_{200}$. Probably, this could be due to the fact that $HCRT_{200}$ augmented body temperature but decreased the total time in SWS. It is possible to postulate that exogenous rise of HCRT-1 within the mPOA could have differentially affected the neural circuits that control these variables, generating an uncoordinated state among them. Besides, as opposed to saline, a correlation between SWS and body temperature was not observed after DMSO administration. This could be cause by the fact that the number of animals was lower for the DORA group (7 vs. 9) and therefore the correlation did not reach significance. Another possible explanation is that DMSO may have a subtle biological effect (Jacob and de la Torre, 2009).

Nursing behavior was not correlated with either SWS or body temperature. This contrasts with the fact that DORA increased the time in nursing and in SWS. It could be speculated that this lack of correlation may be due to the fact that a larger sample size is required to assess if these variables are associated (only six animals were included in these analyses), and also, that we only analyzed the total recording times.

In conclusion, our work shows that HCRT-1 modulates the mPOA of lactating rats to promote wakefulness, whereas DORA promotes both NREM and REM sleep. While the sleeping effects of DORA are accompanied by an increase in the time that mother rats nurse their litter and in the number of milk ejections, without affecting body temperature, the HCRT-1 effect on wakefulness was associated with a slight increase in body temperature. Taken together, our results suggest that the reduction of the endogenous HCRT within the mPOA of lactating rats is important to promote sleep, nursing and milk ejection without affecting body temperature, while the exogenous increment of HCRT mainly increased wakefulness and body temperature. Overall, this study highlights the role of the hypocretinergic system, acting through the mPOA, in the modulation of sleep, maternal behavior and body temperature in an integrated fashion.

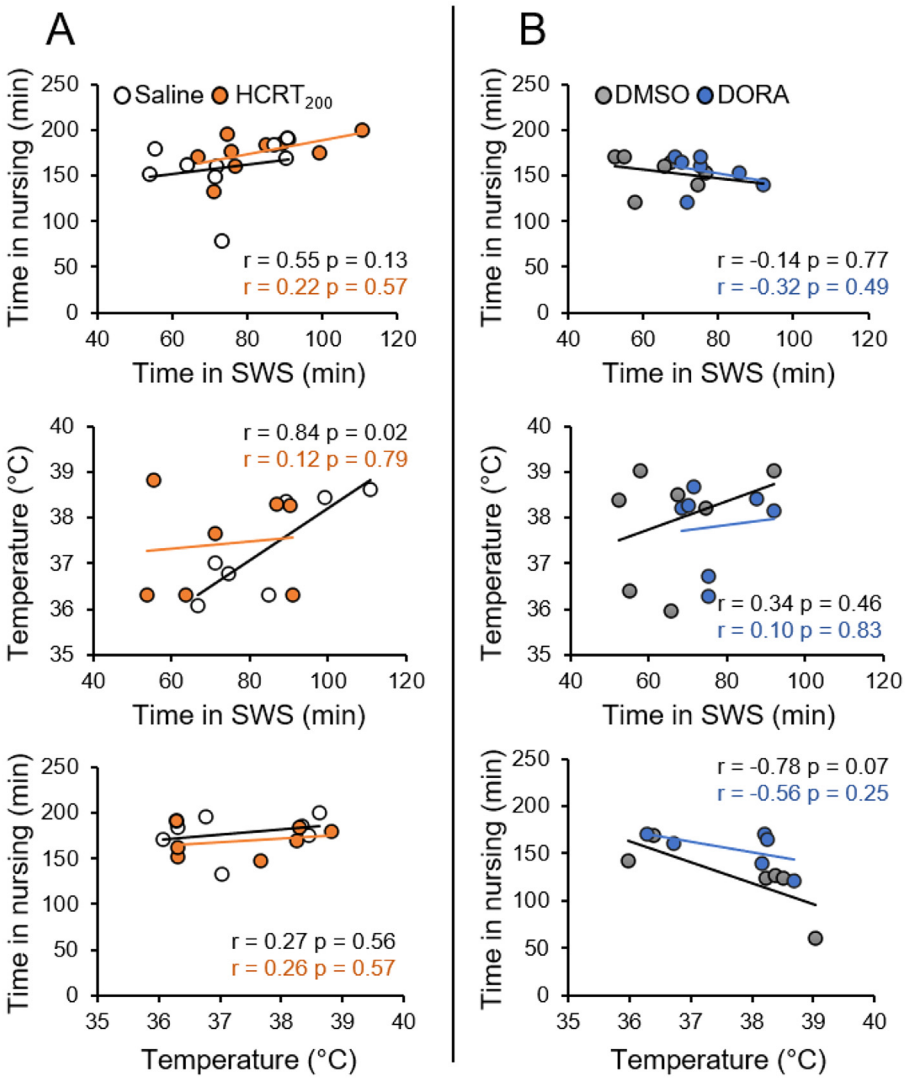


Fig. 8. Correlations between times in SWS, nursing and body temperature. Dot plots show the relationship between the total time in nursing and the total time in SWS, the total time in SWS and body temperature, and the total time in nursing and body temperature, after mPOA administration of saline and $HCRT_{200}$ **(A)**, and DMSO and DORA **(B)**.

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CAPÍTULO III. Caracterización de la actividad de las neuronas del APOm y su modulación por HCRT-1 en ratas lactantes y vírgenes

En este último trabajo nos propusimos estudiar bajo anestesia con uretano, la actividad de las neuronas del APOm de ratas lactantes, realizando una primera descripción de las características electrofisiológicas básicas de esas neuronas, y comparar si muestran variaciones con respecto a las de hembras vírgenes ciclantes. Dados los cambios anátomo-funcionales que experimenta el APOm durante el posparto, hipotetizamos que estos cambios podrían verse reflejados con características distintivas en la actividad de las neuronas. Además, si bien previamente administramos HCRT en el APOm y estudiamos cambios en el comportamiento, desconocíamos hasta ahora el efecto de éstas sobre la actividad individual de las neuronas. Como parte de esta caracterización, estudiamos la actividad de las neuronas en relación a los estados del EEG y el efecto de las HCRT. También, evaluamos si las neuronas relacionadas con el EEG y que responden a HCRT muestran características distintivas. Los resultados mostraron que la actividad basal y características básicas de las neuronas del APOm no varía en el posparto en comparación a hembras vírgenes. Además, observamos que el efecto de las HCRT es heterogéneo, causando efectos diferentes en distintas neuronas, y no difirió entre vírgenes y lactantes. Se destaca que las neuronas que responden a HCRT muestran algunas diferencias electrofisiológicas con respecto a las que no responden a HCRT.

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Electrophysiological characterization of medial preoptic neurons in lactating rats and its modulation by hypocretin-1

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ABSTRACT

The medial preoptic area (mPOA) undergoes through neuroanatomical changes across the postpartum period, during which its neurons play a critical role in the regulation of maternal behavior. In addition, this area is also crucial for sleep-wake regulation. We have previously shown that hypocretins (HCRT) within the mPOA facilitate active maternal behaviors in postpartum rats, while the blockade of endogenous HCRT in this area promotes nursing and sleep. To explore the mechanisms behind these HCRT actions, we aimed to evaluate the effects of juxta-cellular HCRT-1 administration on mPOA neurons in urethane-anesthetized postpartum and virgin female rats. We recorded mPOA single units and the electroencephalogram (EEG) and applied HCRT-1 juxta-cellular by pressure pulses. Our main results show that the electrophysiological characteristics of the mPOA neurons and their relationship with the EEG of postpartum rats did not differ from virgin rats. Additionally, neurons that respond to HCRT-1 had a slower firing rate than those that did not. In addition, administration of HCRT increased the activity in one group of neurons while decreasing it in another, both in postpartum and virgin rats. This study suggests that the mechanisms by which HCRT modulate functions controlled by the mPOA involve different cell populations.

1. Introduction

The medial preoptic area (mPOA) is a crucial hypothalamic region that controls sleep (Benedetto et al., 2021; Vanini and Torterolo, 2021), thermoregulation (Kumar, 2004), and maternal care (Numan, 1974, 2006). During the transition to motherhood and postpartum period, the mPOA undergoes through anatomical and physiological changes that allow the female to adapt to the maternal care of the pups. In this sense, it has been described modifications in the complexity of dendritic spines in maternal mPOA neurons (Shams et al., 2012; Parent et al., 2017), in the expression of hormone receptors such as estradiol, progesterone, prolactin, and oxytocin (Insel, 1990; Wagner and Morrell, 1996; Numan et al., 1999; Mann and Bridges, 2001), as well as in the density of neurotransmitters' receptors, including dopamine, vasopressin, neurotensin, amylin, and GABA (Gammie, 2005; Bosch and Neumann, 2008; Dobolyi, 2009; Akbari et al., 2013; Driessen et al., 2014). In addition, there are changes in the neurotransmitter phenotype of mPOA neurons, such as the expression of melanin-concentrating hormone (MCH) in GABAergic neurons, that only occur during the postpartum period (Rondini et al., 2010). However, it is unknown if these postpartum adaptations lead to electrophysiological changes in mPOA neurons.

mPOA neurons are critical for the normal development of maternal behaviors (Fleming and Walsh, 1994; Lonstein et al., 1998). In this sense, mPOA neurons increase their c-Fos expression (a marker for neuronal activity) when the mother displays active maternal behaviors (Fleming and Walsh, 1994; Lonstein et al., 1998) and remains high for two days after contact with the litter (Stack and Numan, 2000). Most of these active mPOA neurons are GABAergic (Tsuneoka et al., 2013), and recent evidence shows that chemogenetic activation of GABAergic preoptic neurons increases maternal behavior (Dimen et al., 2021). However, the electrophysiological characteristics of mPOA neurons during the postpartum period are mostly unknown. Only Fang et al. (2018) have studied the activity of mPOA neurons in mice at different reproductive states. These authors described that the neuronal firing rate decreases in lactating mothers compared to virgin and post-lactation animals. Given this precedent, together with the neuroanatomic-functional

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changes described above, we hypothesize that the activity of the mPOA neurons in lactating and virgin female rats differ in several electrophysiological parameters.

Sleep-active and wake-active neurons are intermingled within the mPOA of male mice and rats (Szymusiak et al., 1998; Takahashi et al., 2009). While most sleep-active neurons are GABAergic (Gong et al., 2004), Vanini and collaborators recently described a group of glutamatergic neurons in the ventral half of the mPOA, in which chemogenetic activation increases wakefulness and decreases both NREM and REM sleep (Vanini et al., 2020; Mondino et al., 2021). However, there are no studies regarding mPOA neuronal activity in the postpartum period during sleep.

The hypocretinergic (HCRTergic) system contributes to the maintenance of wakefulness and regulates several motivating activities, including maternal behavior (Torterolo et al., 2003, 2011; Harris et al., 2005; D'Anna and Gammie, 2006; Muschamp et al., 2007; Boutrel et al., 2010; McGregor et al., 2011; Rivas et al., 2016, 2021). Hypocretin-1 (HCRT-1) and HCRT-2 (also called Orexins A and B, respectively) are neuropeptides synthesized by neurons located in the postero-lateral hypothalamus, which project to many brain areas, including the mPOA (Peyron et al., 1998; Trivedi et al., 1998). Interestingly, Fos immunoreactivity of HCRTergic neurons as well as the expression of pre-pro-HCRT mRNA and HCRT receptor-1 mRNA, increase during the postpartum period in mice and rats (Wang et al., 2003; Espana et al., 2004).

We have recently shown that HCRT-1 within the mPOA facilitates some active maternal behaviors in postpartum rats, and the blockade of endogenous HCRT in the mPOA promotes nursing and sleep (Rivas et al., 2016, 2021). However, to our knowledge, the effect of HCRT on mPOA unit activity has not been previously reported.

In the present report, as a first step to characterize the in vivo electrophysiology of the mPOA neurons in postpartum and virgin rats, we utilized urethane anesthesia as a proxy for the sleep-wake cycle. Under this anesthesia, there are spontaneous and rhythmic alternations between two different electroencephalographic patterns, a slow-wave state that resembles NREM sleep, and an active state with features of both REM sleep and wakefulness (Clement et al., 2008; Pagliardini et al., 2013; Mondino et al., 2022). Mondino et al. (2022) named these urethane-induced states NREMure and REMure, respectively. In addition, we also evaluated the effect on neuronal activity of juxta-cellular administration of HCRT-1 in both lactating and virgin rats.

2. Materials and methods

2.1. Animals and housing

We utilized fifteen diestrus virgin females and twenty-two primiparous lactating Wistar female rats weighing 230–350 g (days six to eight postpartum). Animals were housed in a temperature-controlled (22 ± 1 °C) room, under a 12-h light/dark cycle (lights on at 6:00 a.m.), with ad libitum access to food and water. The experimental procedures were approved by the Institutional Animal Care Committee (protocol nº 070153–000550–18).

2.2. Stereotaxic surgery

The recordings were made in rats anesthetized with urethane (1.2 g/kg, i.p.), maintaining body temperature at 37.0–37.5 ºC using a heating pad. We positioned the head of the rat in a stereotaxic frame, made a scalp incision, and exposed the skull. According to Paxinos and Watson (2005) coordinates (AP −0.5 mm, L 0.5 mm, H 8.0 – 9.3; from Bregma), we drilled a small hole in the skull to descend a recording electrode into the mPOA. Additional holes were performed to place three stainless-steel screw electrodes for electroencephalogram (EEG) recordings in the following locations: frontal cortex (AP = +3.5, ML = 2.0), occipital cortex (AP = −6.5, ML = 2.0) and cerebellum (AP = −11.0, ML = 0.0; as a reference electrode).

2.3. Unit and EEG recordings

We carried out unit recordings using standard procedures with an Ag-Cl electrode inside a glass micropipette of 5–20 MΩ, filled with 2 M NaCl; we also handmade fabricated double micropipettes for simultaneous recording and juxta-cellular administration of HCRT (Torterolo et al., 1998, 2002; Devera et al., 2015; Pascovich et al., 2020). An Ag-Cl electrode under the neck skin was used as reference for unit recordings. The HCRT-filled micropipette tip had a diameter of 5–20 mm and was separated from the recording electrode tip by 50–150 mm. Neuronal signals were amplified x 1000 by an AC-coupled amplifier (Dagan 2400 A), filtered between 300 Hz and 10 kHz, and digitized at 20 kHz. Single unit activity in the mPOA was acquired and processed using Spike2 software (Cambridge Electronic Design, UK). Baseline firing of mPOA units was recorded for at least 300 s. Afterward, juxta-cellular HCRT-1 (100 μM, BACHEM, Bubendorf, Switzerland) diluted in sterile saline 0.9% (or its vehicle) was applied by pressure pulses of 20 PSI for 200–300 ms (Devera et al., 2015; Pascovich et al., 2020). EEG signals were amplified (x1000), filtered (0.1–500 Hz), sampled (1024 Hz, 16 bits), acquired, and processed using the Spike2 software.

2.4. Histological verification of unit recording sites

At the end of the experiment, the animal was euthanized with an overdose of urethane and perfused with 4% paraformaldehyde. The brain was removed and cut in coronal sections (150 μm) using a vibratome. Brain sections were observed under an optical microscope to localize micropipettes traces according to the neuroanatomical atlas of Paxinos and Watson (2005).

2.5. Electrophysiological recording analysis

We sorted the single units according to amplitude and waveform criteria. We examined the lack of spikes during the refractory period (< 2 ms), confirming the absence of contamination by other units. Then, we analyzed the average waveforms of the action potentials (AP). The APs were mostly biphasic; the AP duration was considered between the beginning of the first phase and the end of second phase, despite a third phase was observed in some cases (Hajos et al., 1995; Devera et al., 2015).

The basal pattern of discharge was analyzed offline by mean and instantaneous frequency, their respective coefficients of variation (SD/mean), interval histogram (IH), and autocorrelation histogram (AH). In both histograms, bins of 1 ms were used. In neurons that its activity was unrelated to the EEG, these parameters were calculated for each recorded unit from the total number of spikes occurring in the first 300 s of stable activity. In neurons whose activity varied according to EEG state, the basal characteristics were calculated during the longest period of stable activity in NREMure (EEG-slow waves state).

Recorded neurons were also categorized in different groups according to:

1. Reproductive state: lactating or virgin.
2. Firing pattern. Taking into account the IH, AH, and the raw recording, four groups of neurons were defined: arrhythmic (neuronal discharge without a rhythm in the AH); rhythmic (rhythmic neuronal discharge profile in the AH); long-duration burst (rhythmic neuronal firing trains separated by intervals longer than 10 s, with highly stereotyped firing seen in the raw recording); predominant interval (neurons with a main interval observable in both IH and AH; these neurons showed burst discharge; Fig. 2).

3. EEG relation. The neurons were classified as those that increase their activity during NREMure (NREMure-ON), REMure (REMure-ON), or their activity was unrelated to the EEG state.
4. HCRT-1 effect. Neurons were categorized according to their response to HCRT-1 administration in increased, decreased, or no-change.

2.6. Statistical analyses

For the statistical analyses we used SPSS 21 (IBM) and described the data as the mean and standard error of the mean (SEM). We employed the Generalized Linear Mixed Models (GLMM) to test the effects of predefined variables on electrophysiological parameters from the single-cell recordings, with gamma distribution and log as link function. For categorical response variables, binomial distribution was used in presence or absence as in burst firing, and multinomial distribution for several categories as in firing patterns of neurons (Agresti, 2003). We used random effects for animals since we recorded several cells in the same rat, but fixed effects for reproductive condition, EEG relationship, and HCRT results. Bonferroni post hoc test (sequential adjusted) was used for pairwise comparisons. Information criteria for the selection of models were based on the −2 log pseudo-likelihood in SPSS.

We used the Mann–Whitney test to compare the firing rate of each neuron during NREMure vs. REMure (windows of 1–5 min depending on the firing rate of the analyzed neurons). We also compared the firing rate before and after HCRT-1 administration with the same approach (Torterolo et al., 2002; Cabrera et al., 2013; Devera et al., 2015; Pascovich et al., 2020). The population means between these conditions were analyzed using the Wilcoxon Signed-Rank Test. The criterion chosen to discard the null hypothesis was $p < 0.05$.

3. Results

3.1. Recording sites

We recorded 192 neurons that, based on microelectrodes tracks reconstructions, were located within the limits of the mPOA (Fig. 1). We excluded seven neurons that were outside this area.

3.2. Electrophysiological characterization of units in the mPOA

One hundred and four neurons were recorded in 21 lactating rats (4.32 ± 0.14 neurons per animal), and 88 neurons in 15 virgin rats (4.86 ± 0.25 neurons per animal). We first compared the electrophysiological characteristics of the neurons between lactating and virgin rats. We did not find any significant differences in the AP duration, mean and instantaneous frequency, or in the mean or instantaneous frequency CVs (Table 1). However, there was a tendency to increase the AP duration and the instantaneous firing frequency in lactating compared to virgin rats. Lactating and virgin rats also presented equal proportions of neurons with burst firing patterns (68% and 60%, respectively), defined as two or more spikes with intervals shorter than 10 ms and a decrease in the amplitude of higher-order spikes (Fig. 2). The patterns of discharge were also non-statistically different between lactating and virgin rats, with a predominance of the arrhythmic type of discharge (57% and 61%, respectively) in both conditions.

3.3. Firing profile of unit recording associated with EEG dynamics

We evaluated 52 mPOA neurons of lactating rats. We found that 29% (n = 15) of these neurons were REMure-ON, 6% (n = 3) were NREMure-ON, while 65% (n = 34) were unrelated to the EEG. In virgin rats, a total of 45 neurons were examined: 13% (n = 6) were REMure-ON, 9% (n = 4) were NREMure-ON and 78% (n = 35) were unrelated to the EEG. This proportion of neurons was not different between lactating and virgin (F = 0.423, p = 0.862). Fig. 3 shows examples of each neuronal type.

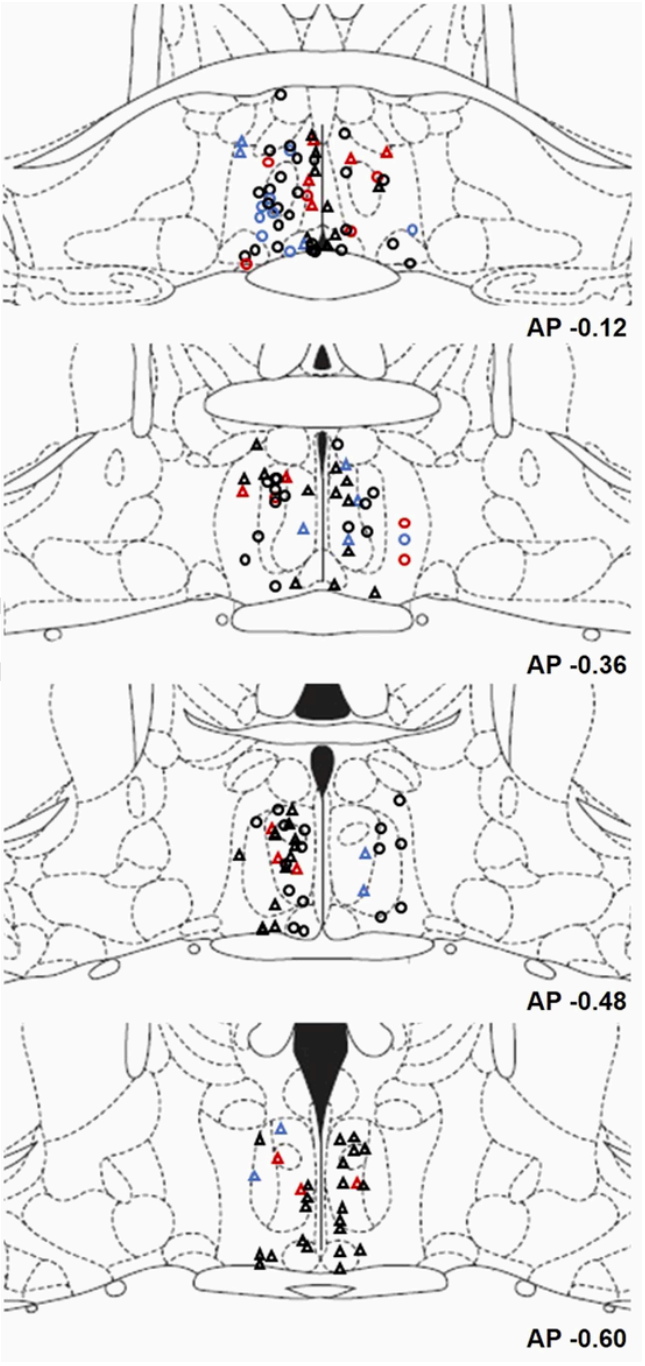

Fig. 1. Reconstruction of the recorded neuron sites at the level of mPOA in lactating (circles) and virgin rats (triangles). The colors indicate if neurons increased (red) or decreased (blue) following juxta-cellular administration of HCRT. In black are represented neurons that either did not change their activity following HCRT administration or were not tested. Representative coronal section plates were taken from Paxinos and Watson (2005); the bottom numbers indicate the distance from Bregma.

The firing pattern of each type of EEG-related neuron is shown in Table 2. In all conditions, the predominant type was arrhythmic. However, note that we did not record rhythmic NREMure-ON neurons, and none of the long-duration burst neurons were related with the EEG. The proportion of neuronal types did not differ between virgin and lactating rats (F = 0.260, p = 0.994).

Table 1
Basic electrophysiological characteristics of mPOA units.

	Lactating	Virgins	GLMM	
	104 neurons	88 neurons	F	*p*
AP duration (ms)	2.52 ± 0.07	2.35 ± 0.08	2.951	0.087
Mean firing rate (Hz)	5.90 ± 0.75	6.07 ± 0.66	0.027	0.869
Mean CV (SD / mean firing rate)	0.95 ± 0.07	0.78 ± 0.06	2.075	0.151
Instantaneous firing frequency (Hz)	19.42 ± 2.07	15.13 ± 1.25	3.071	0.081
IF CV (SD / IF)	1.35 ± 0.07	1.52 ± 0.17	0.114	0.736
Burst firing (n° of neurons, %)	71 (68%)	53 (60%)	1.799	0.181
Firing pattern (n° of neurons, %)				
Arrhythmic	61(57%)	54 (61%)	0.923	0.431
Rhythmic	18 (17%)	9 (10%)		
Long duration burst	9 (9%)	12 (14%)		
Predominant interval	16 (15%)	14 (16%)		

The neurons were recorded from 21 lactating and 15 virgin rats. AP, action potential; CV, coefficient of variation; IF, instantaneous firing frequency; SD, standard error. Generalized Linear Mixed Models (GLMM) were used to test the effects of reproductive condition on each electrophysiological parameter (gamma distribution and log as link function). For categorical response variables, binomial distribution was used in burst firing and multinomial in firing patterns of neurons (four categories).

The basal mean firing rate of neurons related to the EEG varied significantly among groups (GLMM, F = 3.845 p = 0.025). REMure-ON neurons had lower frequencies compared to those of neurons unrelated to the EEG (t = 3.614, p = 0.012), but not compared to NREMure-ON (t = 5.265, p = 0.302; see Fig. 4). We found no differences according to the reproductive condition of animals in the GLMM (F = 0.106, p = 0.746). In addition, AP duration did not vary neither when comparing among the different neuronal types (F = 0.224, p = 0.800), nor when comparing the reproductive condition (F = 3.253, p = 0.074) (Fig. 4).

3.4. Effect of HCRT-1

Fig. 5 shows examples of the effects of HCRT-1 on the firing rate of mPOA neurons. Juxta-cellular pressure injection of HCRT-1 in lactating rats was achieved in 40 neurons of 16 animals. HCRT-1 increased the firing rate in 13 neurons, from 3.28 ± 0.93 Hz to 5.48 ± 1.46 Hz (W = 91.0, p < 0.001), with a latency of 57.6 ± 16.8 s and effect duration of 309.3 ± 79.5 s. HCRT-1 also decreased the firing rate in 13 neurons, from 3.58 ± 1.06 Hz to 1.95 ± 0.78 Hz (W = 91.0, p < 0.001), with a latency of 62.9 ± 11.7 s and effect duration of 551.0 ± 107.4 s. Furthermore, HCRT-1 had no effect on the firing rate in 14 neurons (from 10.23 ± 2.42 Hz to 10.14 ± 2.38 Hz; W = 11.0, p = 0.761).

In virgin rats, we injected HCRT-1 in 34 neurons of 14 animals and found that HCRT-1 increased the firing rate in 14 neurons, from 4.09 ± 1.06 Hz to 7.68 ± 2.40 Hz (W = 105.0, p < 0.001), with a la-

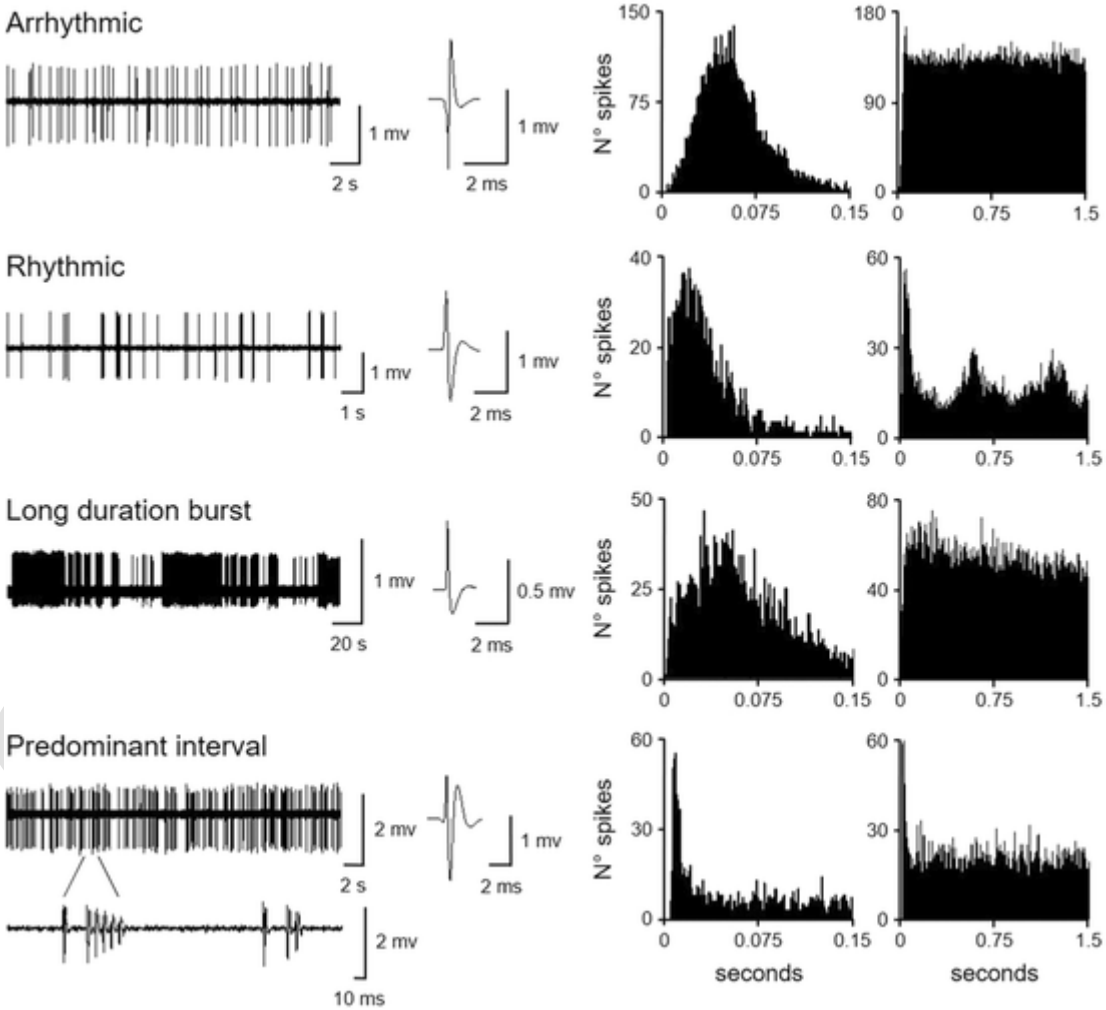

Fig. 2. Examples of four neurons with different patterns of discharge. From left to right: raw recording, action potential waveform average, interval histogram, and autocorrelation histogram. The raw recording of the neuron with a predominant interval is shown in a short time frame to highlight the discharge bursts.

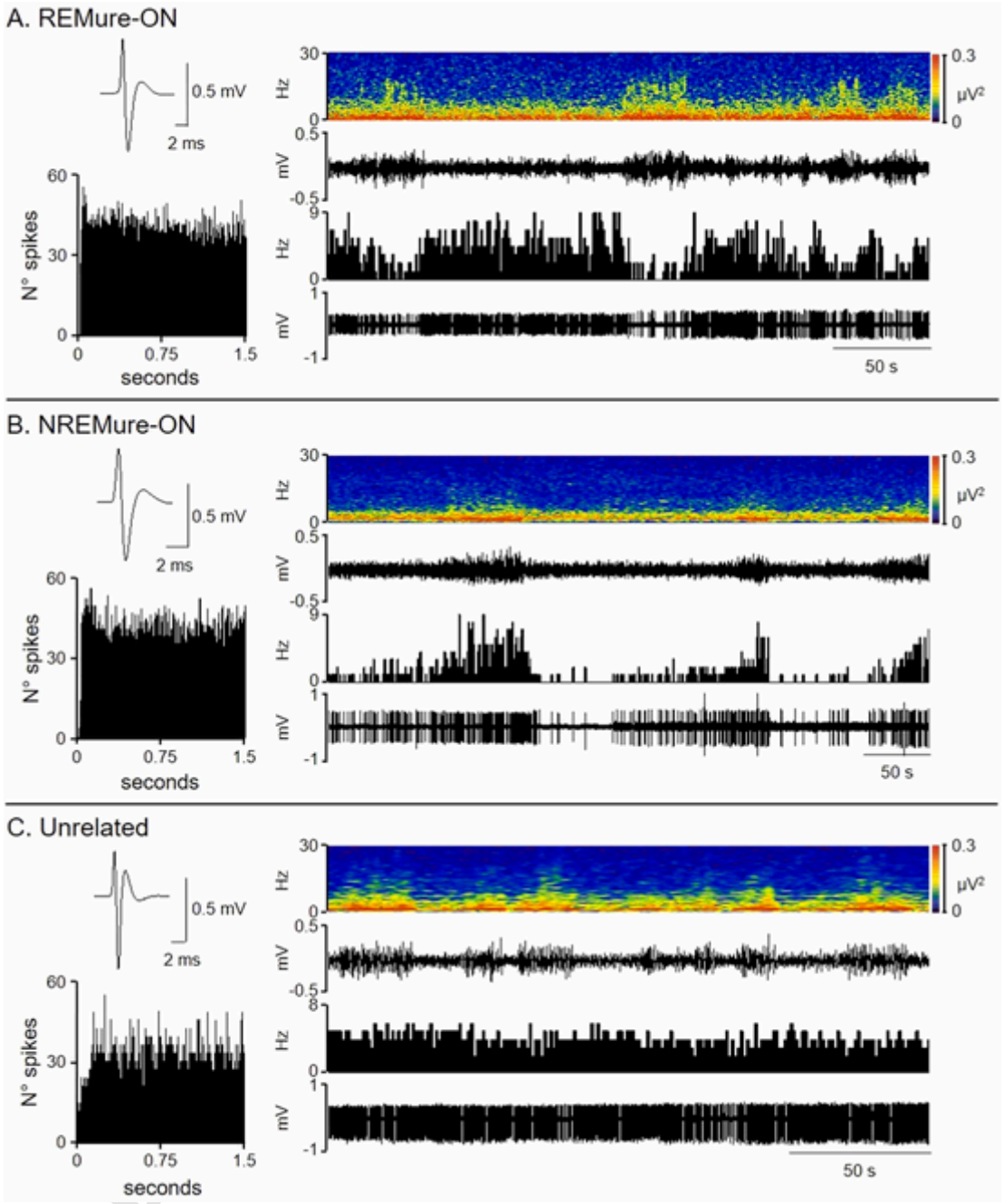

Fig. 3. Relationship between mPOA neuronal activity and EEG state. A. REMure-ON neuron. The neuron increases its firing rate during EEG activation. B. NREMure-ON neuron. This unit increases its activity during EEG with slow waves. C. Neuron unrelated with the EEG state. On the left, the waveform average and autocorrelation histogram are shown for each neuron. On the right, from bottom to top: raw unit recording, firing rate histogram, raw EEG, and EEG spectrogram.

tency of 27.4 ± 13.6 s and effect duration of 188.9 ± 57.7 s. HCRT-1 also decreased the firing rate in 12 neurons, from 5.53 ± 1.46 Hz to 2.81 ± 0.98 Hz ($W = 78.0$, $p < 0.001$) with a latency of 74.6 ± 18.1 s and effect duration of 307 ± 79.5 s. Finally, HCRT-1 had no effect in 8 neurons (from 11.18 ± 3.1110.66 ± 2.97; $W = 24$, $p = 0.109$).

The proportion of neurons that increased, decreased, or did not change their firing rate following HCRT-1 administration was not different between lactating (32%, 32%, and 36%, respectively) and virgin rats (39%, 33%, and 28%, respectively; $F = 0.282$, $p = 0.944$).

As a control of the procedure, we analyzed the juxta-cellular administration of saline solution in five neurons of lactating rats, where any modification in their firing rate was seen (from 8.83 ± 1.45 Hz before to 9.25 ± 1.41 Hz after saline administration; $T = 0.674$, $p = 0.500$).

For both lactating and virgin rats, the highest proportion of neurons was of the arrhythmic type, regardless of the response to HCRT-1. Neither the number nor the proportion of neurons differed between postpartum and virgin rats ($F = 0.785$, $p = 0.723$).

The basal mean firing rate of neurons that responded to HCRT-1 juxta-cellular administration was different from those that did not respond, independently of the reproductive condition of animals (GLMM: HCRT effect, $F = 10.032$, $p < 0.001$; reproductive condition, $F = 1.318$, $p = 0.255$). The basal mean firing rate of neurons that increase ($t = 4.273$, $p < 0.001$) or decrease ($t = 3.392$, $p < 0.001$) their firing rate following HCRT-1 administration was lower than that of neurons that showed no response to HCRT-1 (Fig. 6).

The width of the AP had significant variations in the different groups of neurons that responded or not to HCRT-1, being the repro-

Table 2
Firing pattern of each type of EEG-related mPOA neuron in lactating and virgin rats.

	Lactating			Virgins		
Firing pattern (n° of neurons, %)	REMure-ON	NREMure-ON	unrelated	REMure-ON	NREMure-ON	unrelated
Arrhythmic	8 (61%)	2 (67%)	22 (65%)	6 (100%)	4 (100%)	17 (49%)
Rhythmic	4 (31%)	0	3 (9%)	0	0	4 (11%)
Long duration burst	0	0	6 (18%)	0	0	8 (23%)
Predominant interval	1 (8%)	1 (33%)	3 (9%)	0	0	6 (17%)

No significant differences were found between groups tested with Generalized Linear Mixed Models (GLMM).

ductive condition a non-significant factor in the GLMM (HCRT effect: $F = 4.052$, $p = 0.021$; reproductive condition: $F = 2.787$, $p = 0.099$). The neurons that responded to HCRT-1 by increasing their firing rate had shorter AP duration than those that did not respond to HCRT-1 ($t = -2.803$, $p = 0.007$), without showing significant differences compared to those that decreased their activity ($t = 0.273$, $p = 0.163$) (Fig. 6).

Independently of reproductive condition, of the neurons that increased their firing rate following the microinjection of HCRT-1, 30% were REMure-ON while 0% were NREMure-ON. From the total of the neurons that decreased their activity in response to HCRT-1, 8% were REMure-ON while 15% were NREMure-ON.

4. Discussion

In the present study, through in vivo extracellular single-unit recordings under urethane anesthesia, we analyzed the electrophysiological characteristics of mPOA neurons in lactating and virgin rats. Moreover, we examined the relationship between the unit activity with the EEG and the effects of juxta-cellular administration of HCRT-1. (Table. 3).

4.1. Characteristics of mPOA neurons in lactating and virgin rats

We found no differences between the main electrophysiological characteristics of mPOA neurons between lactating and virgin rats. Although the duration of AP tended to be longer and the mean instantaneous frequency higher in lactating compared with virgin rats, these differences were not significant, probably caused by the great variability in the recorded neurons.

The electrophysiological properties of mPOA neurons have received little attention during the postpartum period. To our knowledge, no other study has recorded the activity of mPOA neurons in lactating rats. In mice, (Fang et al., 2018) reported that spontaneous firing rate of mPOA neurons was lower in lactating compared to virgin females. This difference between studies may be due to different species or methodological procedures since Fang et al. recordings were done in freely moving conditions. In fact, the mean firing rate was considerably lower (3.36 ± 0.46 and 1.89 ± 0.34 spikes/s, in virgin and lactating animals, respectively) than those observed in the present study (6.07 ± 0.66 and 5.90 ± 0.75 spikes/s, virgin and lactating rats, respectively). In addition, Horrell et al. (2019) using in vitro recordings of mPOA neurons from mice males that provide extensive paternal care to their offspring, evidenced that several intrinsic electrophysiological parameters were similar between virgin and father animals.

In agreement with anatomical and neurochemical data (Simerly et al., 1986; Tsuneoka et al., 2013), our data show that mPOA neurons are an electrophysiological heterogeneous group. While we classified most neurons as arrhythmic, there were neurons grouped as "long duration bursts" with rhythmic firing trains separated by long silence intervals; these neurons are consistent with the firing pattern of gonadotropin-releasing hormone (GnRH) neurons of the preoptic area (Suter et al., 2000; Kuehl-Kovarik et al., 2002), suggesting a possible cellular phenotype for these neurons.

4.2. Relationship with the EEG activity

Similarly to our findings, different types of sleep-active neurons (such as NREM-ON, REM-ON, and NREM/REM-ON) have been previously described to coexist within the mPOA (Koyama and Hayaishi, 1994; Suntsova and Dergacheva, 2004; Takahashi et al., 2009). In this sense, in male rats, 14% of recorded neurons were NREM-ON, 26% REM-ON, and 37% NREM/REM-ON (Koyama and Hayaishi, 1994).

For many years, urethane was considered a general anesthetic that resembles the characteristics of physiologic sleep and was utilized as a pharmacological model for its study (Clement et al., 2008; Pagliardini et al., 2013). Hence, as a proxy for the sleep-waking cycle, we utilized urethane anesthesia. We found a higher proportion of REMure-ON neurons (29% and 13%) than NREMure-ON neurons (6% and 9%) in both

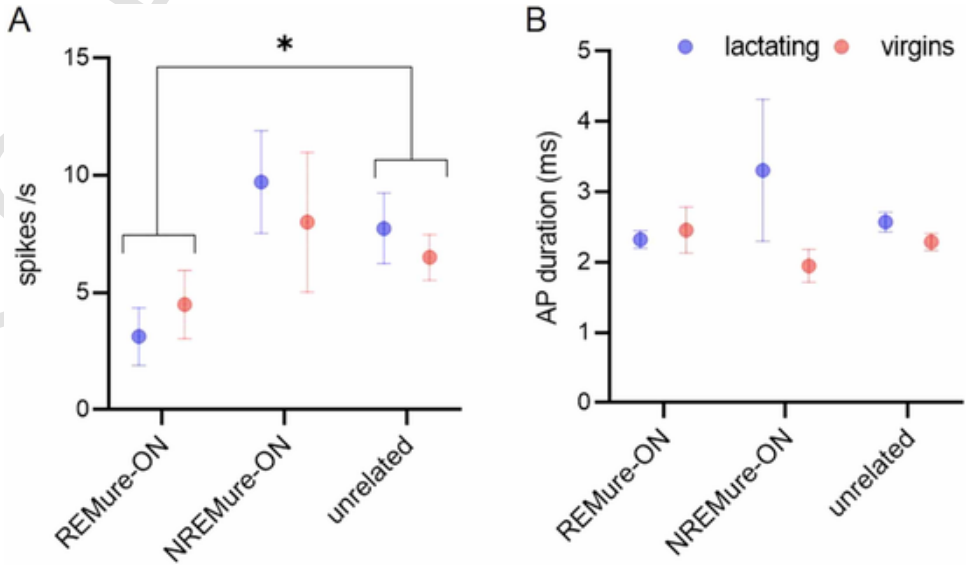

Fig. 4. Mean firing rate (A) and action potential duration (B) of neurons according to their relationship with EEG state and reproductive state. * Indicates $p < 0.05$.

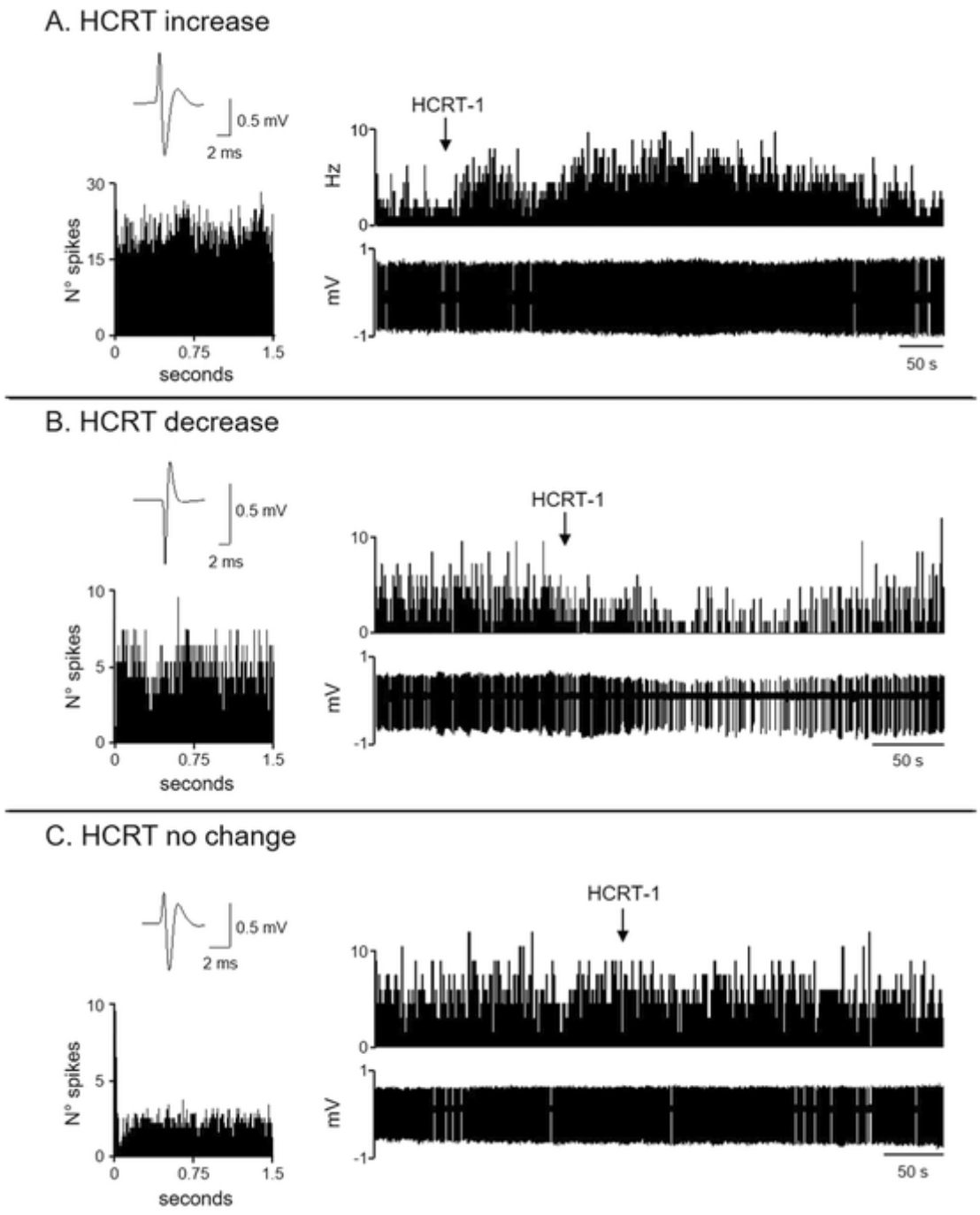

Fig. 5. Effects of juxta-cellular administration of HCRT-1 on the activity of mPOA neurons. The raw recording (bottom) and the frequency histogram (top) of three representative neurons were HCRT either increase (A), decrease (B) or had not changed (C) in the neuronal discharge. The arrow shows the time of microinjection of HCRT-1. On the left, the action potential waveform average and autocorrelation histogram for each neuron are shown.

lactating and virgin rats, respectively. Interestingly, a large percentage of neurons are reported to be active during REM sleep not only in rats (Koyama and Hayaishi, 1994) but also in mice (Takahashi et al., 2009) and cats (Suntsova and Dergacheva, 2004).

Our data exhibit certain differences compared to previous mPOA recordings under urethane anesthesia. Kumar et al. (1989) reported in male rats 13% NREMure-ON and 14% REMure-ON neurons, while (Lincoln, 1969) in the preoptic and anterior hypothalamus of cycling female rats described 27% NREMure-ON and 23% REMure-ON neurons. Besides, Bueno and Pfaff (1976) reported no association between the EEG and the activity of mPOA neurons in ovariectomized rats treated or not with estrogens. Gender, reproductive state, or slight differences in the recording site may account for the differences in the results.

Most EEG-related neurons were of the arrhythmic type, both in lactating and virgin rats. Lincoln (1969) reported that most neurons of the preoptic area and anterior hypothalamus of adult female urethane-anesthetized rats have a random discharge, similar to the arrhythmic neurons of our study. However, the author did not describe in detail the discharge pattern of these neurons. Takahashi et al. (2009) recorded neurons in the preoptic area and basal forebrain across the sleep–wake cycle of male mice. They described that most sleep-ON neurons (that increase their rate both during NREM and REM sleep) fired either in single isolated spikes or in clusters, whereas a small group displayed high

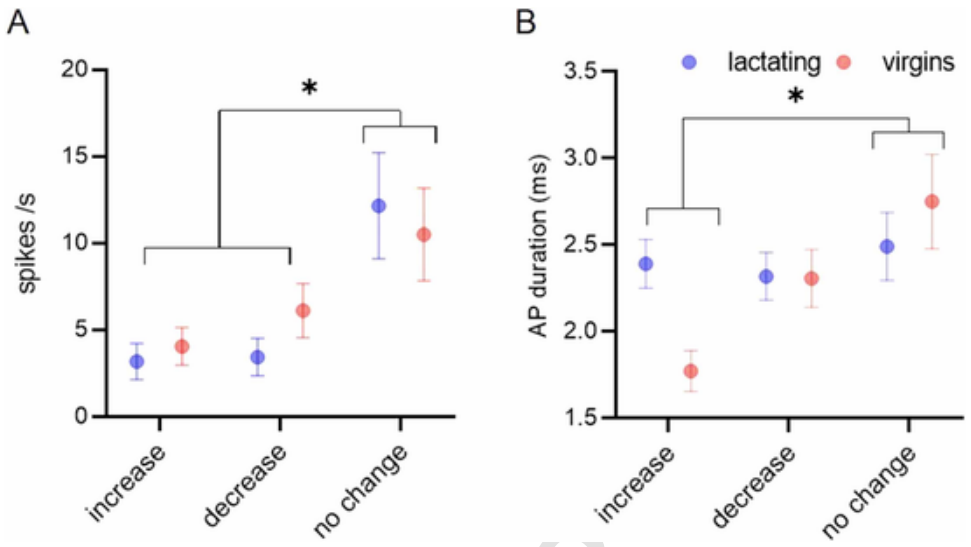

Fig. 6. Basal firing rate (A) and action potential duration (B) of neurons according to the response to HCRT-1. Neurons were categorized either as increase, decrease or not change in their mean firing rate, according to the effect of HCRT-1. * Indicates $p < 0.05$.

Table 3
Firing pattern of each type of mPOA neuron according to its response to HCRT-1 in lactating and virgin rats.

	Lactating			Virgins		
Firing pattern (n° of neurons, %)	HCRT increase	HCRT decrease	HCRT no changed	HCRT increase	HCRT decrease	HCRT no changed
Arrhythmic	11 (92%)	6 (46%)	10 (67%)	13 (93%)	9 (75%)	6 (60%)
Rhythmic	1 (8%)	4 (31%)	2 (13%)	0	1 (8%)	0
Long duration burst	0	0	2 (13%)	0	1 (8%)	4 (40%)
Predominant interval	0	3 (23%)	1 (7%)	1 (7%)	1 (8%)	0

No significant differences were found between groups tested with Generalized Linear Mixed Models (GLMM).

frequency burst discharges. Moreover, REM-ON neurons fired principally in single isolated spikes, while wake/REM-ON neurons fired either in clusters or in bursts, with rhythmic discharges synchronized with rhythmic theta waves (this latter type of neurons were located in the horizontal limb of the diagonal band of Broca or its surrounding areas). However, these data are not necessarily comparable to ours, since they recorded along the entire preoptic area and adjacent basal forebrain of unanesthetized male mice.

Mondino et al. (2022) have recently described differences between NREMure and REMure states and the corresponding natural sleep states, through a detailed analysis of the EEG that included power, coherence, directionality, and complexity. Given these recent findings, it would be particularly relevant to carry out this characterization of mPOA neurons and the effect of HCRT in non-anesthetized animals.

4.3. HCRT-1 effect in mPOA neurons

Our data show that juxta-cellular HCRT-1 either increases or decreases the firing rate of different mPOA neurons. The proportion of these two groups was similar in lactating and virgin rats. Regarding other subareas of the preoptic area, it has previously been reported that HCRT-1 increases neuronal excitability in median preoptic nucleus (MnPO) neurons, either directly through membrane depolarization via postsynaptic receptor or indirectly through the activation of glutamatergic afferents (Kolaj et al., 2008). As no direct inhibitory effects of HCRT-1 have been demonstrated, we hypothesized that the decrease in the firing rate observed in some neurons was due to presynaptic inhibition through the activation of GABAergic terminals. In this sense, HCRT neurons inhibit the firing of MCH and serotonergic neurons indirectly, increasing the activity of GABAergic neurons (van den Pol et al., 1998; Liu et al., 2002; Apergis-Schoute et al., 2015). Therefore, as previously reported in other brain areas, HCRT effect on mPOA neurons could be mediated either by presynaptic or postsynaptic receptors (van den Pol et al., 1998).

Most neurons grouped as "long duration bursts", did not respond to HCRT-1, except for one neuron that decreased its activity. As previously mentioned, these neurons could correspond to the GnRH phenotype. Previously, it has been reported that HCRT-1 causes an inhibitory response in GnRH neurons in brain slices of ovariectomized mice (Gaskins and Moenter 2018). The changes occurring in the activity of these neurons during lactation may account for the differences between the latter report and the present findings (Xu et al., 2009; Liu et al., 2014). To our knowledge, no other work evaluated the effect of HCRT-1 on preoptic neurons.

Neurons that HCRT-1 increased their activity had lower basal firing frequencies and narrow AP, compared to those that did not respond to HCRT-1. This fact suggests that HCRT-1 could be acting in a specific group of neurons. However, neurons in which HCRT-1 increased or decreased their activity did not differ from each other, indicating similarities in their electrophysiological characteristics. Given that most preoptic neurons are GABAergic (Lonstein and De Vries, 2000; Tsuneoka et al., 2013; Dimen et al., 2021), this phenotype would be the most likely candidate for most of the recorded neurons. Intracellular in vitro recordings within the mPOA show that the firing rate of GABAergic neurons (7.5 ± 3.1 Hz) was greater than the non-GABAergic (3.1 ± 2.9 Hz) (Lundius et al., 2010). However, in the present report, the average firing rate of the neurons that responded to HCRT-1 was 3.43 Hz, similar to the reported for non-GABAergic by (Lundius et al., 2010), suggesting that these neurons are more likely to be non-GABAergic. In this regard, a group of glutamatergic neurons whose activation increases wakefulness and decreases both NREM and REM sleep has been described in the mPOA and surroundings areas (Vanini et al., 2020; Mondino et al., 2021). Given that in previous studies we showed that HCRT-1 within the mPOA of lactating rats increases wakefulness and decreases sleep (Rivas et al., 2021), neurons that increase their activity in response to HCRT-1 may be glutamatergic. However, a phenotypic identification is necessary to confirm this hypothesis.

In neurons where HCRT increases its firing rate, 30% were REMure-ON while none was NREMure-ON. Given the wake-promoting role of

HCRT, we might expect that HCRT activates neurons that are wake-ON. Since these recordings were made under anesthesia, we cannot determine if the REMure-ON neurons were either wake-ON or REM-ON, although Mondino et al. (2022) stated that the active state under urethane has more similarities to REM sleep than to wakefulness. On the other hand, from the total of neurons that HCRT decreased its activity, 8% were REMure-ON while 15% were NREMure-ON. The fact that HCRT decreased the activity of NREMure-ON neurons (presumably active NREM neurons) is consistent with the wake-promoting role of HCRT and with the reciprocal inhibition between sleep and wake-promoting systems that have been suggested (Gallopin et al., 2000; Saper et al., 2010; Venner et al., 2019). In the latter sense, reciprocal anatomical and functional connections between VLPO sleep-promoting galaninergic inhibitory neurons and monoaminergic, cholinergic, and lateral hypothalamic wake-promoting neurons have been described (Gallopin et al., 2000; Venner et al., 2019). Interestingly, HCRTergic neurons are also inhibited by sleep-active preoptic neurons (Saito et al., 2013).

Our previous evidence shows that HCRT in the mPOA promote wakefulness, along with some components of maternal behavior, and increase body temperature (Rivas et al., 2016, 2021). However, the specific neuronal networks that mediate these physiological effects, and the type of mPOA neurons involved remain unknown. Since the neurochemical phenotype of mPOA neurons includes a great variety of possible neurotransmitters (Simerly et al., 1986; Tsuneoka et al., 2013), the future identification of recorded neurons in which HCRT-1 modulates its activity is fundamental to understand the functions of the hypocretinergic projections towards the mPOA.

5. Conclusions

The results of our extracellular in vivo recordings indicate that the electrophysiological characteristics of mPOA neurons did not differ between postpartum and virgin rats. Moreover, some of these neurons showed an EEG-related activity, in similar proportions between virgin and lactating rats. In addition, the juxta-cellular HCRT administration increased the activity of one group of neurons while decreasing it in others, both in postpartum and virgin rats. Finally, these HCRT-1-responsive neurons exhibited distinct electrophysiological characteristics from those that did not respond.

This is the first study that shows an electrophysiological characterization of mPOA neurons as well as the effect of HCRT on these neurons in lactating rats. Since the effects of HCRT on mPOA neurons were not the same in all neurons, this study suggests that the mechanisms by which HCRT modulate the different parameters and behaviors controlled by the mPOA include different cell populations.

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CONSIDERACIONES ADICIONALES

Basados en los antecedentes de nuestro primer trabajo, Grieb et al. (2018) estudiaron los niveles de HCRT-1 en el APOm en relación a la edad de las crías (días 7 y 14) y la etapa posparto, sin encontrar variaciones en ningún caso. Nuestro trabajo muestra un efecto levemente mayor en la segunda semana posparto con la administración exógena de HCRT-1, mientras que el antagonista tuvo mayor efecto en la primera semana, sugiriendo que los niveles endógenos de HCRT-1 serían mayores en la primera semana respecto a la segunda. Esta diferencia con lo reportado por Grieb et al. (2018), podría corresponderse con variaciones muy pequeñas en los niveles endógenos, sumado a la variabilidad entre individuos. De hecho, Grieb et al. (2018) encontraron variaciones notorias en los niveles endógenos de HCRT-1 en el APOm. Al agrupar los animales según estos niveles, encontraron que estos se asocian negativamente con la frecuencia de contacto con las crías. Como consecuencia, esto podría asociarse con un mayor estado de alerta en las madres con niveles de HCRT-1 más elevados, lo que disminuye el tiempo de contacto con las crías. Estos hallazgos están en sintonía con lo encontrado en nuestro segundo trabajo, ya que el bloqueo de la HCRT endógena aumentó el tiempo de amamantamiento y el tiempo en contacto con las crías.

Recientemente se han evidenciado cambios en el sistema HCRTérgico hacia el final del período de lactancia. Diniz et al. (2018) mostraron una disminución en el número de neuronas HCRTérgicas y de las neuronas doblemente marcadas con HCRT y Fos durante el proceso de destete. De manera interesante, la disminución en esta población neuronal depende del estímulo de succión de las crías, ya que, si éste se interrumpe, no disminuyen de igual forma (Diniz et al., 2018). Esta evidencia indica que la activación del sistema HCRTérgico juega un rol durante el período de lactancia, y que el estímulo de succión de las crías es una señal para restaurar los niveles de HCRT pre-lactancia. Esto podría ser importante para disminuir el estado de alerta motivacional mediado por las HCRT dirigido al cuidado de las crías y que no ocurran alteraciones en el sueño-y la vigilia posteriormente al período de lactancia.

Aunque en los últimos años se ha comenzado a abordar el estudio de este sistema durante esta etapa fisiológica particular, el rol específico del sistema HCRTérgico aún no ha sido completamente dilucidado. La evidencia aquí mostrada, indica que las HCRT durante la lactancia juegan un rol tanto en la regulación del comportamiento maternal como en la del sueño y la vigilia mediante la modulación del APOm. Sin embargo, no se puede descartar que este sistema cumpla otras importantes funciones durante el posparto, a través de otros circuitos cerebrales, como la regulación de los cambios metabólicos asociados a la hiperfagia lactacional, como ha sido

sugerido (Sun et al., 2003). En este sentido, es muy probable que el sistema HCRTérgico cumpla un rol unificado como ha sido sugerido en la "teoría de supervivencia unificada" para el funcionamiento de este sistema (Chase, 2013), en el que las neuronas HCRTérgicas y sus receptores funcionan para coordinar la actividad de los sitios cerebrales y periféricos que comandan los comportamientos de supervivencia, incluido el reproductivo (Chase, 2013)

Si bien el estudio del APOm ha recibido considerable atención en la regulación del comportamiento maternal, los mecanismos por el cual esta área regula e integra otras variables fisiológicas durante el posparto, han sido poco abordados. En la presente tesis, junto con otros trabajos de nuestro grupo de investigación (Benedetto et al., 2017a; Benedetto et al., 2021), mostramos cómo distintos neurotransmisores actúan en el APOm para regular, simultáneamente, el sueño y la vigilia durante el posparto y distintos aspectos del comportamiento maternal. En relación al control del sueño y vigilia, nuestros trabajos sugieren algunas diferencias en los efectos de los mismos sistemas de neurotransmisión con respecto a estudios previos realizados en machos, indicando que las modificaciones que ocurren en el APOm durante el posparto podrían alterar los circuitos reguladores del sueño y la vigilia. Dado que la literatura está basada principalmente en estudios realizados en machos, en parte estas diferencias podrían deberse a cambios en los circuitos del APOm entre machos y hembras; por ejemplo, relacionados con la expresión de receptores para estrógenos y andrógenos en esta área (Jahan et al., 2015; Simerly et al., 1990). En este sentido, se destaca la importancia de identificar en el APOm cambios asociados al posparto y la lactancia que afecten al sueño y la vigilia.

CONCLUSIONES GENERALES

En esta tesis pudimos concluir que las HCRT modulan el APOm, afectando al comportamiento maternal, el sueño y la vigilia, así como la temperatura corporal de ratas lactantes. Específicamente, mostramos que el sistema HCRTérgico en el APOm promueve el comportamiento maternal activo y que la liberación de HCRT endógena en esta área es necesaria para el correcto despliegue de este comportamiento, durante las primeras dos semanas posparto. Adicionalmente, la HCRT-1 en el APOm promueve la vigilia junto a un leve aumento de la temperatura corporal, sin afectar el comportamiento maternal pasivo; mientras que el bloqueo de la HCRT endógena incrementa tanto el sueño NREM como REM, junto con el tiempo de amamantamiento y las eyecciones lácteas, sin afectar la temperatura corporal de las hembras lactantes. Por otra parte, las características electrofisiológicas básicas de las neuronas del APOm en ratas anestesiadas no difieren entre hembras posparto y vírgenes, posiblemente reflejando la

gran heterogeneidad de las poblaciones neuronales registradas. Por último, las HCRT causan efectos diferentes en distintas neuronas del APOm y las neuronas que responden a HCRT muestran algunas diferencias electrofisiológicas con respecto a las que no responden. En este trabajo, aportamos evidencia novedosa sobre un posible mecanismo en el APOm que modula el comportamiento maternal y el sueño y la vigilia de ratas lactantes.

PERSPECTIVAS

Para profundizar en los circuitos neuronales del APOm que regulan el sueño junto con el comportamiento maternal, sería interesante dilucidar las poblaciones neuronales específicas que median estos comportamientos en ratas lactantes, mediante la activación selectiva de grupos neuronales de un fenotipo determinado, como GABA, glutamato y galanina, con técnicas como quimio u optogenética. A su vez, sería importante identificar las neuronas en que las HCRT actúan para modular estas variables, a través de la activación específica de las terminales HCRTérgicas en el APOm, y la activación o inhibición de grupos neuronales determinados.

Otra perspectiva posible para desarrollar es determinar el efecto de la experiencia maternal en la regulación de las HCRT. Es sabido que hembras primíparas y multíparas presentan diferencias en el comportamiento maternal atribuidas a la experiencia maternal previa. Estas diferencias se han relacionado con determinados sistemas de neurotransmisión, como el glutamatérgico, pero se desconoce si influyen en el efecto de las HCRT, ni si se reflejan en la activación de neuronas de distinto fenotipo en el APOm.

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