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EFECTO DE LA EXPOSICIÓN PERINATAL AL DE-79, MEZCLA DE ÉTERES BIFENILOS OCTABROMADOS, SOBRE EL SISTEMA VASOPRESINÉRGICO EN RATAS ADULTAS

T E S I S

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ABREVIATURAS

*Todas las abreviaturas son por sus siglas en inglés.

ACTH	Hormona adrenocorticótropa
ADH	Hormona antidiurética
ANOVA	Análisis de varianza
AQP-2	Acuaporinas-2
AVP	Arginina vasopresina
BDEs	Éteres bromidifenilos / éteres difenilos bromados
BFRs	Retardantes de flama bromados
BST	Núcleo del lecho de la estría terminal
cDNA	Ácido desoxiribonucleico complementario
cGMP	Guanosín monofosfato cíclico
decaBDEs	Éteres difenilos decabromados / Éteres decabromodifenilos
EDs	Disruptores endócrinos
ELISA	Ensayo por inmunoabsorción ligado a enzimas
eNOS	Sintasa endotelial del óxido nítrico
FRs	Retardantes de flama
GD	Días de gestación
Glu	Glutamato
GPCR	Receptores acoplados a proteínas G
HBCDD	Hexabromociclododecano
IgG	Inmunoglobulina G
IgM	Inmunoglobulina M
iNOS	Sintasa inducible del óxido nítrico
IOD	Densidad óptica integrada
-IR	Inmunorreactividad
L-NAME	NG-nitro-L-arginina-metil éster
M	Mes(es)
ME	Eminencia media
MeA	Amígdala medial
MNCs	Células magnocelulares neuroendócrinas
mRNA	Ácido ribonucleico mensajero
NADPH-d	Nicotinamida adenina dinucleótido diaforasa
NMDA	N-metil-D-aspartato
nNOS	Sintasa neuronal del óxido nítrico

NO	Óxido nítrico
NOS	Sintasa del óxido nítrico
octaBDEs	Éteres difenilos octabromados / Éteres octabromodifenilos
OVLT	Órgano vascular de la lámina terminal
PBBs	Difenilos polibromados
PBDEs	Éteres difenilos polibromados / Éteres polibromodifenilos
PCBs	Policlorobifenilos / Bifenilos policlorados
PCR	Reacción en cadena de la polimerasa por transcripción reversa en punto final
PNCs	Células parvocelulares neuroendócrinas
PnD	Días postnatales
pentaBDEs	Éteres difenilos pentabromados / Éteres pentabromodifenilos
POPs	Contaminantes orgánicos persistentes
PP	Hipófisis/pituitaria posterior
PVN	Núcleo Paraventricular del hipotálamo
Q-PCR	Reacción en cadena de la polimerasa cuantitativa (en tiempo real)
RNA	Ácido ribonucleico
rRNA	Ácido ribonucleico ribosomal
RT-PCR	Reacción en cadena de la polimerasa con transcriptasa reversa
SCN	Núcleo supraquiasmático
SFO	Órgano subfornical
SON	Núcleo Supraóptico del hipotálamo
TBBPA	Tetrabromobisfenol
USA	Estados Unidos de América

RESUMEN

Los éteres bifenilos polibromados (PBDEs) son contaminantes ambientales persistentes y bioacumulables distribuidos mundialmente; se usan como retardantes de flama y se encuentran en productos de consumo como equipos eléctricos, materiales de construcción, revestimientos, textiles y espuma de poliuretano. Los PBDEs se detectan en la mayoría de las muestras biológicas, incluidas la sangre, el tejido adiposo y la leche materna. Estos compuestos se consideran neurotóxicos y disruptores endocrinos por sus importantes efectos biológicos como son las alteraciones en el crecimiento, en la reproducción y sobre la actividad del eje hipotálamo-pituitario-adrenal.

La vasopresina (AVP), uno de los neuropéptidos secretados por el sistema hipotalámo-neurohipofisiario, tiene un papel clave en el mantenimiento de la homeostasis hidroelectrolítica, que es vital. Además, es un blanco conocido para la mezcla comercial de pentaBDEs DE-71 y para los compuestos halogenados estructuralmente similares, los bifenilos policlorados. Sin embargo, aún se desconocen los posibles efectos adversos de las mezclas que contienen compuestos de octaBDEs, como el DE-79, en el sistema AVPérgico.

El objetivo de este estudio es examinar los efectos de la exposición perinatal al DE-79 en el sistema AVPérgico. Para lo cual, las ratas gestantes se expusieron al DE-79 vía oral desde el día 6 de la gestación hasta el día 21 posnatal en dosis 0 (control), 1.7 (baja) ó 10.2 (alta) mg/kg/día. Las crías macho fueron divididas en 2 grupos a los 3 meses de edad: el grupo normosmótico, de animales con acceso ad libitum a agua corriente y el grupo hiperosmótico que fue sometido al reto de carga salina (acceso ad libitum a solución salina

al 2%, 20g NaCl/l, durante 4 días). Se evaluaron alteraciones en la osmorregulación (osmolalidad sérica) y en la liberación sistémica de AVP, así como la inmunorreactividad (AVP-IR) y la expresión génica de AVP en los núcleos paraventricular y supraóptico hipotalámicos. Para dilucidar un posible mecanismo de los efectos de DE-79 en el sistema AVPérgico, se investigaron tanto la inmunorreactividad (nNOS-IR) como la expresión de mRNA de la sintasa neuronal de óxido nítrico (nNOS) en los mismos núcleos hipotalámicos.

Los resultados mostraron que la exposición perinatal al DE-79 produjo alteraciones en la AVP-IR, la expresión de mRNA y la liberación sistémica de AVP en la edad adulta en condiciones normosmóticas y más evidentemente bajo un estímulo hiperosmótico. La nNOS-IR y la expresión de mRNA de nNOS también se vieron afectadas en los mismos núcleos. Observamos una desregulación homeostática, por lo que los organismos no están preparados para responder a nuevos retos fisiológicos. Dado que el óxido nítrico (NO) es un regulador de la AVP, proponemos que las alteraciones en el NO podrían ser un mecanismo subyacente a la alteración del sistema AVPérgico después de la exposición perinatal de DE-79 que conduce a déficits en la osmorregulación.

ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are globally distributed persistent and bioaccumulative environmental pollutants; they are used as flame retardants and are found in consumer goods such as electrical equipment, construction materials, coatings, textiles and polyurethane foam (furniture padding). PBDEs are detected in most biological samples, including human blood, adipose tissue, and breast milk and are considered as neurotoxicants and endocrine disruptors with important biological effects such as alterations in growth, reproduction, and on the activity of the hypothalamus-pituitary-adrenal axis.

The vasopressin (AVP), one of the neuropeptides secreted by the hypothalamic-neurohypophysial system, plays a key role in the maintenance of the vital water homeostasis. Also, it is a known target for pentaBDEs mixture (DE-71) and the structurally similar chemicals, polychlorinated biphenyls. However, the potential adverse effects of mixtures containing octaBDE compounds, like DE-79, on the AVPergic system are still unknown.

The present study aims to examine the effects of perinatal DE-79 exposure on the AVPergic system. Dams were dosed from gestational day 6 to postnatal day 21 at doses of 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day, male offspring were divided in two groups at 3-months of age: the normosmotic group, where animals had ad libitum access to tap water and the hyperosmotic group that was subjected to the salt loading challenge (ad libitum access to 2% saline solution, 20 g NaCl/l, for 4 days). Male offspring were later assessed for alterations in osmoregulation (i.e. serum osmolality and systemic AVP release),

and both AVP immunoreactivity (AVP-IR) and gene expression in the hypothalamic paraventricular and supraoptic nuclei. Additionally, to elucidate a possible mechanism for the effects of DE-79 on the AVPergic system, both neuronal nitric oxide synthase immunoreactivity (nNOS-IR) and its mRNA expression levels were determined in the same hypothalamic nuclei.

We detected disturbances in AVP-IR, mRNA expression and systemic release in adulthood under normosmotic conditions and more evidently under hyperosmotic stimulation. nNOS-IR and its mRNA expression were also affected in the same nuclei. The results showed that perinatal exposure to DE-79 produced homeostatic dysregulation, which does not allow organisms to be prepared to respond to upcoming physiological challenges. Since nitric oxide is an AVP regulator, we propose that disturbances in NO could be a mechanism underlying the AVPergic system disruption following perinatal DE-79 exposure leading to osmoregulation deficits.

1. INTRODUCCIÓN

1.1 Contaminación y medio ambiente

La contaminación ambiental es un problema mundial y es responsable de una cantidad alarmante de enfermedades. Los contaminantes ambientales son un grupo de compuestos químicos que se encuentran presentes en el aire, agua, suelo, lluvia, sedimentos, productos alimenticios marinos y terrestres (Colborn et al., 1993). Para algunos productos químicos, el compuesto intermediario metabolizado es aún más tóxico que el compuesto original. La estabilidad química es una característica deseable para un producto químico industrial, sin embargo, esto da lugar a compuestos que son altamente resistentes a la degradación, por lo que se acumulan y forman parte de los denominados "contaminantes orgánicos persistentes" (WHO, 2010; Zawatski and Lee, 2013). En los últimos años, ha quedado claro que los contaminantes y los productos químicos producidos en masa tienen efectos significativos en la salud de humanos y animales (León-Olea et al., 2014). Por lo anterior, los gobiernos mundiales han dirigido su atención a la generación y difusión de información ambiental. Sin embargo, la información sobre las diferentes sustancias químicas es aún limitada. Además, estas sustancias se usan para fabricar productos que usamos diariamente y que desempeñan un papel importante en nuestra calidad de vida. Un ejemplo de éstas, son los retardantes de flama.

1.2 Retardantes de flama

Los incendios accidentales causan pérdidas importantes de salud pública y en términos económicos. En respuesta a esto, la industria química desarrolló retardantes de flama (FRs)

comerciales: productos químicos activados por calor que reducen la capacidad de ignición y por lo tanto disminuyen el proceso de inflamabilidad, limitando la cantidad de calor liberado. Estos se adicionan a una amplia gama de textiles, plásticos, materiales de construcción y equipos electrónicos utilizados para fines industriales y domésticos (D'Silva et al., 2004). El uso de sustancias con propiedades retardantes de flama representa una drástica caída en la incidencia de incendios ocurridos en los últimos 30 años (Costa and Giordano, 2007). La combustión incompleta resultante del uso de FRs y de los materiales que los contienen pueden producir subproductos negativos como el monóxido de carbono, dioxinas y furanos halogenados, entre otros (Nelson, 1998; U.S. EPA, 2014).

Los FRs contienen uno o más de los siguientes elementos: cloro, bromo, aluminio, boro, nitrógeno, fósforo o silicio (Levchik, 2007). Generalmente se dividen de acuerdo a los grupos que contienen halogenos, fósforo, nitrógeno y los FRs inorgánicos (Birnbaum and Staskal, 2004; U.S. EPA, 2014). Los halogenados son la clase más diversificada de los FRs. Hay cuatro compuestos halogenados que actúan eficientemente como FRs y su eficiencia se incrementa con el tamaño de la molécula: yodo > bromo > cloro > flúor (Alaee et al., 2003; De Wit, 2002; Levchik, 2007; Rocha-Gutiérrez et al., 2015). Los compuestos fluorados son estables, pero el halógeno se libera después de que ocurre la combustión y los compuestos yodados no son estables. Los compuestos halogenados más empleados son los clorados y bromados (Alaee et al., 2003; Levchik, 2007). Los FRs clorados (como los bifenilos policlorados -PCBs-) presentan una acción retardante de flama efectiva, pero diversos estudios encontraron efectos nocivos para el medio ambiente y los seres vivos, por lo que fueron sustituidos por los compuestos bromados (Costa and Giordano, 2007; Darnerud et

al., 2001; Kodavanti and Curras-Collazo, 2010; Rocha-Gutiérrez et al., 2015). Los FRs bromados (BFRs; como los éteres difenilos polibromados –PBDEs–, difenilos polibromados –PBBs–, hexabromociclododecano –HBCDD-, tetrabromobisfenol –TBBPA–, entre otros) representan el mayor grupo del mercado debido a su bajo costo y alta eficiencia de rendimiento (Birnbaum and Staskal, 2004; Segev et al., 2009; U.S. EPA, 2014). Los BFRs tienen la capacidad para liberar átomos activos de bromo (radicales libres) en la fase gaseosa a medida que el material se descompone en el fuego. Estos átomos de bromo saturan las reacciones químicas que se producen en la llama, reduciendo el calor generado y disminuyendo o incluso impidiendo el proceso de combustión (Levchik, 2007; WHO/IPCS, 1994). Los éteres difenilos polibromados (PBDEs) fueron los primeros BFRs en ser detectados en el medio ambiente en 1979 (de Carlo, 1979) y la primera vez que fueron reportados como contaminantes ambientales fue en los primeros años de los 80s (Andersson and Blomkvist, 1981).

1.3 Éteres difenilos polibromados

Los éteres difenilos polibromados (PBDEs) son una clase de retardantes de flama bromados que se adicionan a plásticos, espumas de poliuretano, textiles y equipo electrónico. Cuando se produce un incendio, los PBDEs interfieren con el proceso de combustión, retardando así la ignición e inhibiendo la propagación del fuego (U.S. EPA, 2010).

1.3.1 Propiedades químicas y físicas

Una molécula de éter difenilo consiste en dos anillos de seis átomos de carbono cada uno, donde un carbono en cada anillo está unido al mismo átomo de oxígeno. La cantidad de bromo y el tiempo permitido para la reacción controlan el grado de bromación en la molécula de éter bromodifenilo (Ecology et al., 2006). Los PBDEs incluyen 209 diferentes isoformas teóricas o congéneres. Los congéneres varían en función de la posición y del número de bromos (1-10) unidos a los dos anillos de carbono (Birnbaum and Staskal, 2004; Ecology et al., 2006). Basándose en el número de sustituyentes de bromo, hay 10 grupos homólogos de congéneres de PBDEs (de monobromados hasta decabromados; ATSDR, 2017). Los PBDEs tienen una estructura molecular similar a otros compuestas halogenados, los bifenilos policlorados (PCBs), con la diferencia de que los PBDEs tienen un grupo éter uniendo los anillos bencénicos y tienen bromos en vez de cloros (**Fig. 1**).

Los PCBs fueron prohibidos en el mercado por su alta toxicidad. Los PBDEs, como los PCBs, no se fijan en el producto polimérico a través de enlaces químicos, por lo tanto, pueden filtrarse en el ambiente. Son contaminantes orgánicos persistentes, se bioacumulan en el medio ambiente y seres vivos, y se biomagnifican en la cadena alimenticia (Birnbaum and Staskal, 2004; Costa and Giordano, 2007; Darnerud et al., 2001; Rocha-Gutiérrez et al., 2015).

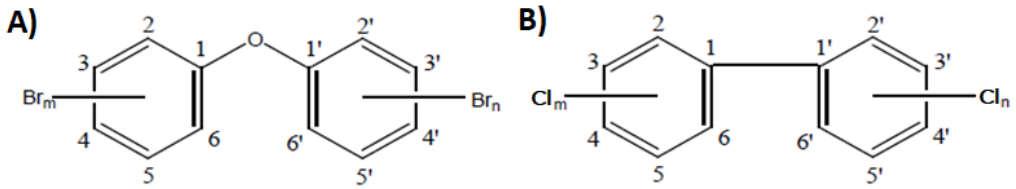

Figura 1. Estructura química de PBDEs y PCBs. Representación gráfica de la estructura química general de **(A)** los éteres difenilos polibromados (PBDEs) y **(B)** los bifenilos policlorados (PCBs), donde m + n = 1 a 10 (modificado de WHO/IPCS, 1994; Rocha-Gutiérrez et al., 2015).

Tres mezclas comerciales de PBDEs se produjeron y utilizaron. Estas se nombraron por el número promedio de bromos adjuntos a la estructura del éter, siendo mezclas de deca-, octa- y pentabromados (ATSDR, 2017; Rocha-Gutiérrez et al., 2015). La mezcla comercial que se usa actualmente es la de éteres difenilos decabromados (decaBDEs: DE-83R, Saytex 102E), que puede debromarse metabólicamente o en el medio ambiente en formas más biodisponibles, las otras dos mezclas comerciales son de éteres difenilos octabromados (octaBDEs: DE-79) y éteres difenilos pentabromados (pentaBDEs: DE-60F, DE-61, DE-62, DE-71; Alaee et al., 2003; ATSDR, 2017; Birnbaum and Staskal, 2004). La composición general de las mezclas comerciales de los PBDEs se encuentra en la **Tabla 1**.

Tabla 1. Composición general de las mezclas comerciales de PBDEs

	Porcentaje de congénere (%)							
Producto comercial	Tri-BDEs	tetra-BDEs	penta-BDEs	hexa-BDEs	hepta-BDEs	octa-BDEs	nona-BDEs	deca-BDEs
PentaBDE	<1	24-38	50-60	4-8				
OctaBDE				10-12	43-44	31-35	10-11	<1
DecaBDE							<3	97-98

Porcentajes de congéneres de éteres bromodifenilos (BDEs) presentes en cada una de las mezclas comerciales. Modificado de ATSDR, 2017; Ecology et al., 2006; WHO/IPCS, 1994

Los PBDEs comerciales tienen una alta resistencia a la degradación física, química y biológica. El punto de ebullición de los PBDEs está entre 310 y 425 °C y su presión de vapor es baja a temperatura ambiente. Son lipofílicos, su solubilidad en agua es baja, especialmente para los compuestos bromados superiores. (Darnerud et al., 2001; WHO/IPCS, 1994). Las propiedades físicas se resumen en la **Tabla 2**.

Tabla 2. Propiedades físicas de las mezclas comerciales de PBDEs

Característica	PentaBDEs	OctaBDEs	DecaBDEs
Peso molecular	564.8	801.5	959.2
Color	Claro (ambar a amarillo pálido)	Blancuzco	Blancuzco
Estado físico	Líquido altamente viscoso	Polvo	Polvo
Presión de vapor	$3.5x10^{-7}$ mmHg (25°C)	$4.9×10^{-8}$ mmHg (21°C)	3.2x10-8 mmHg
Punto de fusión (°C)	92 (BDE-99); 97-98 (BDE-100); -7 a -3 (comercial)	~ 200	290-306
Punto de ebullición (°C)	>300 (d)	>330 (d)	>320 (d)
Densidad (g/ml)	2.25-2.28 (25°C)	2.76-2.8	3-3.25
Solubilidad en agua (µg/l)	13.3	<1 ppb (25°C)	20-30 ?
Solubilidad en solventes orgánicos	10 g/kg (metanol); miscible en tolueno	20 g/l (acetona); 200 g/l (benceno); 2g/l (metanol) todos a 25°C	---
Log K_{ow}	6.5-7.0	6.29	10

Símbolos: --- sin información; (d) descompone; ? dato no confiable.
Fuente: modificado de ATSDR, 2017; Darnerud et al., 2001; ENVIRON International Corporation, 2003a, 2003b; WHO/IPCS, 1994.

1.3.2 Producción y usos

La producción comercial de los PBDEs inició en los 70s (ATSDR, 2017; WHO/IPCS, 1994). Se usan, únicamente, como retardantes de flama, se adicionan en concentraciones de 5 a 30% a diferentes polímeros, resinas, conectores eléctricos, plásticos como poliestireno de alto impacto, entre otros (Darnerud et al., 2001; WHO/IPCS, 1994).

Las mezclas comerciales de pentaBDEs y octaBDEs fueron, voluntariamente, retiradas del mercado de la Unión Europea a finales del 2004 y de Estados Unidos de América (USA) en 2006 (ATSDR, 2017; Costa and Giordano, 2007; U.S. EPA, 2010). En el 2008 la Unión Europea restringió el uso de mezclas de decaBDEs (EC, 2014). A pesar de los esfuerzos por eliminar a los penta- y octaBDEs, una extensa gama de productos en el mercado los contiene. Además, existe evidencia de la debromación del BDE 209 (principal componente de la mezcla comercial de decaBDEs) en congéneres menos bromados como el BDE 183 (principal componente de la mezcla comercia de octaBDEs) y otros nona- y octaBDEs (ver revisiones de Santos et al., 2016; Zhao et al., 2018). Lo anterior explica la existencia de PBDEs menos bromados en el medio ambiente y en los seres vivos.

Las mezclas comerciales de pentaBDEs se usaron, principalmente, por la industria de muebles y tapicería como aditivo en la espuma de poliuretano para cojines y colchones (ENVIRON International Corporation, 2003a). Las mezclas de octaBDEs en la electrónica y las industrias plásticas, se adicionaron al acrilonitrilo butadieno estireno (ABS) usado en carcasas de equipos de oficina de uso común como máquinas de escribir, CPUs, terminales de video y fotocopiadoras (ATSDR, 2017; ENVIRON International Corporation, 2003b; U.S. EPA, 2010). Las mezclas de decaBDEs se usan, principalmente, en productos electrónicos como computadoras, alambres y cableado; y en menor medida en tejidos y tapicería (ATSDR, 2017).

1.3.3 Toxicocinética

Las rutas de entrada de los PBDEs en los seres humanos y animales se dan por ingesta, inhalación y absorción dérmica. La vía oral es la más eficaz. Estudios indican que la administración por sonda nasogástrica, en vehículos lipófilicos, presenta un rango de absorción de 70-85% para tetra-, penta- y hexaBDEs, y un 10-26% para decaBDEs (ATSDR, 2017; Hakk and Letcher, 2003; Riu et al., 2008). Además. los PBDEs se transfieren de madres embarazadas al feto en desarrollo, así como de la leche materna a los lactantes (ATSDR, 2017; Chen et al., 2014a; Eskenazi et al., 2013; Li et al., 2013, 2017; López et al., 2006; WHO, 2010). Se distribuyen a todos los tejidos y se acumulan preferentemente en la grasa (ATSDR, 2017; Costa and Giordano, 2014; Staskal et al., 2006).

La hidroxilación oxidativa de los PBDEs es la principal transformación metabólica que ocurre en humanos y animales de laboratorio. Los compuestos hidroxilados pueden tener propiedades toxicológicas diferentes o de mayor magnitud que los compuestos originales (Costa and Giordano, 2014; Gross et al., 2015). Se desconocen las rutas metabólicas detalladas de los PBDEs, pero se propone que el citocromo P450 participa en la formación de estos metabolitos hidroxilados (ATSDR, 2017; Gross et al., 2015; Staskal et al., 2006).

La vida media es de aproximadamente 15 días para los decaBDEs, pero es más prolongado (> 90 días) para los congéneres menos bromados (Costa and Giordano, 2014; Thuresson et al., 2006). Los PBDEs se excretan principalmente a través de las heces y la orina (ATSDR, 2017; Costa and Giordano, 2014; Hakk and Letcher, 2003; Riu et al., 2008; Staskal et al., 2006).

1.3.4 Exposición humana

En general, los PBDEs entran en contacto con el medio ambiente durante su fabricación, así como durante el proceso de fabricación de los productos que los incluyen como aditivos (Kodavanti and Curras-Collazo, 2010; Król et al., 2012; U.S. EPA, 2010). Estos contaminantes son liberados en el aire, el agua y la tierra para después entrar en la cadena alimenticia. Son compuestos orgánicos lipofílicos y persistentes, se bioacumulan y biomagnifican en la cadena alimenticia acuática y terrestre (U.S. EPA, 2010; WHO, 2010). Se distribuyen a nivel mundial a través del aire y de las corrientes oceánicas, viajando largas distancias a través de intercambios aire-agua y ciclos que involucran lluvia, nieve y partículas secas. Por lo tanto, se encuentran en lugares alejados de los sitios industriales o de las zonas donde fueron liberados. Los PBDEs tienen alta afinidad para enlazarse a partículas, por lo que se presentan en altos niveles en sedimentos, lodos residuales y partículas de polvo (Akortia et al., 2016; Alaee et al., 2003; Darnerud et al., 2001; Dewailly et al., 1999; Xiao et al., 2012; WHO, 2010). En la **Fig. 2** se muestra de manera general el trayecto y destino de los PBDEs y las vías de exposición.

La ingesta de polvo doméstico contaminado (y en menor grado la inhalación y absorción dérmica al polvo) representa entre el 80 y el 90% de las exposiciones totales de PBDEs de la población general (ATSDR, 2017; Johnson-Restrepo and Kannan, 2009; Jones-Otazo et al., 2005; WHO, 2010; Wu et al., 2007). El resto de la exposición se da por ingesta de alimentos contaminados. Estudios realizados en Japón, España, Suecia y USA han demostrado la presencia de PBDEs en diferentes alimentos correspondiente a una ingesta diaria de 26 a 150 ng/kg/día, los mayores niveles se encontraron en pescados, seguidos por carne roja y

luego productos lácteos (Andersson and Blomkvist, 1981; Bocio et al., 2003; Boucher et al., 2018; Darnerud et al., 2001; Schecter et al., 2006; Wu et al., 2007).

La exposición ocupacional es otra vía importante, los trabajadores involucrados en la manufactura y producción de productos a los que se les adicionan PBDEs están expuestos a concentraciones más altas de éstos. Las personas que viven cerca de sitios de disposición de desechos peligrosos pueden estar expuestas a los PBDEs al respirar polvo contaminado (Athanasiasou et al., 2008; ATSDR, 2017; SEMARNAT, 2013). Se encontraron niveles de PBDEs 4 a 10 veces más altos en aire cerca de una trituradora de plásticos en comparación con otras localizaciones de una planta desmanteladora de electrónicos (Sjödin et al., 2001). Se encontraron mayores concentraciones de PBDEs en sangre de trabajadores dedicados al desmantelamiento de productos electrónicos de una instalación de reciclaje electrónico de Suecia y en los involucrados en el reciclaje de espuma y en la instalación de alfombras en los USA (Julander et al., 2005; Stapleton et al., 2008). Además, se encontraron niveles altos de PBDEs en leche materna de mujeres que vivían cerca de una planta de reciclaje de desechos electrónicos en China y en niños que trabajaban y/o vivían cerca de un basurero en Nicaragua (Athanasiasou et al., 2008; Li et al., 2017).

La exposición a los PBDEs inicia desde la gestación, pues la placenta no representa una barrera efectiva contra los PBDEs (Leonetti et al., 2016; Li et al., 2013; Shin et al., 2016). Los congéneres de BDEs pasan a través de la placenta a diferentes tasas de penetración (Chen et al., 2014b; Shin et al., 2016). Posteriormente, la exposición ocurre a través de la leche materna (Ali et al., 2013; Antignac et al., 2009; Eskenazi et al., 2013; Kodavanti et al., 2010; Król et al., 2012; Li et al., 2013; Roosens et al., 2010). En cuanto a reportes de ingesta

estimada diaria de PBDEs por lactancia, Canadá presentó un promedio de 280 ng/kg peso/día (Jones-Otazo et al., 2005), mientras que el promedio de China fue de 51.7 ng/kg peso/día (Chen et al., 2014b), en Indonesia fue de 6.0 ng/kg peso/día (Sudaryanto et al., 2008) y en Taiwan de 20.6 ng/kg peso/día (Chao et al., 2007).

En comparación con los adultos, los niños tienen mayor exposición y susceptibilidad a los PBDEs. Los bebés, niños y adolescentes consumen más alimentos y líquidos por kilogramo de peso corporal que los adultos, aumentando la exposición por ingesta. Además, tienen mayores índices de inhalación y una mayor proporción de superficie corporal a peso corporal, resultando en un aumento en la exposición por inhalación y a través de la dermis. El comportamiento normal de los niños, como arrastrarse por el suelo y poner sus manos y objetos en la boca, resulta en una exposición extra no experimentada por los adultos (ATSDR, 2017; U.S. EPA, 2010; WHO, 2010). La habilidad de los niños para absorber, metabolizar y desintoxicar sustancias químicas difiere de la de los adultos. El feto, el lactante y el niño son susceptibles debido al rápido desarrollo de sus órganos y sistemas, niveles reducidos de ciertas enzimas detoxificantes y depósitos de grasa más pequeños para secuestrar a los PBDEs (ATSDR, 2017; Damstra, 2002; Ek et al., 2012; Guzelian et al., 1992; WHO, 2010). Johnson-Restrepo y Kannan (2009) estimaron las dosis de exposición diaria a PBDEs por ingesta de polvo/contacto dérmico, inhalación y dieta (incluyendo la ingesta de leche materna en lactantes) en diferentes edades en población abierta de USA (Estados Unidos de América). Las dosis de exposición diaria reportadas fueron: lactantes < 1 año de edad con 86.4 ng/kg/día (el 91% por consumo de leche materna), infantes de 1 a 5 años de

edad con 13.3 ng/kg/día, escolares de 6 a 11 años de edad 5.3 ng/kg/días, adolescentes de 12 a 19 años de edad con 3.5 ng/kg/día y adultos >20 años de edad con 2.9 ng/kg/día.

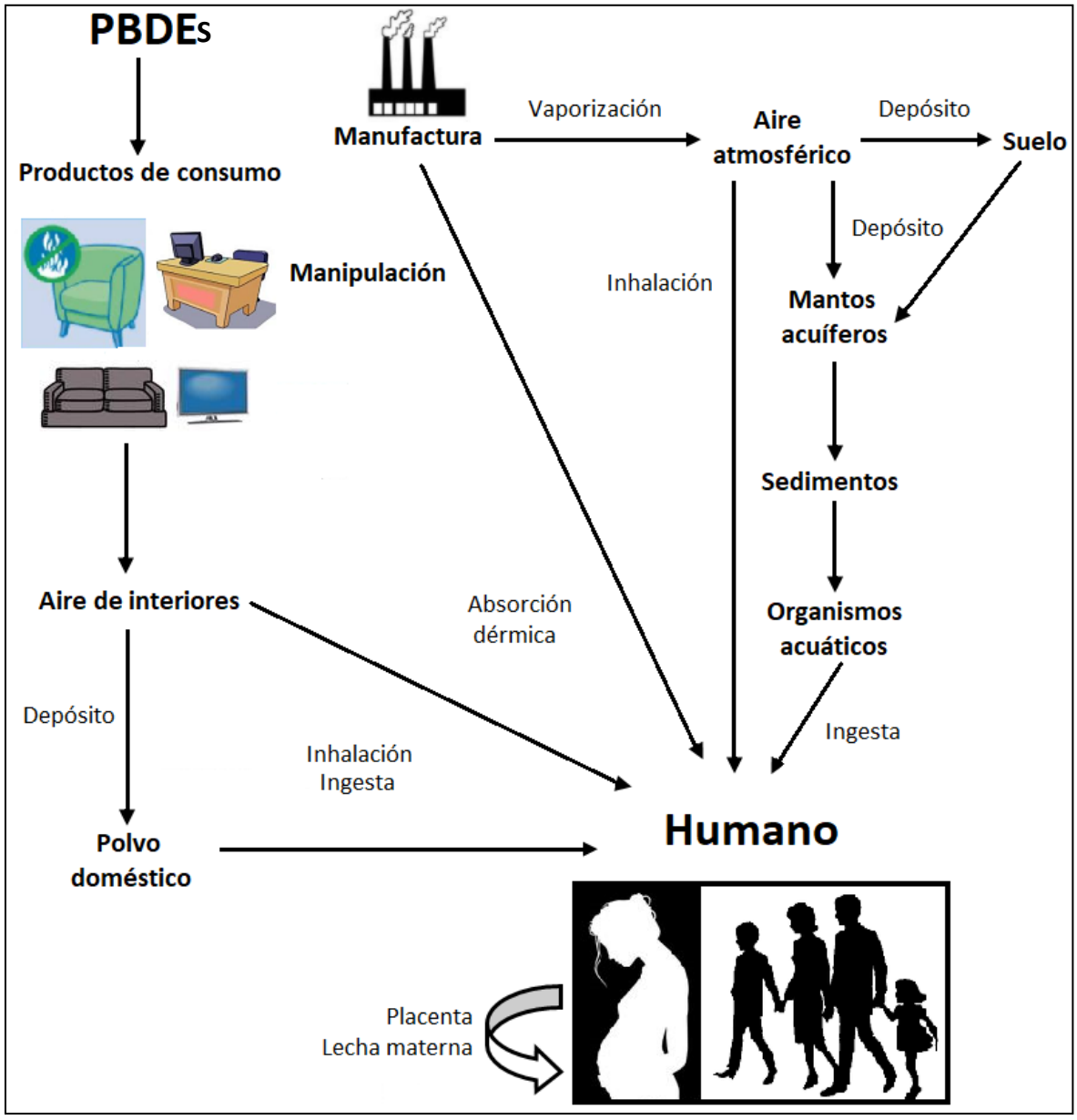

Figura 2. Esquema de trayecto y destino de los PBDEs. Los éteres difenilos polibromados (PBDEs) que entran al ambiente provienen de la producción, uso y disposición final de los artículos que los contienen. Las principales fuentes de contaminación son aparatos electrónicos y muebles. Las vías de exposición en el humano son la ingesta, inhalación, absorción dérmica, transplacentaria y por leche materna. (Modificado de Król et al., 2012).

1.3.5 Situación en México

Los Estados Unidos Mexicanos no son, ni han sido, productores de PBDEs y se desconoce la demanda y consumo en el país. Sin embargo, los contienen una gran variedad de productos, fabricados en USA y otros países, que son importados a México, principalmente aparatos electrónicos, automóviles, muebles y ropa (Rocha-Gutierrez and Lee, 2013). Una vez que la vida útil de estos productos termina, son desechados en sitios de disposición de residuos, que, en la mayoría de los casos, no cumplen con la normatividad correspondiente. Además, la basura electrónica de países desarrollados es importada ilegalmente a países en vías de desarrollo, como México (ATSDR, 2017; SEMARNAT, 2013). Por lo tanto, la población mexicana está expuesta constantemente a los PBDEs.

Los trabajos de investigación relacionados con la presencia o monitoreo de contaminantes emergentes en México aún son escasos. El primer estudio para determinar a los PBDEs en muestras humanas reportó niveles altos en sangre de mujeres de una zona urbana de San Luis Potosí (López et al., 2006), estos niveles están por arriba de los reportados en Europa y Japón (Ryan and Patry, 2002); y en leche materna de mujeres de una población rural de la Huasteca Potosina (López et al., 2006), con niveles por arriba de lo reportando en madres de Corea del Sur (Shin et al., 2016) y de Taiwan (Chao et al., 2007), pero por debajo de los que presentaron madres australianas (Toms et al., 2007), americanas (Johnson-Restrepo et al., 2007), chinas (Chen et al., 2014b) y suecas (López et al., 2006).

En cuanto a la exposición en niños mexicanos, se han realizado estudios en diferentes poblaciones urbanas y rurales de ciudades con actividad industrial, agrícola con uso de

plaguicidas y de eliminación de desechos (Ochoa-Martinez et al., 2016; Orta-García et al., 2018; Perez-Maldonado et al., 2009; 2017) donde se encontraron niveles por debajo de los encontrados en niños de Nicaragua que trabajaban en un basurero (Athanasiasou et al., 2008) y en niños de población general de USA (Erkin-Cakmak et al., 2015; Eskenazi et al., 2013; Wu et al., 2015); y por arriba de lo reportado en niños de población general de Alemania (Link et al., 2012), China (Zhang et al., 2011), Dinamarca (Knudsen et al., 2017) y de Paquistán (Ali et al., 2013).

En una comparativa con niveles de PBDEs en adultos, los niveles detectados en la Ciudad de México (Orta-García et al., 2014) se encuentran por debajo de lo reportado para USA (ATSDR, 2017; Eskenazi et al., 2013), pero por arriba de los reportados en Asia y Europa (Antignac et al., 2009; Fromme et al., 2015; Garí and Grimalt, 2013; Kalantzi et al., 2011; Kim et al., 2012; Roosens et al., 2010; Tay et al., 2019).

1.3.6 Efectos adversos

Los efectos de la exposición a los PBDEs están relacionados con la edad de exposición, la latencia de la exposición, el congénere o la mezcla de congéneres de PBDEs, la dinámica dosis-respuesta y los efectos latentes a largo plazo (Diamanti-Kandarakis et al., 2009). La mayoría de la información sobre los efectos adversos de los PBDEs y sus metabolitos proviene de estudios en animales, pues no se cuenta con información definitiva sobre los efectos en la salud que provocan en humanos. Sin embargo, estudios recientes han evaluado la asociación entre las concentraciones de PBDEs en diferentes tejidos en humanos y posibles efectos en la salud (ATSDR, 2017).

En el sistema inmune, estudios en humanos han asociado la disminución del número de linfocitos circulantes con el incremento en los niveles de PBDEs en suero de niños (14-19 años) de los Países Bajos (Leijs et al., 2009). Estudios en animales adultos con exposición aguda (menor de 14 días) a PBDEs reportan la disminución del peso del timo (Fowles et al., 1994), del número total de esplenocitos y células T CD4+ y CD8+ (Thuvander and Darnerud, 1999). La exposición intermedia (15-364 días) a decaBDEs, reporta un aumento significativo de células T CD4+ y CD8+ (Liu et al., 2012) y a BDEs menos bromados provoca el aumento significativo de la incidencia de hiperplasia esplénica y del peso del bazo (Liu et al., 2012; Maranghi et al., 2013; Martin et al., 2007). En animales con exposición crónica (>364 días) se observa el aumento en la incidencia de hematopoyesis esplénica, fibrosis esplénica e hiperplasia linfoide (NTP, 1983). Los datos en animales sugieren que la exposición oral a PBDEs durante el desarrollo puede conducir a la inmunosupresión, pues diferentes estudios describen la alteración en el peso del timo y del bazo y la disminución de las IgM e IgG en suero (ATSDR, 2017; Fowles et al., 1994; Hong et al., 2010; Watanabe et al., 2008).

Con respecto a efectos neurotóxicos, los estudios en humanos han relacionado niveles altos de PBDEs con alteraciones en la actividad motora (ver revisión de Gibson et al., 2018). Eskenazi et al. (2013) relacionaron la exposición a los PBDEs en el útero y durante la infancia con alteraciones en el desarrollo neuroconductual. Otros estudios, asociaron las altas concentraciones séricas de PBDEs con un menor IQ y desarrollo mental y psicomotor en niños (Gibson et al., 2018; Herbstman et al., 2010). Estudios in vitro demuestran que la exposición de PBDEs interfiere con las vías de transducción de señales en neuronas granulares del cerebelo de rata al afectar a la proteína quinasa C (PKC) y a la homeostasis

del calcio (Kodavanti et al., 2005; Kodavanti and Derr-Yellin, 2002; Kodavanti and Ward, 2005). Estos eventos de señalización intracelular están asociados con el desarrollo neuronal y la función de aprendizaje y memoria; funciones que están alteradas en animales expuestos a PBDEs, tanto en la edad adulta, como en gestación y lactancia. Estudios en animales expuestos a los PBDEs durante la vida adulta encontraron deterioro de la atención y control inhibitorio, y alteraciones en la memoria y el aprendizaje (ATSDR, 2017; Yan et al., 2012). En animales expuestos a PBDEs en períodos de rápido crecimiento y desarrollo cerebral se encontraron: aberraciones permanentes por comportamiento espontáneo, capacidad de habituación y alteraciones en la memoria y el aprendizaje (Eriksson et al., 2002; Kodavanti and Loganathan, 2014; Viberg et al., 2006).

Los PBDEs fueron catalogados como disruptores endócrinos (EDs), lo que significa que son sustancias que al ser absorbidas por el cuerpo imitan, modifican o bloquean los sistemas hormonales y homeostáticos, que permiten al organismo comunicarse con su entorno y responder a él (ATSDR, 2017; Diamanti-Kandarakis et al., 2009; Kodavanti and Curras-Collazo, 2010; Waye and Trudeau, 2011; WHO, 2010). Los efectos más estudiados como EDs han sido en relación con las hormonas reproductivas y tiroideas, pero se han encontrado que alteran diferentes sistemas hormonales.

Diversos estudios en humanos relacionan la exposición de PBDEs con la disminución en la movilidad y concentración de espermatozoides y con menor edad de la menarquia (Abdelouahab et al., 2011; Chen et al., 2011). Además, en animales expuestos (en la edad adulta o durante la gestación y lactancia) se encontraron efectos anti-androgénicos; retraso en la pubertad en ratas macho y hembra; disminución en la foliculogénesis y en la

concentración de estradiol en suero en ratas hembra; y disminución del crecimiento de los tejidos dependientes de andrógenos en ratas macho (Kodavanti et al., 2010; Lilienthal et al., 2006; Stoker et al., 2005; Talsness et al., 2008). Las hormonas tiroideas (T3 y T4) son similares estructuralmente con los metabolitos producidos por los PBDEs en el organismo, lo que facilita su unión a los receptores de estas hormonas, por lo que la tiroides es un objetivo de preocupación para la exposición a PBDEs (Kodavanti and Curras-Collazo, 2010; León-Olea et al., 2014; Talsness et al., 2008). Diversos estudios en hombres adultos expuestos ocupacionalmente a los PBDEs reportan que los niveles de T4, T3 y TSH se encuentran alterados (Abdelouahab et al, 2011; Hagmar et al, 2001; Johnson et al., 2013; Meeker et al., 2009; Turyk et al, 2008). Estudios en roedores muestran la disminución en los niveles séricos de T4 posterior a la exposición de PBDEs en la edad adulta (Fowles et al., 1994; Hallgren and Darnerud, 2002) y por la exposición durante el desarrollo (Kodavanti et al., 2010; Kuriyama et al., 2007; Szabo et al., 2009). De la misma manera, estudios en aves, peces y anfibios expuestos a PBDEs durante el desarrollo encontraron alteraciones en los niveles de T4 (Fernie et al., 2005; Kodavanti and Curras-Collazo, 2010; Nugegoda and Kibria, 2017). Otros efectos, relacionados con la exposición de animales a PBDEs, incluyen alteraciones hepáticas como esteatosis microvesicular y macrovesicular hepática, aumento en la actividad de enzimas uridin difosfato-glucuroniltransferasa, etoxi-resorufina-O-desetilasa y pentoxiresorufina-O-desetilasa (UDPGT, EROD y PROD; Bruchajzer et al., 2010, 2011; Fowles et al., 1994; Szabo et al., 2009), hipertrofia hepatocelular, aumento de las proteínas totales y del colesterol sérico, disminución de la relación albúmina/globulina (Fattore et al 2001; Öberg et al., 2010), entre otros.

La exposición a los PBDEs puede tener manifestaciones diferentes según el momento de exposición: en el desarrollo intrauterino, neonatal o en la edad adulta (Colborn et al 1993). La gestación y lactancia (periodo perinatal) son etapas críticas del desarrollo debido al rápido crecimiento y desarrollo de órganos y sistemas que se lleva a cabo. En mamíferos, la mayoría de los sistemas de órganos completan su desarrollo estructural durante el período de organogénesis (Grandjean and Landrigan, 2006). La barrera placentaria es importante para la protección del producto durante la gestación, además, la barrera hematoencefálica provee una protección adicional, tanto en la vida prenatal como posnatal (Ek et al., 2012). Por lo tanto, contaminantes como los PBDEs, que son compuestos lipofílicos, capaces de cruzar ambas barreras, interfieren con los procesos de desarrollo de animales y humanos (Chen et al., 2014b; Kodavanti and Loganathan, 2014). La exposición a PBDEs durante etapas críticas del desarrollo puede producir cambios fisiológicos intensificados que persistan hasta la adultez tardía y que se transfieran a generaciones posteriores (Kodavanti and Curras-Collazo, 2010; Patisaul and Adewale, 2009). Se reportó que la presencia de xenobióticos en el cerebro durante un período definido de este proceso de maduración es un factor clave para los efectos en la etapa adulta (Eriksson et al., 2002; Viberg et al., 2008).

Estudios recientes observaron que la exposición durante las etapas críticas del desarrollo al DE-71 (mezcla de pentaBDEs) provoca la acumulación de diferentes congéneres de PBDEs en varios tejidos. Estos congéneres cruzan las barreras placentaria y hematoencefálica, causando alteraciones en las hormonas tiroideas y reproductivas, en el sistema vasopresinérgico y en el comportamiento (Kodavanti et al., 2010; Kodavanti and Curras-Collazo, 2010; Mucio-Ramírez et al., 2017). Diferentes congéneres y mezclas comerciales de

PBDEs han mostrado que la exposición durante las etapas críticas del desarrollo produce efectos neuroendócrinos (Dingemans et al., 2007; Kodavanti, 2005; Mucio-Ramírez et al., 2017; Viberg et al., 2006, 2008). Sin embargo, aún existe la necesidad de realizar estudios de toxicidad más exhaustivos, especialmente con la exposición a mezclas comerciales específicas durante etapas críticas del desarrollo; y estudiar los efectos en diferentes sistemas, como el sistema vasopresinérgico.

1.4 Sistema Vasopresinérgico

La Arginina Vasopresina (AVP) es un péptido que se aisló en la década de los 50 (Acher and Chauvet, 1954; du Vigneaud et al; 1953); se compone de 9 aminoácidos con un puente disulfuro entre los residuos de cisteína en las posiciones 1 y 6 (**Fig. 3**). La mayoría de los mamíferos tienen al aminoácido arginina en la posición 8 (arginina vasopresina -AVP-), excepto los porcinos que tienen lisina en vez de arginina (lisina vasopresina -LVP-; Kasting, 1988; PhD PSGB, 2000). La AVP se libera a la circulación sistémica y estimula órganos distantes, por lo que es una hormona, aunque también tiene efectos como neurotransmisor/neuromodulador en el sistema nervioso central independiente de sus efectos hormonales (De Wied, 1997; Morris et al, 1987; Neumann and Landgraf, 2012; Verbalis, 2013).

Las principales funciones de la AVP en los mamíferos son: el equilibrio hidroelectrolítico al mediar la antidiuresis y conservar la osmolaridad, por lo que es también denominada hormona antidiurética (ADH); y la vasoconstricción para preservar la presión arterial contra la hipovolemia (Hanoune, 2009a; Leng et al., 1999; Verbalis, 2013).

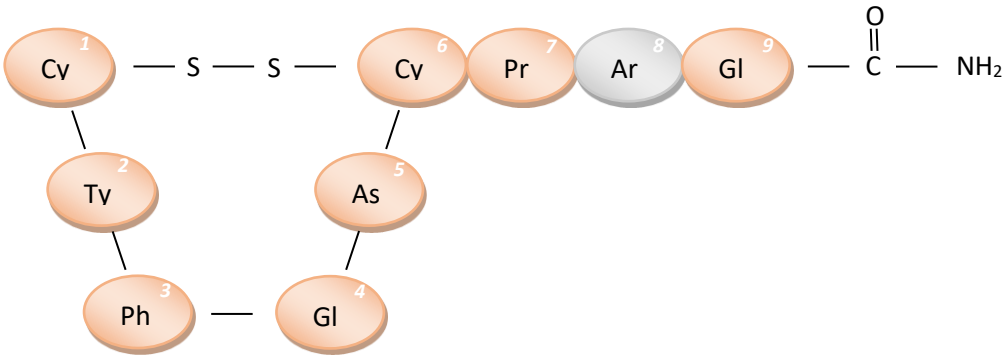

Figura 3. Estructura de la AVP. Se observa la estructura del nonapéptido arginina vasopresina (AVP) con los 9 aminoácidos (abreviados) que la conforman.

1.4.1 Producción y liberación de AVP

La AVP se sintetiza, principalmente, en las células magnocelulares (MNCs) y parvocelulares neuroendócrinas (PNCs) de los núcleos paraventricular (PVN) y supraóptico (SON) del hipotálamo (Brownstein et al., 1980; Buijs et al., 1980; Morris, 2013). En la rata, las células que sintetizan AVP en estos núcleos aparecen desde los 13 y 17 días de gestación (GD), siendo identificables con marcadores inmunohistoquímicos para AVP en los GD 16 y 18 (Buijs et al., 1980; Ifft, 1972). Las fibras vasopresinérgicas (AVPérgicas) fuera del hipotálamo aparecen alrededor del GD 17 (De Vries et al., 1981).

En las MNCs la AVP se encuentra en vesículas secretoras que son enviadas a las terminales axónicas en la neurohipófisis, donde, de manera calcio dependiente, se libera a la circulación sistémica y por medio de la unión a sus receptores regula diferentes procesos fisiológicos como el balance osmótico y la presión arterial. Por otro lado, las PNCs del PVN, proyectan sus axones a la eminencia media (ME), donde a través del plexo capilar del sistema porta hipotalámico-hipofisiario viajan a la adenohipófisis para estimular la

secreción de la hormona adrenocorticótropa (ACTH); al tronco encefálico, a la médula espinal y a otras regiones cerebrales. Existen pequeñas poblaciones de neuronas AVPérgicas en el núcleo del lecho de la estría terminal (BST), amígdala medial (MeA) y núcleo supraquiasmático (SCN), cuyas proyecciones siguen diferentes vías cerebrales donde la AVP actúa como neurotransmisor, involucrada en ritmos circadianos, termorregulación, nocicepción, regulación cardiovascular e ingesta de agua (Buijs, 1978; De Vries and Miller, 1999; Kodavanti and Curras-Collazo, 2010; Ludwig and Leng, 2006; Morris, 2013; Rood and De Vries, 2011; **Fig. 4**).

Desde la neurohipófisis, la AVP se libera al torrente sanguíneo en respuesta a estímulos osmóticos (como la deshidratación, hiperosmolaridad) y no osmóticos (como la hipovolemia, hipotensión, hipoglicemia; Brownstein et al., 1980; Murphy et al., 2016; Ohbuchi et al., 2015; Verbalis, 2013). Además, tiene una liberación somatodendrítica en las MNCs del PVN y SON durante la activación osmótica, que modula la liberación de la AVP sistémica al actuar como una señal de retroalimentación (Ludwig and Leng, 2006; Murphy et al., 2016).

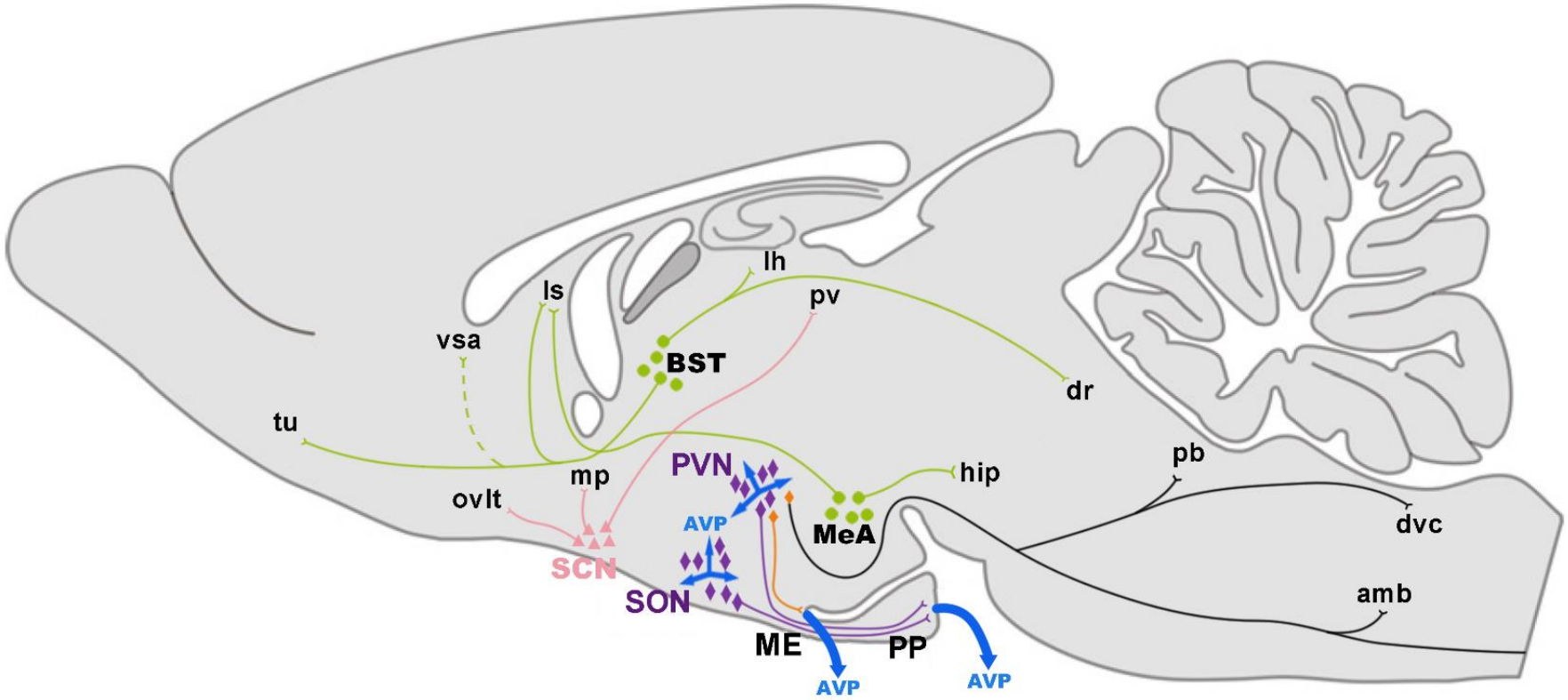

Figura 4. Esquema de las principales proyecciones del sistema vasopresinérgico. Las neuronas magnocelulares (MNCs) de los núcleos paraventricular (PVN) y supraóptico (SON; rombos morados) sintetizan vasopresina (AVP) y la secretan a la circulación sistémica desde los terminales axónicos (flechas azules gruesas) en la hipófisis posterior (PP), además de la secreción desde los somas y dendritas intrínseca a los núcleos (flechas azules finas). Las neuronas parvocelulares (PNCs; rombos naranjas) del PVN proyectan fibras AVPérgicas a la eminencia media (ME), donde la AVP actúa en el eje hipotálamo-pituitario-adrenal (línea naranja), además dan lugar a proyecciones que descienden hacia el tronco cerebral autónomo y la médula espinal (líneas negras). Neuronas del núcleo supraquiasmático (SCN) proyectan al órgano vascular de la lámina terminal (ovlt), áreas preópticas mediales (mp), periventricular (pv), entre otros. Proyecciones AVPérgicas del núcleo del lecho de la estría terminal (BST) y de la amígdala medial (MeA) llegan a regiones del procencéfalo y al tallo cerebral asociados al comportamiento social, aprendizaje, memoria, función cardiovascular y reproductiva.

Otras abreviaturas: tubérculo olfatorio (tu), área septal ventral (vsa), septo lateral (ls), núcleo habenular lateral (lh), hipocampo ventral (hip), núcleo dorsal del rafe (dr), núcleo parabraquial (pb), complejo dorsal vagal (dvc), y núcleo ambiguo (amb). Adaptado de De Vries and Miller, 1999; Kodavanti and Curras-Collazo, 2010.

1.4.2 Receptores de AVP

Los efectos de la AVP están mediados por receptores acoplados a proteínas G (GPCR) y son conocidos como V_{1a}, V_{1b} y V_2. Los receptores V_{1a} y V_{1b} se acoplan a la proteína Gq y siguen la vía de la fosfolipasa C, mientras que los receptores V_2 se acoplan a la proteína Gs y siguen la vía del adenilato cliclasa. Los receptores V_{1a} son los más abundantes, se localizan en las células musculares lisas de la vasculatura donde median vasoconstricción; también se han descrito en corazón, vejiga, riñón, hígado, cerebro y plaquetas. Los receptores V_{1b} están localizados principalmente en la adenohipófisis (específico para las células corticotropas) mediando la producción de ACTH; también se ha detectado en tejidos periféricos (riñón, timo, corazón, pulmón, bazo y útero). Los receptores V_2 se encuentran principalmente en las células del sistema renal de ductos colectores donde median la reabsorción de agua (Carrillo et al., 2003; Hanoune, 2009b; Teruyama, 2014).

1.4.3 Osmorregulación

Diversos mecanismos fisiológicos están involucrados en mantener el balance entre la ingesta y excreción de agua y sales. Dentro de estos mecanismos, el control de la sed y la secreción de AVP tienen el papel más importante regulando los cambios en la osmolaridad plasmática en mamíferos (Bourque, 1998; McKinley et al., 2004; Spinelli et al., 1987). La respuesta apropiada de la AVP depende de: osmoreceptores especializados ubicados en la región del hipotálamo anterior, el órgano subfornical (SFO) y el órgano vasculoso de la lámina terminal (OVLT); y de las propias MNCs osmosensitivas en los núcleos hipotalámicos (Bourque, 2008; McKinley et al., 2004).

Incrementos tan pequeños como el 1% (aproximadamente 3 mOsm/kg) en la osmolaridad plasmática son suficientes para estimular a las células osmorreceptoras que envían señales a las neuronas de los núcleos hipotalámicos estimulando la liberación de la AVP almacenada en las vesículas de la neurohipófisis a la circulación sistémica. La AVP sistémica se une a los receptores V_2 específicos en la parte final de los túbulos distales y en los conductos colectores del riñón provocando en estos, la formación de canales de agua por la agrupación de las acuaporinas-2 (AQP-2). Lo anterior resulta en un incremento en la reabsorción de agua en el organismo y la disminución del volumen urinario para recuperar el equilibrio hidroelectrolítico (Bourque, 1998; Hall, 2015; McKinley and Johnson, 2004; Verbalis, 2013).

1.4.4 El óxido nítrico como regulador de la AVP

El óxido nítrico (NO) es una molécula gaseosa, reactiva, liposoluble y muy difusible. Difunde de su sitio de síntesis por gradiente de concentración hacia sus células blanco donde se combina con su receptor, la enzima guanilatociclasa soluble. El NO se libera de la conversión de L-arginina a L-citrulina, esta reacción es catalizada por la sintasa del óxido nítrico (NOS), requiere del cofactor nicotinamida adenina dinucleótido fosfato reducida (NADPH) y es calcio-calmodulina dependiente (Knowles et al., 1989; Knowles and Moncada, 1994). La NOS tiene tres isoformas: la endotelial (eNOS), que es dependiente de la calmodulina; la neuronal (nNOS), se encuentra principalmente en las neuronas, es de acción rápida y calcio-calmodulína-dependiente; y la inducible (iNOS) o macrofágica, se encuentra en los macrófagos y se sintetiza de novo ante determinados agentes agresores (Mayer, 1995; Snyder, 1992; Werner-Felmayer et al., 1991). La nNOS es la isoforma más prominente

en las MNCs del PVN y SON (Bredt et al., 1990; Eliasson et al., 1997; Rodrigo et al., 1994). Se describió ampliamente la coexpresión de la nNOS y AVP en estos núcleos (Nylén et al., 2001a, 2001b; Sánchez et al., 1994).

El NO es una de las moléculas que regula la actividad del sistema neuroendócrino magnocelular y regula la AVP sistémica y somatodendrítica (Gillard et al., 2007; Kadekaro et al., 2006; Kadowaki et al., 1994; Ota et al., 1993; Reid, 1994). Estudios in vitro e in vivo reportaron que, tanto la nNOS como la AVP, son blanco de tóxicos como los PCBs y PBDEs (Coburn et al., 2015; Currás-Collazo, 2011; Kodavanti and Curras-Collazo, 2010; Sharma and Kodavanti, 2002). La liberación de AVP se regula indirectamente por las vías de la noradrenalina y del glutamato (Glu), que modulan la actividad aferente de las MNCs durante estímulos como la hiperosmolaridad, lo que lleva al incremento de NO por el aumento de calcio intracelular que activa la expresión de la calmodulina y la nNOS. A su vez, el NO aumenta la liberación de Glu a través de los receptores de N-metil-D-aspartato (NMDA), y junto con los receptores ionotrópicos de Glu activados por calcio, estimula la liberación de AVP somatodendrítica (Bains and Ferguson, 1997; Gillard et al., 2007; Kadekaro, 2006; Komori et al., 2010). Los congéneres de los PCBs alteran la vía glutamato-NO-cGMP y la homeostasis intracelular del calcio (Kodavanti, 2005; Llansola et al., 2007).

Nuestro grupo reportó que la exposición a PCBs durante la gestación o durante la etapa adulta disminuye la actividad de la NOS (Coburn et al., 2015), lo que pudiera estar relacionado con la alteración de la liberación sistémica y somatodendrítica de AVP. Además, en ratas adultas la exposición durante la gestación y lactancia (GD 6 – PnD 21) a la mezcla pentabromada, DE-71, altera las funciones de AVP relacionadas con la regulación

cardiovascular (Shah et al., 2011) y disminuye la inmunorreactividad y expresión de mRNA de AVP en el hipotálamo durante un estrés osmótico (Mucio-Ramírez et al., 2017).

Los mecanismos por los que los PBDEs alteran al sistema vasopresinérgico aún no se conocen a detalle, ni los efectos específicos de las mezclas comerciales octabromadas sobre el sistema AVPérgico. Por lo anterior, es de nuestro interés conocer los efectos de la exposición perinatal al DE-79 (mezcla octabromada) en el sistema AVPérgico de ratas adultas. Además, determinar si la nNOS está afectada en los núcleos hipotalámicos productores de AVP, ya que, podría ser uno de los mecanimos por los cuales exista una falta de regulación del sistema vasopresinérgico.

2. RAZONAMIENTO CIENTÍFICO

2.1 Planteamiento del problema

Los PBDEs pueden ser liberados al ambiente durante el proceso de producción e incorporación los productos de consumo humano y, durante el uso y disposición de los productos que los contienen. Lo anterior, aunado a que son lipofílicos y no biodegradables, provoca la contaminación del aire, suelo, agua, biota y de los seres vivos.

La vasopresina participa no sólo en la vasoconstricción y la antidiuresis. Tiene múltiples funciones reguladoras a través de sus tres receptores en diferentes tipos de células del sistema nervioso central, periférico y en diferentes órganos. Estas funciones involucran la regulación de diferentes hormonas, la participación en la nocicepción, la cognición y el comportamiento social (memoria, ansiedad o estrés). Por lo tanto, es relevante el estudio de cómo se afecta el sistema vasopresinérgico con los PBDEs, para obtener una mejor comprensión de la neurotoxicidad y las alteraciones endocrinas que producen estos compuestos.

El conocimiento de la toxicidad de los PBDEs durante el desarrollo es limitado. Por lo tanto, es imperativo continuar investigando los efectos que provoca la exposición durante etapas críticas del desarrollo en los ejes neuroendócrinos menos estudiados como es el sistema vasopresinérgico.

Resultados previos nuestros y de otros grupos demostraron que la respuesta fisiológica ante un estrés osmótico, como la deshidratación, produce un aumento de la

inmunorreactividad a la hormona AVP y a la nNOS en el PVN y SON del hipotálamo de la rata. Sin embargo, en ratas expuestas perinatalmente a los PCBs, al ser sometidas a deshidratación en la edad adulta, la inmunorreactividad a la AVP y a la nNOS disminuye significativamente. También disminuye la liberación de AVP somatodendrítica y aumenta su liberación plasmática por arriba de lo normal, lo que altera la homeostasis hidroelectrolítica. No se sabe si los PBDEs y específicamente los compuestos octabromados, como la mezcla comercial DE-79, tienen un efecto sobre el sistema vasopresinérgico, por lo que nos interesa conocer el efecto de la exposición perinatal a estos compuestos sobre el contenido de AVP y nNOS, su síntesis y la liberación sistémica de AVP y si estos efectos persisten en la etapa adulta.

2.2 Hipótesis

La exposición perinatal al DE-79:

- Afectará el contenido y la expresión del mRNA de la AVP en los núcleos PVN y SON hipotalámicos, así como su liberación sistémica, en condiciones basales y en presencia de un estímulo hiperosmótico, lo que repercutirá en el mantenimiento de la osmolaridad plasmática en ratas adultas.
- Afectará el contenido y la expresión del mRNA de la nNOS en los núcleos PVN y SON hipotalámicos en condiciones basales, así como, en presencia de un estímulo hiperosmótico en ratas adultas.

2.3 Objetivos

2.3.1 Objetivo general

Estudiar el efecto de la exposición perinatal al DE-79 sobre el sistema vasopresinérgico en ratas adultas.

2.3.2 Objetivos específicos

- Estudiar si la exposición perinatal a estos contaminantes tiene un efecto sobre la inmunorreactividad a AVP y nNOS en PVN y SON en ratas de 3 meses de edad, normosmóticas y sometidas a un estímulo hiperosmótico.
- Estudiar si la exposición perinatal a estos contaminantes tiene un efecto sobre la expresión del mRNA de AVP y nNOS en PVN y SON en ratas de 3 meses de edad, normosmóticas y sometidas a un estímulo hiperosmótico.
- Analizar en estos grupos si existen cambios en los niveles de AVP y de la osmolaridad sérica.

3. MATERIAL Y MÉTODOS

3.1 Animales de experimentación

Se utilizaron ratas de la cepa Wistar procedentes del bioterio del Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz (INPRFM), Ciudad de México. Todos los animales estuvieron en cajas individuales de plástico y se mantuvieron bajo condiciones controladas de laboratorio en un ciclo de luz:obscuridad (12:12 horas), temperatura controlada y con libre acceso a agua y alimento (rat chow estándar).

Todos los experimentos se hicieron de acuerdo con las guías de los Institutos Nacionales de Salud para el cuidado y uso de animales de laboratorio y con la aprobación del Comité de Ética y el Comité de Investigación del INPRFM (proyecto NC.143290.0).

3.2 Sustancia química

Se utilizó la mezcla comercial de octaBDEs DE-79 (lote 8525DG01A; Great Lakes Chemical Corporation, West Lafayette, IN), donada por el Dr. P.R.S. Kodavanti (USEPA). Los congéneres principales que contiene la mezcla DE-79 son: BDE-175/183 (42 %), -197 (22.2 %), -207 (11.5 %), y -196 (10.5 %; La Guardia et al., 2006). Hanari et al (2006) reportan la presencia de bifenilos bromados, dioxinas y furanos en esta mezcla.

3.3 Exposición perinatal al DE-79

Las ratas Wistar hembras (con un peso de 220-250 g) se aparearon con machos de la misma cepa (con un peso de 250-300 g). Se determinó que las hembras estaban embarazadas por presencia de tapón vaginal y aumento de peso posterior. Las ratas

gestantes se expusieron al DE-79 vía oral, se les dio de comer frituras de maíz (palomitas) con las dosis: 0 (control), 1.7 (baja) ó 10.2 (alta) mg/kg/día de DE-79 disuelto en aceite de maíz (vehículo) de los 6 días de gestación (GD) a los 21 días postnatales (PnD; **Fig. 5**). El volumen de cada dosis con aceite de maíz se ajustó con base en los cambios del peso de las hembras gestantes. Las dosis baja y alta se seleccionaron para igualar en una base molar a las dosis de la mezcla de difenilos policlorados (PCBs), tóxico químicamente similar, Aroclor 1254 (1 y 6 mg/kg/día), del que se tiene información extensa de estudios in vitro e in vivo (Fan et al., 2010; Kodavanti, 2005; Kodavanti et al., 2010; Kodavanti and Ward, 2005). A los 4 PnD, las camadas fueron ajustadas a 8 crías por camada en todos los grupos, con un número igual de hembras y machos cuando fue posible. Las crías tratadas y control se destetaron a los 22 PnD. Se agruparon por dosis en condiciones estándar de bioterio, hasta el mes de edad, cuando fueron sexadas. A los 3 meses de edad, máximo 2 crías macho de cada camada fueron asignados al azar a los diferentes grupos experimentales (**Tabla 3**).

3.4 Estímulo hiperosmótico: carga salina (salt-loading)

Como estímulo hiperosmótico se utilizó la técnica denominada carga salina (salt-loading), que es la ingesta de solución hipertónica para inducir la hiperosmolaridad en el organismo. El objetivo de esta técnica fue determinar si la exposición perinatal al DE-79 altera el contenido y la síntesis de AVP y nNOS en el PVN y SON y la liberación sistémica de AVP con la activación fisiológica. Dimos de beber solución salina al 2% (20g de NaCl/l de agua corriente) ad libitum durante los 4 días previos al sacrificio a un grupo de animales, de 3 meses de edad, de todas las dosis, formando el grupo hiperosmótico (Dai and Yao, 1995;

Kadowaki et al., 1994; Mucio-Ramírez et al., 2017). A los animales restantes, que incluyeron todas las dosis de DE-79, se les dio de beber agua corriente ad libitum, formando el grupo normosmótico. Se evaluaron los pesos de los animales antes y después del reto osmótico, los animales que perdieron el 10 % o más de peso corporal se consideraron deshidratados (grupo hiperosmótico) y se incluyeron en los grupos experimentales. Se confirmó el estado hiperosmolar con la medición de la osmolaridad sérica.

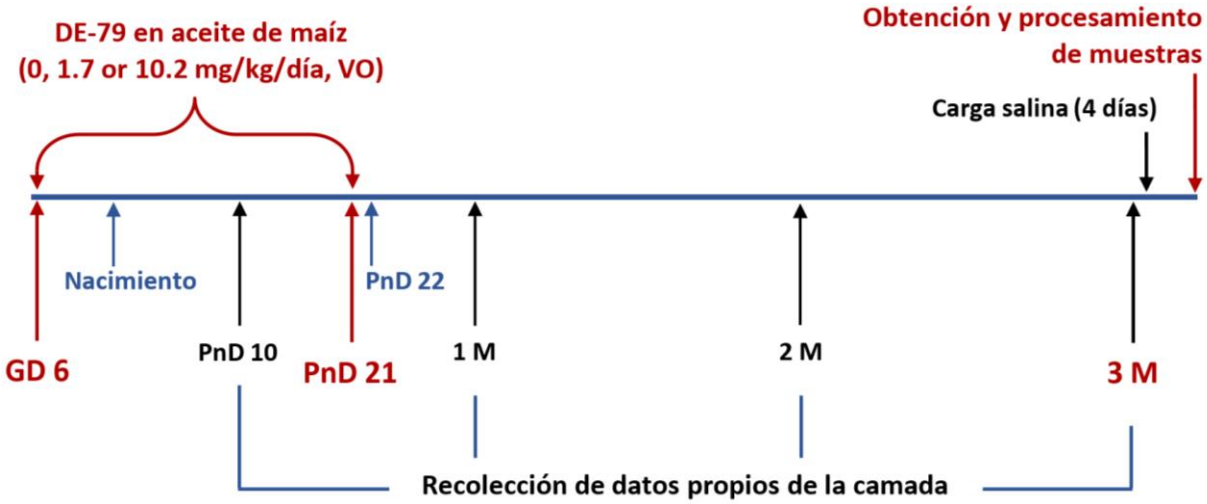

Figura 5. Modelo de dosificación y de ensayo. Paradigma temporal de la exposición perinatal del DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día. La exposición fue del día gestacional (GD) 6 al día posnatal (PnD) 21. Las crías machos se destetaron y separaron a los 22 PnD. A los 3 meses (M) de edad, un grupo de ratas de todas las dosis se expusieron a un estímulo hiperosmótico (carga salina, grupo hiperosmótico; ad libitum NaCl al 2% por 4 días previos al sacrificio), el otro grupo (normosmótico) tuvo acceso a agua corriente ad libitum. Animales de todas las dosis y condiciones osmóticas se asignaron al azar a diferentes grupos de análisis (Inmunorreactividad, expresión de mRNA), se obtuvieron y procesaron las muestras. Se midió la AVP y osmolaridad sérica de todos los grupos. De las camadas se analizó la tasa de mortalidad durante los periodos de 0 a 10 PnD, de 10 PnD a 1 M, de 1 a 2 M y de 2 a 3 M de edad, el peso de todas las crías a los 10 PnD y el peso de los machos a los 1, 2 y 3 M de edad.

3.5 Grupos Experimentales

Las ratas Wistar macho expuestas perinatalmente a dosis control, baja o alta de DE-79 (0, 1.7 y 10.2 mg/kg/día, respectivamente), tanto normosmóticas como hiperosmóticas, fueron asignadas al azar a los grupos de análisis de: inmunofluorescencia de AVP y nNOS, en el PVN y SON hipotalámicos (n = 3-6 por subgrupo); y reacción en cadena de la polimerasa por transcripción reversa en punto final (PCR) de los genes de AVP y nNOS, para determinar la expresión de mRNA en el PVN y SON (n = 3-4 por subgrupo). Se obtuvo sangre de todos los animales para los grupos de análisis de: osmolaridad sérica para estudiar la osmorregulación (n = 6-8 por subgrupo); y ensayo por inmunoabsorción ligado a enzimas (ELISA) de AVP en suero para examinar la liberación a la circulación sistémica (n = 6-7 por subrupo; **Tabla 3**).

Tabla 3. Grupos experimentales y tamaño de muestra

Condición osmótica	**DE-79** (mg/kg/día) GD 6 - PnD 21	**Grupos de análisis**									
		AVP-IR (n)		**nNOS-IR** (n)		**AVP-mRNA** (n)		**nNOS-mRNA** (n)		**AVP sérica** (n)	**Osmolaridad sérica** (n)
		PVN	SON	PVN	SON	PVN	SON	PVN	SON		
Normosmóticos (agua corriente)	0 (control)	5	5	4	4	3	4	3	4	7	8
	1.7 (baja)	4	4	5	4	4	4	4	3	6	7
	10.2 (alta)	6	5	5	5	4	3	4	3	7	8
Hiperosmóticos (carga salina)	0 (control)	4	4	3	3	4	4	4	3	6	8
	1.7 (baja)	4	5	5	5	4	4	4	4	6	6
	10.2 (alta)	5	5	6	5	4	3	4	3	6	8

Animales de 3 meses de edad que fueron expuestos al DE-79, del día gestacional (GD) 6 al día postnatal (PnD) 21, en dosis 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día. Distribución por condición osmótica (grupos normosmótico –agua corriente ad libitum– e hiperosmótico –NaCl al 2% ad libitum por 4 días–) y por tamaño de muestra (n) de los grupos de análisis (inmunorreactividad a vasopresina y sintasa neuronal de óxido nítrico –AVP-IR, nNOS-IR–; expresión de mRNA de AVP y nNOS; AVP sérica; y osmolaridad sérica.

3.6 Obtención y análisis de muestras

3.6.1 Datos de las camadas

Las camadas incluyen a todos los animales nacidos de las madres tratadas y controles, no todas las crías se usaron en los experimentos de esta tesis. Los datos que se analizaron fueron: el peso de las crías a los 10 PnD, y peso de las crías machos a los 1, 2 y 3 M de edad, la tasa de mortalidad (machos y hembras) durante los períodos específicos de 0 a 10 PnD, 10 PnD a 1 M, 1 M a 2 M y 2 M a 3 M de edad.

3.6.2 Osmolaridad sérica

La osmolaridad es la concentración de solutos en un peso de agua dado (Rasouli, 2016), por lo tanto, la osmolaridad sérica mide la concentración de todos los solutos (e.g. electrolitos, proteínas) en el suero.

Se obtuvo sangre troncal (de los animales procesados para expresión de mRNA) o sangre cardiaca (de los animales procesados para inmunorreactividad). Se separó el suero por centrifugación a 1,500 x g por 18 min a 4 °C. El suero se retiró y se almacenó a -80 °C hasta su uso. La osmolaridad sérica se midió por triplicado con un osmómetro de vapor (Wescor Vapro 5600, South Logan, UT). Los resultados se reportaron como el promedio ± error estándar (SEM) en mOsm/kg y en porcentaje del control normosmótico (100 %).

3.6.3 Inmunorreactividad a AVP y nNOS en PVN y SON hipotalámicos

La técnica de inmunofluorescencia se utilizó para analizar la distribución e indirectamente (datos semicuantitativos) el contenido de AVP y nNOS en los núcleos PVN y

SON de ratas perinatalmente expuestas al DE-79 en diferentes dosis bajo condiciones normosmóticas o hiperosmóticas.

Todos los animales se sacrificaron previa anestesia con pentobarbital sódico (63 mg/kg peso). Las ratas se fijaron mediante perfusión intracardiaca (IC). La perfusión se realizó con 150-200 ml de una solución de cloruro de sodio (NaCl) al 0.9 % con heparina al 0.1 % (Pisa® Farmacéutica, México), seguida de 250-350 ml del fijador de paraformaldehido al 4% (Sigma Chemical Co., St. Louis, MO) en una solución amortiguadora de fosfatos de sodio, 0.1 M, pH 7.4, con 0.9 % de NaCl (PBS). Posteriormente, se extrajeron los cerebros, se postfijaron en el mismo fijador por 2-4 horas y se crioprotegieron en sacarosa al 30% a 4°C hasta su uso. Se obtuvieron cortes coronales de 30 µm en micrótomo de congelación por deslizamiento (Leitz, Grand Rapids, MI), de los núcleos PVN y SON que corresponden a Bregma de -0.80 a -2.12 mm (Paxinos and Watson, 2007). Los cortes obtenidos se almacenaron en cajas multipozos (24 pozos), donde se separaron en series de 6 cortes por pozo, se almacenaron en PBS y azida de sodio al 0.01 % hasta su uso. Para esta técnica se utilizaron 3 pozos alternos (18 cortes) por animal.

Se usó inmunofluorescencia de doble marcaje para detectar AVP (n = 4-6 por subgrupo) y nNOS (n = 3–5 por subgrupo), la técnica se describe a continuación:

- Los cortes que utilizamos se lavaron con PBS 0.01M 3 veces por 10 min a temperatura ambiente y en agitación continua.
- Se permeabilizaron y se bloquearon los sitios activos con una solución de bloqueo: PBS-tritón X-100 al 0.3 % (Merck, cat. 12298), albúmina sérica bovina al 3 % (USB,

cat. 108670), gelatina de pescado al 1 % (Sigma, cat. G-7765) y suero normal de burro al 3 % (Sigma, cat. D-9663) por 1 hora a temperatura ambiente y bajo agitación continua.

- Se incubaron con los anticuerpos primarios anti-AVP (anticuerpo monoclonal PS-41 hecho en ratón, donado por el Dr. H. Gainer, NIH; dilución 1:500; Ben-Barak et al., 1985; Whitnall and Gainer, 1985) y anti-nNOS (anticuerpo policlonal C-terminal hecho en conejo, Immunostar; a dilución 1:500; Eliasson et al., 1997; Sanchez-Islas et al., 2014) en solución de bloqueo durante 72 horas a 4 °C, y posteriormente, 1 hora a temperatura ambiente, todo el tiempo en agitación continua.
- Terminada la incubación de los anticuerpos primarios, se realizaron 3 lavados con PBS-tritón al 0.3% (PBS-T) por 10 min cada lavado a temperatura ambiente y en agitación continua.
- Se realizó la incubación de los anticuerpos fluorescentes secundarios Alexa 488 anti-ratón y Alexa 555 anti-conejo (Invitrogen Corp., Carlsbad, CA; a dilución 1:250 para cada uno) en solución de bloqueo por 2 horas a 37 °C en cámara húmeda, posteriormente durante 20 min a temperatura ambiente, todo el tiempo en agitación continua.
- Una vez terminada la incubación, los cortes se lavaron 3 veces en PBS-T por 10 min cada vez, seguidos de 2 lavados en PBS 0.01 M por 5 minutos cada lavado.
- Por último, las secciones se montaron en portaobjetos gelatinados con medio de montaje Antifade Kit (Molecular Probes) para su observación y análisis en microscopio confocal.

Todos los cortes se analizaron con un microscopio Confocal Zeiss 510 META después de obtener el espectro de emisión de cada fluoróforo. Se capturaron imágenes digitalizadas de las áreas de interés con un lase Ar/488nm, 1 unidad airy de diámetro de pinhol y 1 de ganancia, a 10 x para que el núcleo completo estuviera presente en una fotografía. Las imágenes fueron convertidas a escala de grises (0-255) y el fondo fue sustraído. Posteriormente, se midió la densidad óptica integrada (IOD) de cada imagen por medio del programa Image Pro-Plus (Diagnostics Instruments 4.5, Media Cybernetics, MD, USA). Se analizaron 6-8 cortes por rata que incluyeron imágenes representativas de los núcleos completos (se obtuvieron al menos 3 imágenes de la parte anterior, media y posterior de de cada núcleo). La IOD se reportó como el promedio ± SEM en unidades arbitrarias. Los valores de IOD para la inmunorreactividad a AVP y a nNOS (AVP-IR y nNOS-IR, respectivamente) no deben tomarse como un índice lineal de las concentraciones intracelulares de los péptidos, aun así, los valores observados de IOD de ambos péptidos reflejan cambios en el contenido de AVP y nNOS en PVN y SON.

3.6.4 Expresión del mRNA de AVP y nNOS en PVN y SON hipotalámicos

La medición del mRNA se utilizó para evaluar la expresión génica de la AVP y la nNOS en núcleos hipotalámicos de ratas perinatalmente expuestas al DE-79 en diferentes dosis bajo condiciones normosmóticas o hiperosmóticas.

Ratas de todas las condiciones experimentales se sacrificaron por decapitación rápida con guillotina previa anestesia con pentobarbital sódico (63 mg/kg peso). Se obtuvieron los cerebros y permanecieron a – 80 °C hasta su uso. La disección de las regiones hipotalámicas

se realizó de forma manual con los cerebros congelados sobre una caja de petri con hielo seco pulverizado, se obtuvieron cortes coronales, de alrededor de 1 mm de espesor, de las áreas del PVN y SON que corresponden a Bregma de -0.80 a -2.12 mm (Paxinos and Watson, 2007). De las rebanadas se obtuvieron las áreas del PVN y SON hipotalámicos, siguiendo la técnica de sacabocado (Palkovits and Brownstein, 1988) y se mantuvieron en congelación hasta su uso.

Para la determinación de la expresión genética, se llevó a cabo la extracción del RNA total del PVN y SON por el método de tiacinato de guanidina (Chomczynski and Sacchi, 1987). La calidad de la extracción fue verificada por medio de electroforesis con un gel de agarosa al 1.5 % determinando la integridad de las subunidades 28S y 18S del rRNA, la relación de la densidad de las bandas debió ser mayor a 1.5, de lo contrario las muestras se descartaron. La expresión de mRNA de AVP (n = 3–4/subgroupo) y nNOS (n = 3–4/subgroupo) y el gen de referencia, subunidad 18S del rRNA, en el PVN y SON fue semicuantificada por PCR (Jaimes-Hoy et al., 2008). El número de ciclos para cada prueba fue optimizado usando 25 pmol de AVP, nNOS o 18S y 0.5 µl de Taq DNA polimerasa (Biotecnologías Universitarias, UNAM). En la **tabla 4** se muestran las secuencias de los oligonucleótidos analizados y el gen control. Las condiciones finales de ciclos, temperaturas y tiempos para ambos núcleos fueron: 25 ciclos para AVP, 29 ciclos para nNOS y 18 ciclos para 18S; cada uno de 1 min a 94 °C, 1 min a 64 °C y 1 min a 72 °C, todos los cDNA tuvieron una extensión de tiempo final de 10 min a 72 °C.

Tabla 4. Secuencias de oligonucleótidos utilizados

Gen	Secuencia sentido	Secuencia antisentido	PM
AVP	5′ CACCTCTGCCTGCTACTTCC 3′	5′ GGGCAGGTAGTTCTCCTCCT 3′	200
nNOS	5′ TGACTCTTGGGCTACGATGC 3′	5′ GGTGGAAGGGGGCTTAAGTG 3′	202
18S	5′ ATGGCCGTTCTTAGTTGGTG 3′	5′ CGCTGAGCCAGTTCAGTGTA 3′)	219

Los oligonuclótidos fueron elaborados en la Unidad de Síntesis de Oligonucleótidos del Instituto de Biotecnología de la UNAM.

Los productos se separaron utilizando electroforesis en gel de agarosa al 2 % y se tiñeron con bromuro de etidio (0.25 %) para medir la densidad óptica (OD) con el software Advanced American Biotech Imaging. Los resultados se obtuvieron dividiendo la OD del gen de interés entre la del 18S. La OD se reportó como el promedio ± SEM expresada en porcentaje del control normosmótico (100 %).

3.6.5 Vasopresina sérica

La medición de la AVP sérica se utilizó para evaluar la liberación sistémica de AVP de ratas perinatalmente expuestas al DE-79 en diferentes dosis bajo condiciones normosmóticas o hiperosmóticas.

La sangre troncal o cardiaca obtenida de los animales se centrifugó a 1,500 x g por 18 min a 4 °C para obtener el suero. Las muestras de suero se almacenaron a -80 °C hasta su uso. Inicialmente, las muestras se delipidaron al añadirles el volumen equivalente de una mezcla de butanol y éter etílico (40:60), la mezcla se agitó con el vórtex. Posteriormente, las muestras se centrifugaron a 8,000 x g por 5 min. La capa orgánica superior se descartó y la capa acuosa restante se usó para la extracción de AVP. A la capa acuosa se le adicionó

2 veces su volumen de acetona fría, la mezcla se agitó con el vórtex y se centrifugó a 12,000 x g por 20 min. Al sobrenadante de las muestras se añadió 5 veces su volumen éter de petróleo, la mezcla se agitó con el vórtex y las muestras se centrifugaron a 10,000 x g por 10 min. La capa superior de éter se descartó y la capa de proteína acuosa se secó bajo nitrógeno gaseoso. El producto final se reconstituyo con buffer de ensayo. Las concentraciones de AVP se midieron por duplicado con el kit comercial de ensayo por inmunoabsorción ligado a enzimas (ELISA) Arg8-Vasopressin (Enzo Life Sciences, Farmingdale, NY; Coburn et al., 2005). La sensibilidad del ensayo es de 2.84 pg/ml. Los resultados de la cuantificación de AVP sérica se reportaron como el promedio ± SEM en pg/ml y en porcentaje del control normosmótico (100%).

3.6.6 Análisis Estadístico

Los resultados de mortalidad se analizaron con una taza cruda de mortalidad (número total de defunciones entre la muestra total; factor de expansión 100); y los de peso por edad se analizaron por medio un análisis de varianza (ANOVA) de 1 vía (tratamiento con DE-79). La inmunofluorescencia, PCR y osmolaridad se analizaron con un ANOVA de 2 vías (tratamiento con DE-79 – activación osmótica) cuando los datos cumplieron los supuestos de distribución normal/equidad de varianza. Cuando los datos no cumplieron con los supuestos (AVP sérica y nNOS-IR en ambos núcleos), los datos fueron transformados mediante $\log_{10}$. Las diferencias significativas entre los grupos se analizaron con una prueba post hoc de Holm-Sidak. En todos los casos se consideró la diferencia como estadísticamente significativa con un nivel alfa de $p \leq 0.05$. El análisis estadístico se realizó con el software SigmaPlot 12.3 (Systat Software, Inc).

4. RESULTADOS

4.1 Datos de las camadas

En el estudio de las camadas, primero, se analizó el número de nacimientos por camada (**tabla 5**), donde no encontramos diferencias significativas entre los grupos analizados.

Tabla 5. Promedio de nacimientos por camada

Dosis DE-79 (mg/kg/día)	No. Ratas madre	No. Nacimientos	Promedio
0 (control)	39	372	9.54
1.7 (baja)	32	292	9.13
10.2 (alta)	39	399	10.23

Esta muestra incluye todas las crías de las madres control y tratadas, no todas las crías se utilizaron para los experimentos de esta tesis.

La exposición perinatal al DE-79 resultó en una tendencia a aumentar la tasa de mortalidad de los grupos de animales de dosis baja y alta (13.56 % y 14.34 %, respectivamente) comparado con controles desde el 0-10 PnD (11.09 %) y este incremento en la tasa persistió hasta 1 M de edad en el grupo de dosis alta (11.3 % para dosis alta vs 9.10 % del control; **Fig. 6**). En cuanto al peso corporal en los animales estudiados (**Fig. 7**), el análisis por ANOVA de 1 vía mostró que, a los 10 PnD, hay diferencias significativas entre los grupos de diferentes dosis ($F_{2,759} = 13.586$, $p < 0.001$). Las comparaciones múltiples expusieron un aumento de peso en los animales de dosis baja con respecto a los controles y a los de dosis alta ($p < 0.001$ en ambos casos).

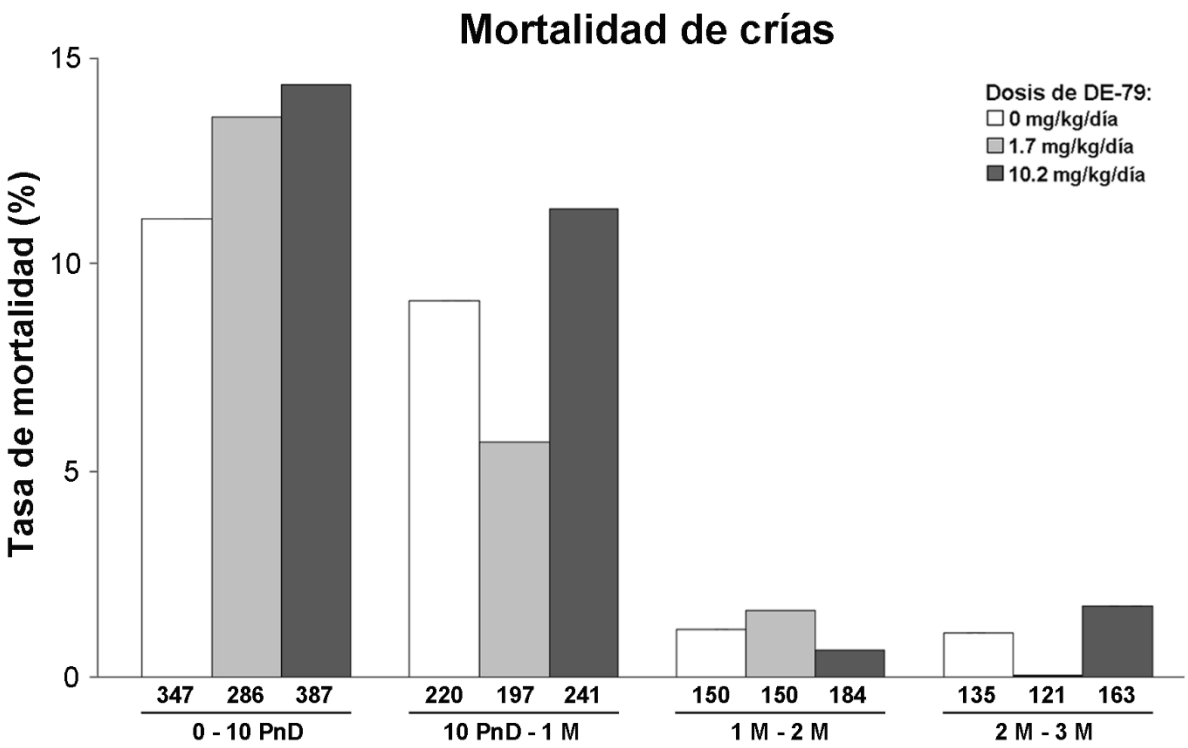

Figura 6. Mortalidad de las crías. Análisis de mortalidad de las ratas (hembras y machos) expuestas perinatalmente al DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día. El conteo de muertes se refiere a los períodos del nacimiento a los 10 días posnatales (PnD), 10 PnD a 1 mes (M), 1 M a 2 M y 2 M a 3 M de edad. Las ratas que recibieron la dosis alta tuvieron la mortalidad más alta hasta el primer mes de edad. Las barras representan el porcentaje (%) de los valores totales de las muertes de las ratas expuestas perinatalmente. Los números debajo de las barras representan el tamaño de la muestra (n).

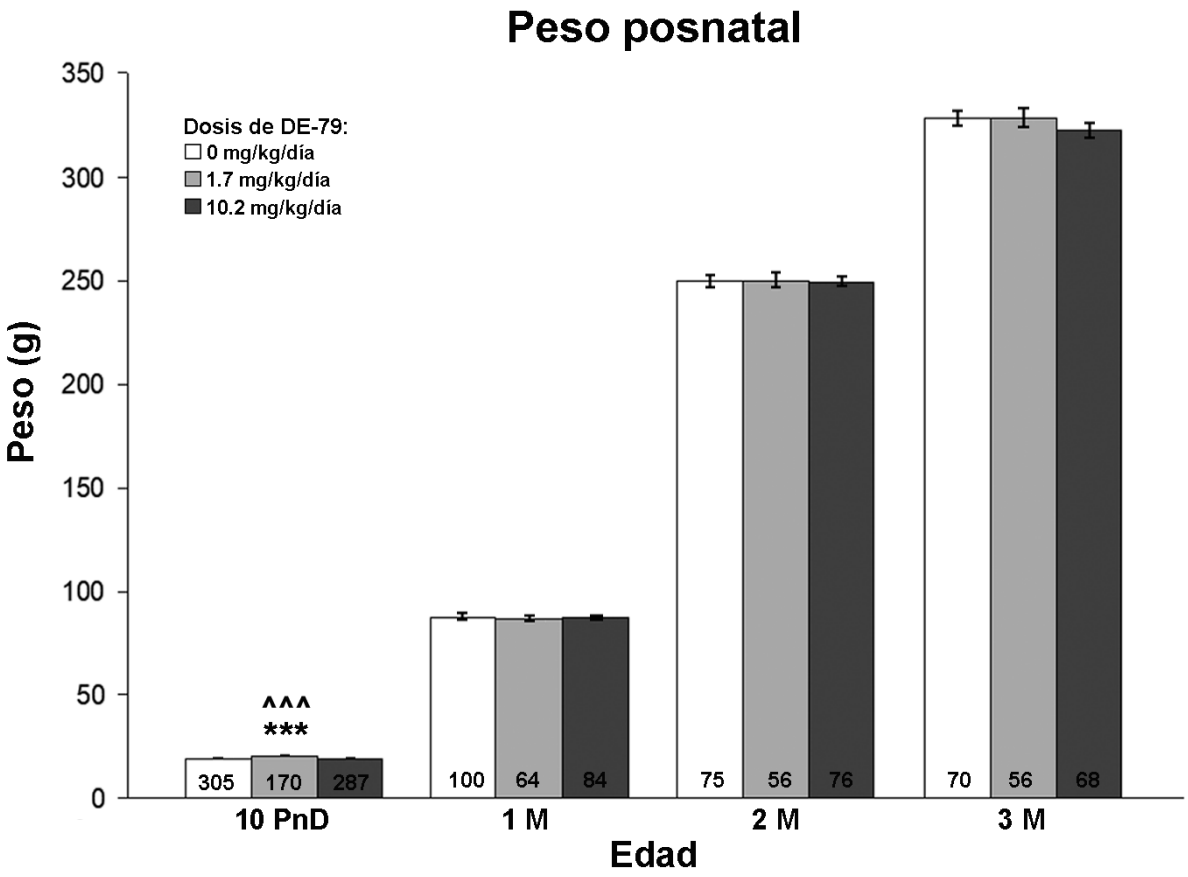

Figura 7. Peso posnatal. Análisis de los pesos de las ratas expuestas perinatalmente al DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día a los 10 días posnatales (PnD), 1 mes (M), 2 M y 3 M de edad. Se observa aumento en el peso de las crías del grupo de dosis baja en comparación al grupo control únicamente a los 10 PnD. Las barras representan los valores promedio ± s.e.m. de los pesos (g) y los números dentro de ellas indican el tamaño de muestra (n). Esta muestra incluye hembras y machos para 10 PnD y solo machos para 1, 2 y 3 M. Los símbolos representan la significancia estadística determinada por ANOVA de 1 vía y test post-hoc de Holm-Sidak.
(*) comparado con el grupo control de la misma edad; (^) comparado con la dosis alta de la misma edad; ***, ^^^ = $p \leq 0.001$.

4.2 La exposición perinatal al DE-79 altera la osmolaridad sérica en el adulto

La osmolaridad se midió en el suero de ratas adultas, expuestas perinatalmente al DE-79, bajo condiciones normosmóticas o hiperosmóticas. El análisis por ANOVA de 2 vías (**Tabla 6**) no mostró interacción entre los factores de condición osmótica y dosis ($F_{2,39}$ = 0.329, p = 0.722), pero se encontró una diferencia estadísticamente significativa entre los grupos del factor condición osmótica ($F_{1,39}$ = 43.053, p < 0.001). Las comparaciones múltiples de la prueba pos-hoc mostraron un incremento significativo en la osmolaridad en respuesta al estímulo hiperosmótico en todas las dosis (p < 0.001 para los grupos control y dosis alta y p = 0.003 para dosis baja). Entre los grupos hiperosmóticos se encontró un incremento dosis dependiente no significativo y entre los grupos normosmóticos no hay diferencias.

Tabla 6. Osmolaridad en suero

DE-79 (mg/kg/día)	**Normosmóticos** (agua corriente)		**Hiperosmóticos** (carga salina)	
	mOsm/kg	Porcentage (%)	mOsm/kg	Porcentage (%)
0 (n=8, n=8)	311.5 ± 2.3	100 ± 0.7	342 ± 6.7***	109.8 ± 2.1***
1.7 (n=7, n=6)	317.3 ± 3.5	101.9 ± 1.1	346.4 ± 8.9**	111.2 ± 2.9**
10.2 (n=8, n=8)	316.5 ± 2.9	101.6 ± 0.9	354.5 ± 8.9***	113.8 ± 2.9***

Osmolaridad medida en suero en condiciones normosmótica (agua corriente ad libitum) e hiperosmótica (NaCl al 2% ad libitum, por 4 días) en ratas de 3 meses de edad expuestas perinatalmente al DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día. El número debajo de las dosis expresa el tamaño de la muestra (n) correspondiente a normosmótico e hiperosmótico, respectivamente. Los valores están expresados en promedio ± s.e.m en mOsm/kg y en porcentaje del control normosmótico (100%). Los símbolos representan la significancia estadística determinada por ANOVA de 2 vías y test post-hoc de Holm-Sidak. (*) comparado con el grupo normosmótico de la misma dosis; ** = p ≤ 0.01; *** = p ≤ 0.001.

4.3 La exposición perinatal al DE-79 altera la AVP en el adulto

4.3.1 Inmunorreactividad a AVP en PVN y SON

La inmunofluorescencia para AVP se analizó por medio de la densidad óptica integrada (IOD) de la inmunorreactividad de AVP (AVP-IR) en imágenes de cortes coronales de los núcleos PVN (**Fig. 8A-F**) y SON (**Fig. 8G-L**) de ratas expuestas perinatalmente al DE-79 a dosis de 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día, bajo condiciones normosmóticas (**Fig. 8A, B, C, G, H, I**) o hiperosmóticas (**Fig. 8D, E, F, J, K, L**).

Las imágenes mostraron una distribución uniforme de la inmunorreactividad (IR) en soma y en fibras en cada núcleo del grupo control bajo condiciones normosmóticas (**Fig. 8A, G**) y un incremento fisiológico en la AVP-IR bajo el estímulo hiperosmótico (**Fig. 8D, J**). También, observamos un aparente incremento en el volumen celular de las neuronas AVPérgicas en ambos núcleos de los grupos controles hiperosmóticos, como se describió previamente (Johnson et al., 2015; Zhang et al., 2001). Los animales normosmóticos de dosis baja presentaron un incremento en la AVP-IR similar al de los controles hiperosmóticos (**Fig. 8B, H, vs D, J**), mientras que en los de dosis alta se observó un decremento en la AVP-IR (**Fig. 8C, I vs A, G**). Finalmente, los animales hiperosmóticos de la dosis baja y los de la dosis alta no presentaron el aumento fisiológico esperado, comparados con sus grupos normosmóticos correspondientes (**Fig. 8E, F, K, L**).

El análisis estadístico con ANOVA de 2 vías mostró la interacción significativa entre los factores condición osmótica y dosis en PVN y SON ($F_{2,22}$ = 6.298, p = 0.007 y $F_{2,22}$ = 6.623, p = 0.006, respectivamente); y una diferencia estadísticamente significativa en la AVP-IR entre las dosis en ambos núcleos ($F_{2,22}$ = 14.912, p < 0.001 para PVN y $F_{2,22}$ = 16.182, p < 0.001 para SON). Las comparaciones múltiples (**Fig. 8a, b**) expusieron que, en los grupos normosmóticos, la dosis baja tuvo una AVP-IR significativamente mayor comparada con el control en PVN y SON (p = 0.033 y p = 0.028, respectivamente) y comparada con la dosis alta (p < 0.003 para PVN y SON). El reto hiperosmótico llevó a un incremento fisiológico significativo de la AVP-IR en los grupos control en PVN y SON (p < 0.001 y p = 0.002, respectivamente). Sin embargo, ni los grupos de dosis baja, ni de alta respondieron a la activación hiperosmótica. En cambio, el grupo de dosis alta mostró un decremento estadísticamente significativo de la AVP-IR en ambos núcleos (p < 0.001) comparado con la del control hiperosmótico. En conclusión, la AVP-IR entre grupos normosmóticos fue mayor en la dosis baja y la respuesta fisiológica al reto hiperosmótico, no se presentó en los animales tratados con dosis baja o alta.

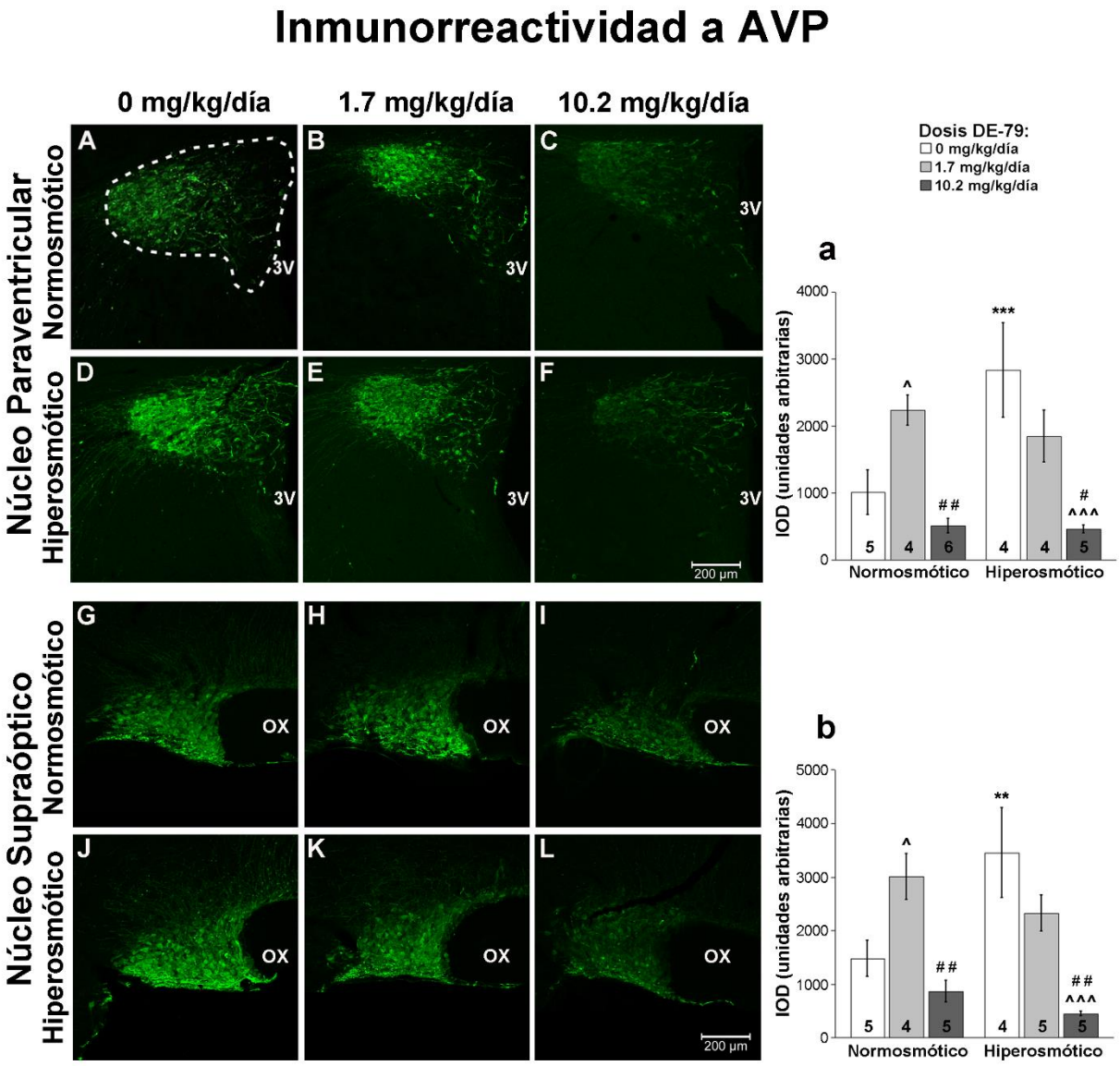

Figura 8. Efectos de la exposición perinatal al DE-79 en la inmunorreactividad a AVP (AVP-IR) en ratas macho adultas. Izquierda: Panel de imágenes representativas de cortes coronales en confocal de PVN (arriba; imágenes **A** a **F**) y SON (abajo; imágenes **G** a **L**) con AVP-IR. Análisis en ratas perinatalmente expuestas al DE-79 en dosis 0 (control; **A, D, G, J**); 1.7 (baja; **B, E, H, K**) o 10.2 mg/kg/día (alta; **C, F, I, L**); y en ratas normosmóticas (**A, B, C, G, H, I**) o hiperosmóticas (**D, E, F, J, K, L**). La línea punteada señala la región de interés usada para medir la IOD (densidad óptica integrada). Derecho (a y b): Representación gráfica del análisis de IOD por núcleo. Las barras representan los valores promedio ± s.e.m. y los números dentro de ellas indican el tamaño de muestra (número de ratas, de las que, en promedio, fueron analizados por rata 6 cortes bilaterales). Los símbolos representan la significancia estadística determinada por ANOVA de 2 vías y test post-hoc de Holm-Sidak. (*) hiperosmótico comparado con normosmótico de la misma dosis; (^) dosis baja/alta comparada con el control del mismo grupo (normo−/hiperosmótico); (#) dosis alta comparada a la dosis baja del mismo grupo (normo−/hiperosmótico); ^, # = p ≤ 0.05; **, # # = p ≤ 0.01; ***,^^^ = p ≤ 0.001. Abreviaturas: tercer ventrículo (3V); quiasma óptico (OX).

4.3.2 Expresión del mRNA de AVP en PVN y SON

La expresión del mRNA de AVP de PVN y SON se normalizó con 18S, el gen de referencia, y se analizó como porcentaje de los controles normosmóticos (**Fig. 9**). Las mediciones se hicieron en condiciones normosmóticas o bajo un reto hiperosmótico en PVN y SON de ratas adultas expuestas perinatalmente al DE-79 a dosis de 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día.

El análisis con ANOVA de 2 vías mostró una interacción significativa entre los factores condición osmótica y dosis en ambos núcleos ($F_{2,17} = 49.452$, $p < 0.001$ para PVN y $F_{2,16} = 11.55$, $p < 0.001$ para SON). Además, observamos una diferencia estadísticamente significativa en el promedio de la expresión de mRNA de AVP entre los grupos del factor condición osmótica en PVN ($F_{1,17} = 29.798$, $p < 0.001$) y entre dosis en SON ($F_{2,16} = 5.834$, $p = 0.013$). En las comparaciones múltiples encontramos un incremento significativo de la expresión de mRNA con el grupo de dosis alta comparado con el control y el de dosis baja en los grupos normosmóticos de PVN ($p < 0.001$ para ambos) y en SON ($p = 0.001$ dosis alta vs control; $p = 0.023$ dosis alta vs baja). La activación hiperosmótica incrementó la expresión de mRNA de AVP en el control comparado con su normosmótico solo en PVN ($p < 0.001$), y en la dosis baja comparado con su homólogo normosmótico en ambos núcleos ($p < 0.001$ para PVN; $p = 0.040$ para SON). Por otra parte, los grupos de dosis alta no tuvieron respuesta ante el reto hiperosmótico, resultando en un decremento significativo de la expresión de mRNA en PVN y en SON ($p < 0.001$) comparado con su respectivo grupo normosmótico. Entre los grupos hiperosmóticos, la dosis alta presentó un decremento significativo

comparado con el control en PVN ($p < 0.001$) y comparado con la dosis baja en ambos núcleos ($p < 0.001$ para PVN y $p = 0.006$ para SON).

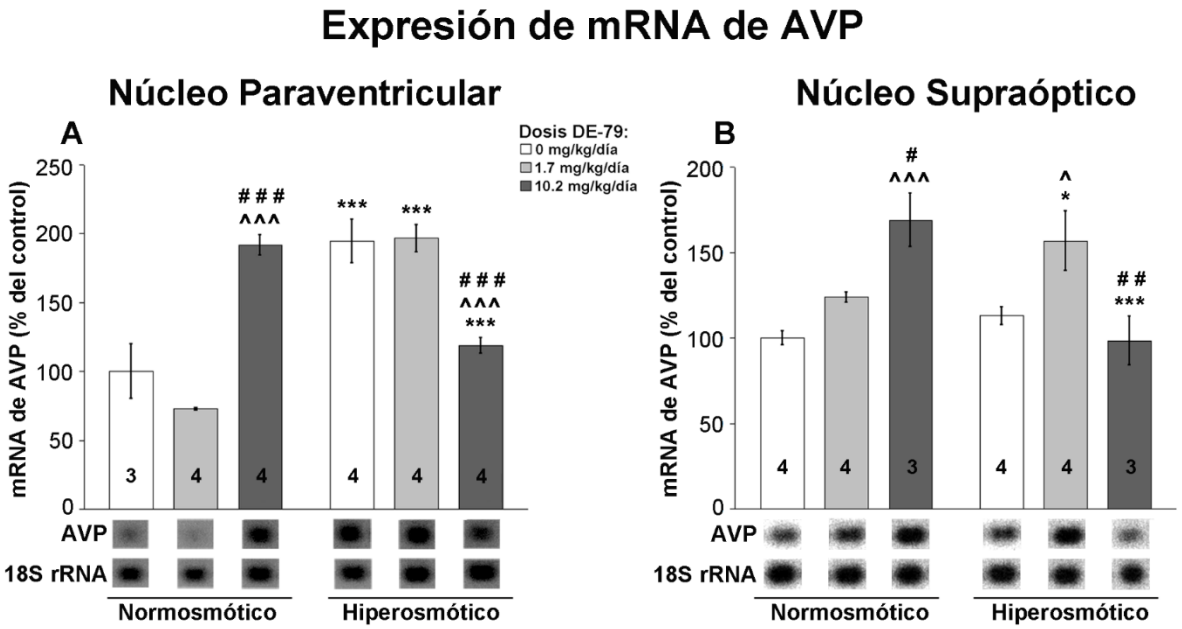

Figura 9. Efectos de la exposición perinatal al DE-79 en la expresión de mRNA de AVP en ratas macho adultas. El análisis de PVN (**A**) y SON (**B**) se llevó a cabo en ratas perinatalmente expuestas al DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 mg/kg/día (alta) normosmóticas o hiperosmóticas. Las barras representan los valores promedio ± s.e.m. expresados como porcentaje del control normosmótico (100%) y los números dentro de ellas indican el tamaño de muestra (n). Debajo de cada barra se encuentra un ejemplo de las bandas de electroforesis de AVP y 18S. Se hizo un análisis estadístico de densitrometría (densidad óptica) para el mRNA de AVP normalizado con el rRNA de 18S. Los símbolos representan la significancia estadística determinada por ANOVA de 2 vías y test post-hoc de Holm-Sidak. (*) hiperosmótico comparado con normosmótico de la misma dosis; (^) dosis baja/alta comparada con el control del mismo grupo (normo−/hiperosmótico); (#) dosis alta comparada a la dosis baja del mismo grupo (normo−/hiperosmótico); *, ^, # = $p \leq 0.05$; # # = $p \leq 0.01$; ***, ^^^, # # # = $p \leq 0.001$.

4.3.3 Niveles séricos de AVP

En la sangre recolectada de los animales de todos los grupos se midieron los niveles de AVP en suero bajo condiciones normosmóticas e hiperosmóticas. El análisis con ANOVA de 2 vías, en los datos transformados logarítmicamente (**Fig. 10**), mostró una interacción significativa entre los factores condición osmótica y dosis ($F_{2,32} = 4.082$, $p = 0.026$). Además, observamos una diferencia estadísticamente significativa entre los grupos del factor condición osmótica ($F_{1,32} = 11.371$, $p = 0.002$). La prueba de Holm-Sidak de comparaciones múltiples mostró un incremento fisiológico significativo en el grupo control hiperosmótico comparado con su grupo normosmótico respectivo ($p < 0.001$), que correspondió a un incremento de 2.5 veces de los niveles de AVP aproximadamente. Entre los grupos normosmóticos encontramos un aumento estadísticamente significativo de los grupos de dosis baja y alta comparados con el control ($p = 0.029$ para dosis baja vs control y $p = 0.008$ para dosis alta vs control), esto correspondió a un incremento de AVP en suero de alrededor de 2 veces. Por otra parte, los grupos hiperosmóticos de dosis baja y alta no presentaron cambios estadísticamente significativos comparados con sus respectivos normosmóticos, por lo tanto, no hubo una respuesta en los niveles de AVP en suero después de la activación hiperosmótica.

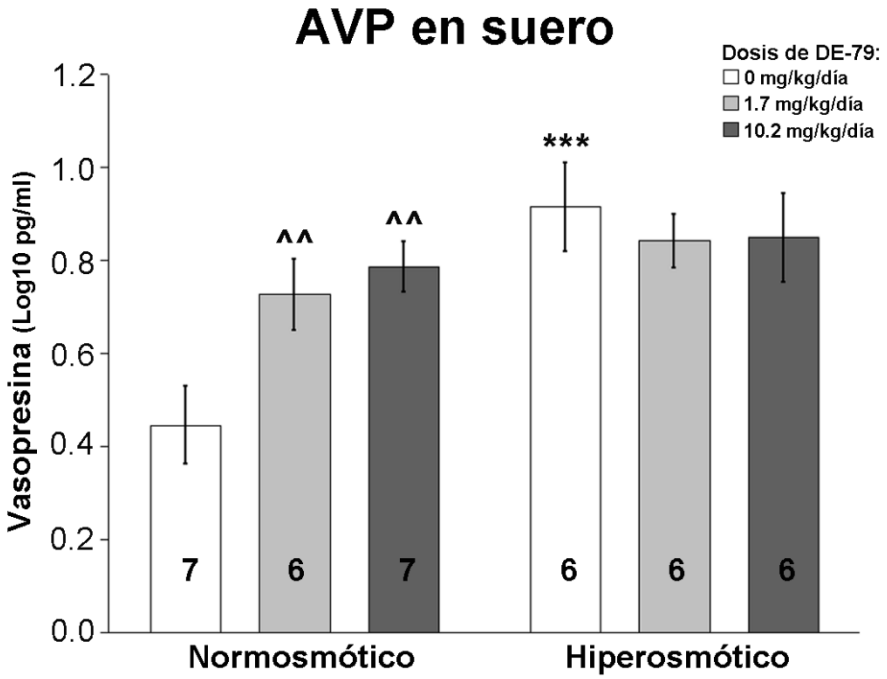

Figura 10. Efectos de la exposición perinatal a DE-79 en la AVP sérica en ratas macho adultas. La AVP en suero se analizó en ratas de 3 meses de edad, perinatalmente expuestas al DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 mg/kg/día (alta), bajo condiciones normosmóticas o hiperosmóticas. Las barras representan los promedios ± s.e.m. de los datos transformados logarítmicamente en pg/ml y los números dentro de ellas indican el tamaño de muestra (n). Los símbolos representan la significancia estadística determinada por ANOVA de 2 vías y test post-hoc de Holm-Sidak. (*) hiperosmótico comparado con normosmótico de la misma dosis; (^) dosis baja/alta comparada con el control del mismo grupo (normo−/hiperosmótico); ^^ = $p \leq 0.01$; *** = $p \leq 0.001$.

4.4 La exposición perinatal al DE-79 afecta a la nNOS en el PVN y SON en el adulto

4.4.1 Inmunorreactividad a nNOS en PVN y SON

Analizamos la inmunorreactividad a nNOS (nNOS-IR) de las imágenes de inmunofluorescencia de PVN (**Fig. 11A-F**) y SON (**Fig. 11G-L**) de ratas expuestas perinatalmente al DE-79 a dosis de 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día, bajo condiciones normosmóticas (**Fig. 11A, B, C, G, H, I**) o hiperosmóticas (**Fig. 11D, E, F, J, K, L**).

Las imágenes mostraron la distribución de la nNOS-IR en soma y en fibras en cada núcleo del grupo control bajo condiciones normosmóticas (**Fig. 11A, G**) y un incremento fisiológico en la IR bajo el estrés hiperosmótico (**Fig. 11D, J**). En el grupo normosmótico de dosis baja se observó un aparente aumento de la nNOS-IR en SON (**Fig. 11H vs G**), mientras que el de dosis alta mostró una aparente disminución en PVN (**Fig. 11C vs A**), ambos comparados contra el control. Entre los grupos hiperosmóticos, los de dosis baja y alta no presentaron el incremento fisiológico de nNOS-IR (**Fig. 11E, F, K, L**) comparados contra su contraparte normosmótica (**Fig. 11B, C, H, I**).

El análisis estadístico de los datos, transformados logarítmicamente, de la IOD con una ANOVA de 2 vías mostró la interacción significativa entre los factores condición osmótica y dosis en PVN y SON ($F_{2,22}$ = 5.844, p = 0.009 y $F_{2,20}$ = 9.655, p = 0.001, respectivamente). Además, observamos una diferencia estadísticamente significativa entre los grupos del factor dosis en ambos núcleos ($F_{2,22}$ = 8.471, p = 0.002 para PVN y $F_{2,20}$ = 9.326, p=0.001 para SON); y entre los grupos del factor condición osmótica solo en SON ($F_{1,20}$ = 23.324, p < 0.001). Las comparaciones múltiples (**Fig. 11a, b)** revelaron diferencias entre los grupos normosmóticos de dosis baja y control de ambos núcleos, estadísticamente significativo solo en SON (p = 0.008). La estimulación hiperosmótica generó un aumento fisiológico de la nNOS-IR en los grupos controles en PVN y SON (p = 0.006 y p < 0.001, respectivamente), mientras que no se encontró esta respuesta en los grupos de dosis baja y alta. Además, entre los grupos hiperosmóticos, se observó un decremento significativo de la nNOS-IR en los de dosis baja (p = 0.024 en PVN y p = 0.038 en SON) y dosis alta (p < 0.001 en PVN y SON), ambos comparados con el grupo hiperosmótico control.

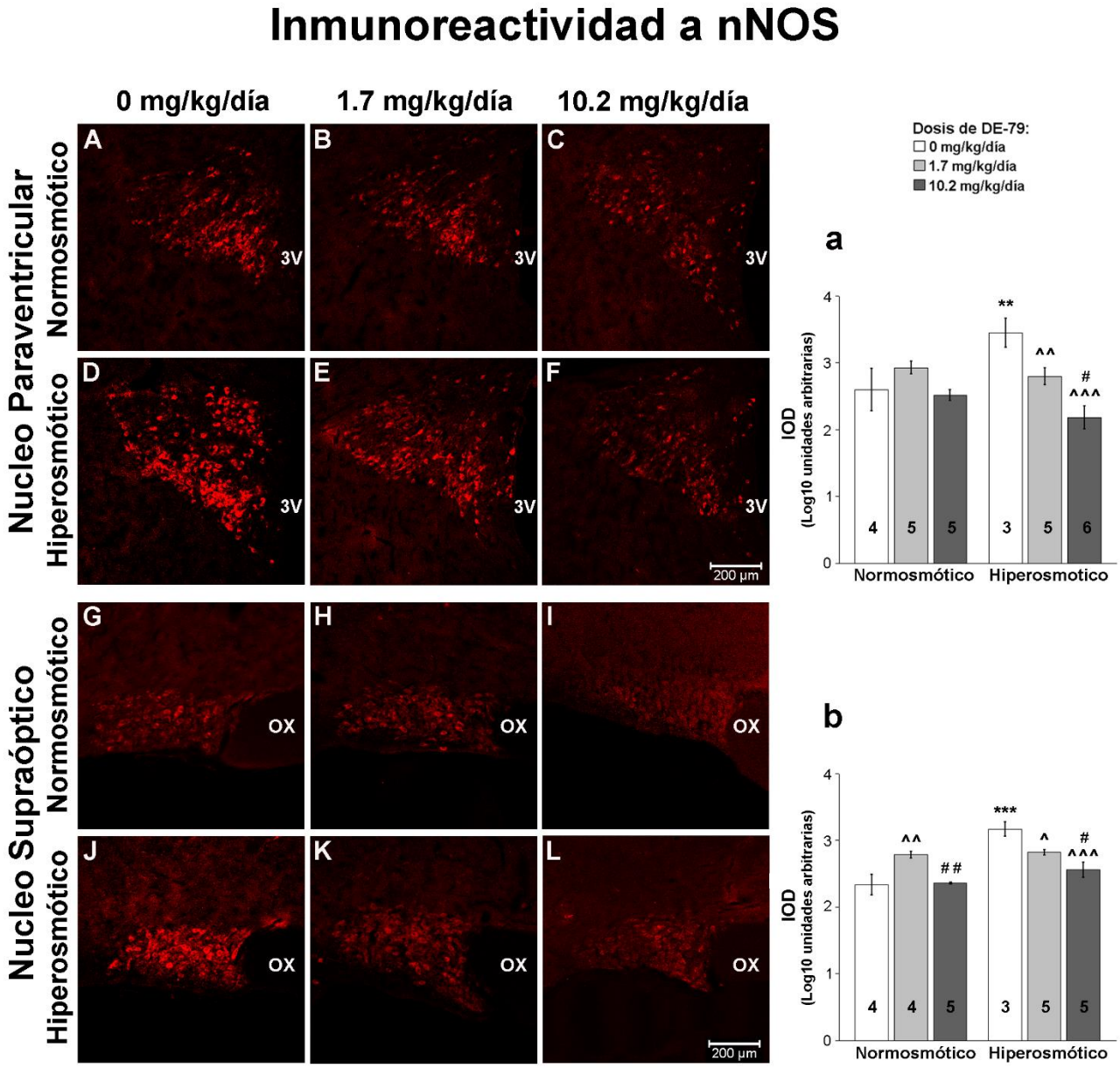

Figura 11. Efectos de la exposición perinatal al DE-79 en la inmunorreactividad a nNOS (nNOS-IR) en ratas macho adultas. Izquierda: Panel de imágenes confocales representativas de cortes coronales de PVN (arriba; imágenes **A** a **F**) y SON (abajo; imágenes **G** a **L**) con nNOS-IR. Análisis en ratas perinatalmente expuestas al DE-79 en dosis 0 (control; **A, D, G, J**); 1.7 (baja; **B, E, H, K**) o 10.2 mg/kg/día (alta; **C, F, I, L**); y en ratas normosmóticas (**A, B, C, G, H, I**) o hiperosmóticas (**D, E, F, J, K, L**). Derecho (**a** y **b**): Representación gráfica del análisis de IOD (densidad óptica integrada) por núcleo. Las barras representan los valores promedio ± s.e.m de los datos transformados logarítmicamente y los números dentro de ellas indican el tamaño de muestra (número de ratas, de las que, en promedio, fueron analizados bilateralmente 6 cortes por rata). Los símbolos representan la significancia estadística determinada por ANOVA de 2 vías y test post-hoc de Holm-Sidak. (*) hiperosmótico comparado con normosmótico de la misma dosis; (^) dosis baja/alta comparada con el control del mismo grupo (normo−/hiperosmótico); (#) dosis alta comparada a la dosis baja del mismo grupo (normo−/hiperosmótico); ^, # = $p \leq 0.05$; ^^, ##,** = $p \leq 0.01$; ***,^^^ = $p \leq 0.001$. Abreviaturas: tercer ventrículo (3V); quiasma óptico (OX).

4.4.2 Expresión del mRNA de nNOS en PVN y SON

La expresión del mRNA de nNOS en PVN y SON se obtuvo de los grupos de ratas macho adultas en condiciones normosmóticas o bajo un reto hiperosmótico en PVN y SON de ratas adultas expuestas perinatalmente al DE-79 a dosis de 0 (control), 1.7 (baja) o 10.2 (alta) mg/kg/día. Los datos se normalizaron con el gen de referencia 18S, y se expresaron como porcentaje de los controles normosmóticos (**Fig. 12**).

El análisis con ANOVA de 2 vías indicó una interacción significativa entre los factores condición osmótica y dosis en ambos núcleos ($F_{2,17} = 34.99$, $p < 0.001$ para PVN y $F_{2,14} = 4.392$, $p = 0.033$ para SON). Además, observamos una diferencia estadísticamente significativa entre los grupos del factor condición osmótica en ambos núcleos ($F_{1,17} = 92.157$, $p < 0.001$ para PVN y $F_{1,14} = 12.557$, $p = 0.003$ para SON); así como entre los grupos del factor dosis ($F_{2,17} = 14.014$, $p < 0.001$ para PVN y $F_{2,14} = 5.03$, $p = 0.023$ para SON).

En las comparaciones múltiples observamos un incremento significativo de la expresión de mRNA de nNOS en el grupo normosmótico de dosis baja en comparación con el control en PVN ($p = 0.004$). La activación hiperosmótica incrementó la expresión de mRNA en el grupo control comparado con su normosmótico en ambos núcleos ($p < 0.001$); mientras que el grupo de dosis baja mostró incremento con el estímulo hiperosmótico, estadísticamente significativo solo en PVN ($p = 0.015$) y el grupo de dosis alta no presentó ningún aumento. Entre los grupos hiperosmóticos, los grupos de dosis baja y alta presentaron un decremento de la expresión al mRNA de nNOS comparada con la del grupo control, significativa en PVN ($p < 0.001$ vs dosis baja y alta) y en SON ($p = 0.01$ vs dosis baja; $p = 0.048$ vs dosis alta).

Además, también en el grupo hiperosmótico del PVN, la expresión de mRNA del grupo de dosis alta fue significativamente menor que la del de dosis baja (p = 0.004). Nuevamente, los grupos de dosis baja y alta no mostraron el aumento fisiológico esperado en la expresión de mRNA de nNOS como el control.

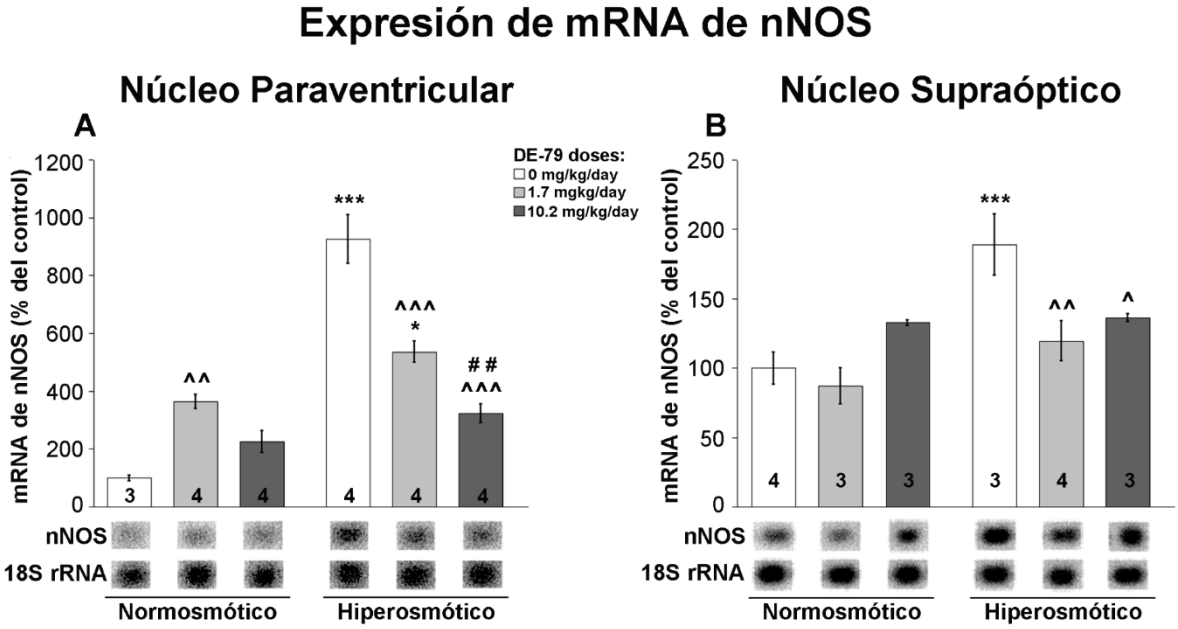

Figura 12. Efectos de la exposición perinatal al DE-79 en la expresión de mRNA de nNOS en ratas macho adultas. El análisis de PVN (**A**) y SON (**B**) se llevó a cabo en ratas perinatalmente expuestas al DE-79 en dosis 0 (control), 1.7 (baja) o 10.2 mg/kg/día (alta) normosmóticas o hiperosmóticas. Las barras representan los valores promedio ± s.e.m. expresados como porcentaje del control normosmótico (100%) y los números dentro de ellas indican el tamaño de muestra (n). Debajo de cada barra se encuentra un ejemplo de las bandas de electroforesis de nNOS y 18S. Se hizo un análisis estadístico de densitrometría (densidad óptica) para el mRNA de nNOS normalizado con el rRNA de 18S. Los símbolos representan la significancia estadística determinada por ANOVA de 2 vías y test post-hoc de Holm-Sidak. (*) hiperosmótico comparado con normosmótico de la misma dosis; (^) dosis baja/alta comparada con el control del mismo grupo (normo−/hiperosmótico); (#) dosis alta comparada a la dosis baja del mismo grupo (normo−/hiperosmótico); *, ^ = p ≤ 0.05; ^^, # # = p ≤ 0.01; ***, ^^^ = p ≤ 0.001.

5. DISCUSIÓN

En este estudio demostramos que la exposición perinatal a la mezcla comercial de octaBDEs, DE-79, altera el contenido y los niveles de mRNA de AVP y nNOS en el PVN y SON hipotalámicos en la edad adulta. Estas alteraciones en la AVP y la nNOS tienen un efecto sobre el balance hidroelectrolítico, lo cual afecta el mantenimiento de la homeostasis.

Los efectos adversos producidos por los retardantes de flama bromados están relacionados con el congénere al que se expone, la latencia de la exposición y la edad a la exposición (Diamanti-Kandarakis et al., 2009). Se ha establecido que los PBDEs promueven mayor disrupción endócrina cuando el organismo se expone durante etapas tempranas del desarrollo que cuando la exposición es durante la edad adulta (Chen et al., 2014a; Coburn et al., 2015; Eriksson et al., 2002; Kodavanti, 2005; León-Olea et al., 2014).

En este estudio, la exposición al DE-79 fue durante la etapa perinatal, del día gestacional (GD) 6 al día postnatal (PnD) 21, y los experimentos se realizaron a los 3 meses de edad (adulto joven). En los datos las camadas observamos una mayor tasa de mortalidad de los animales tratados con DE-79 en comparación con los controles, hasta el primer mes de edad; **Fig. 6**), por lo tanto, estamos analizando a las crías menos afectadas que lograron sobrevivir. No sabemos la razón del incremento de la mortalidad, pero se puede deber a múltiples causas, desde efectos en las madres, hasta disrupción endócrina de las crías como la alteración del sistema tiroideo que es esencial para el desarrollo, así como de otras hormonas, incluyendo la AVP que también está involucrada en el desarrollo en etapas

tempranas (Boer, 1985; Ji et al., 2019; Kodavanti and Curras-Collazo, 2010; Moog et al., 2017; Zelena et al., 2009).

Nuestros resultados muestran que las ratas adultas expuestas perinatalmente al DE-79 presentan una desregulación del sistema vasopresinérgico en condiciones normosmóticas y no son capaces de tener una respuesta fisiológica adecuada a un estímulo hiperosmótico.

5.1 La exposición perinatal al DE-79 afecta la osmorregulación en el adulto

La exposición perinatal al DE-79, en dosis baja y alta, resultó en valores de osmolaridad sérica más altos en condiciones normosmóticas, comparados con el control. Como se esperaba, el reto hiperosmótico (carga salina) resultó en un incremento fisiológico en la osmolaridad sérica de aproximadamente 30 mOsm/kg (9.8%) en los grupos controles (**Tabla 6**). Sin embargo, los grupos hiperosmóticos de dosis bajas y altas mostraron una osmolalidad sérica aún mayor en comparación con el grupo hiperosmótico control. Aunque estas diferencias no fueron estadísticamente significativas son biológicamente significativas, pues, bajo condiciones fisiológicas, pequeños incrementos de 3 mOsm/kg (alrededor del 1%) son suficientes para producir incrementos medibles en la concentración de AVP sérica, y de aumentar la reabsorción de agua en el riñón, así como la sensación de sed (Baylis, 1987; Verbalis, 2013). En este estudio, los efectos de la exposición perinatal al DE-79 en la osmorregulación fueron similares a lo reportado con la exposición de PCBs y pentaBDEs (Coburn et al., 2015; Mucio-Ramírez et al., 2017; Shah et al., 2011).

Las alteraciones en la osmorregulación sugieren desregulación del sistema AVPérgico, por lo que analizamos la expresión de mRNA y la AVP-IR de los núcleos hipotalámicos productores de AVP, así como la liberación sistémica de AVP.

5.2 La exposición perinatal al DE-79 afecta la regulación del sistema AVPérgico en el adulto

La respuesta a una carga salina (estímulo hiperosmótico) encontrada en nuestros grupos control, es el aumento fisiológico de la osmolaridad sérica, un marcado incremento de la AVP-IR (**Fig. 8**) y de la expresión del AVP-mRNA (**Fig. 9**) en el PVN y SON hipotalámicos y un incremento de los niveles de AVP circulante (**Fig. 10**), como ya se ha reportado (Dai and Yao, 1995; Johnson et al., 2015; Landgraf et al., 1988; Mucio-Ramírez et al., 2017).

Entre los grupos normosmóticos, el grupo de dosis baja mostró un incremento significativo en la AVP-IR en ambos núcleos comparados con el grupo control (**Fig. 8B, H, a, b**). Este incremento fue en el rango del que presentó el grupo control hiperosmótico, por lo que parecería que la dosis baja de PBDE-79 actuó como un factor estimulante similar a la estimulación hiperosmótica. Sin embargo, al someterlo al estímulo hiperosmótico no hubo cambios adicionales en la AVP-IR (**Fig. 8B, E, H, K, a, b**). Por otro lado, los grupos de dosis alta normosmóticos mostraron los niveles de AVP-IR, más bajos y la respuesta al desafío hiperosmótico redujo aún más la AVP-IR (**Fig. 8C, F, I, L, a, b**). Estas respuestas son similares a las obtenidas en ratas adultas expuestas perinatalmente a una mezcla comercial de PCBs o mezcla de pentaBDEs (30 mg/kg/día de Aroclor 1254 y DE-71, respectivamente; Mucio-Ramírez et al., 2017) que no presentaron una respuesta a la estimulación hiperosmótica. Para determinar si las anormalidades en la AVP-IR de animales tratados con PBDEs pueden

deberse a una desregulación de la síntesis de AVP, estudiamos la expresión de mRNA de AVP.

Los grupos de dosis baja incrementaron la expresión de mRNA de AVP, similar a los grupos control, en respuesta al desafío hiperosmótico en comparación con su contraparte normosmótica (**Fig. 9A**). La dosis alta mostró una expresión elevada de mRNA de AVP en condiciones normosmóticas, en comparación con el control, pero no presentó una respuesta hiperosmótica (**Fig. 9A, B**). Lo anterior sugiere que la exposición perinatal al DE-79 actúa como un estresor en condiciones normosmóticas, pero el sistema no puede responder a un segundo estímulo, como el desafío hiperosmótico. Los efectos de dosis altas son diferentes a los encontrados en ratas adultas con exposición perinatal a Aroclor 1254 (30 mg/kg/día), donde las ratas tratadas en condiciones normosmóticas no mostraron un aumento en la expresión de mRNA. Sin embargo, ninguno de los dos tratamientos (Aroclor 1254 o dosis alta de DE-79) presentaron el aumento fisiológico esperado en respuesta al estímulo hiperosmótico, lo que sugiere un blanco común y, posiblemente, un mecanismo similar para PCBs y PBDEs.

Sobre la liberación sistémica de AVP (**Fig. 10**), los animales normosmóticos expuestos a dosis baja y alta mostraron un aumento estadísticamente significativo en la AVP sérica en comparación con el control, lo que sugiere que los PBDEs actúan como un estímulo para la liberación de AVP. Sin embargo, después del estímulo hiperosmótico, los animales expuestos a dosis baja y alta presentaron las mismas concentraciones que sus contrapartes normosmóticas, lo que sugiere que alcanzaron el máximo de liberación. Los altos valores de osmolaridad, encontrados en los grupos hiperosmóticos de dosis baja y alta, pueden

deberse a la liberación restringida de AVP sistémica en estos animales. Los resultados de la liberación sistémica contrastan con lo descrito por Coburn et al. (2005), donde el desafío hiperosmótico aumentó la liberación sistémica de AVP en ratas expuestas a PCBs en comparación a las ratas control. Estas discrepancias pueden deberse a diferencias en el diseño experimental como: 1) su estímulo hiperosmótico agudo (NaCl 3.5 M intraperitoneal) vs nuestro estímulo crónico en animales expuestos, donde el sistema AVPérgico podría responder después de un desafío agudo pero no después de un estímulo prolongado como la carga salina; 2) la exposición a PCBs en adultos (30 mg/kg/día de Aroclor 1254 durante 15 días durante la edad adulta) contra la exposición perinatal al DE-79, donde los organismos en desarrollo son más susceptibles a las sustancias tóxicas (Coburn et al., 2015; Kodavanti, 2005).

En suma, estos resultados mostraron que, tanto la dosis baja como alta del DE-79, tienen efectos sobre el sistema AVPérgico según la dosis, pero la magnitud de los efectos no es dosis-dependiente. En condiciones normosmóticas, la dosis baja de DE-79 se comportó como un estímulo hiperosmótico: mostró una AVP-IR alta, al nivel de los animales con carga salina, y niveles séricos altos de AVP y una expresión normal de mRNA de AVP, la razón de esto último no es clara; pero en nuestro estudio la expresión normal de mRNA, aparentemente, es suficiente para aumentar la AVP-IR en los núcleos PVN y SON y la liberación sistémica. Mientras que la dosis alta, también en condiciones normosmóticas, se acompaña de un incremento en la expresión de mRNA de AVP y de liberación sistémica a expensas de un marcado decremento en la AVP-IR en el PVN y SON hipotalámicos (disminución en las reservas). Por lo tanto, ambas dosis produjeron alteraciones en

condiciones normosmóticas, actuando como estresores. Sin embargo, después de un estímulo hiperosmótico, los grupos de dosis baja mantienen sus niveles altos, aunque no presentaron una respuesta adicional con el estímulo, en comparación con sus respectivos grupos normosmóticos. Los grupos de dosis alta no presentan la respuesta al reto hiperosmótico y tienen los niveles más bajos de los grupos hiperosmóticos en la expresión de mRNA y la AVP-IR, posiblemente debido al agotamiento del sistema y para evitar la caída de los niveles séricos de AVP. A pesar de que los niveles séricos se mantienen en el grupo de dosis alta, éste presentó los niveles más altos de osmolaridad sérica, que implica alteraciones importantes en la homeostasis. Estos resultados podrían explicarse por una alteración en otros reguladores del sistema AVPérgico, uno de ellos es el sistema del NO. En consecuencia, estudiamos la nNOS-IR y la expresión del mRNA.

5.3 La exposición perinatal al DE-79 compromete la activación fisiológica de la nNOS durante el estímulo hiperosmótico en el adulto

En este estudio, observamos, como otros han reportado, que el desafío hiperosmótico produjo un aumento en la inmunoreactividad de nNOS (nNOS-IR) y la expresión de mRNA de nNOS en el PVN y SON hipotalámicos de los grupos control (Kadowaki et al., 1994; Ueta et al., 1995; Villar et al., 1994). Además, reportamos la alteración de la respuesta hiperosmótica tanto en la nNOS-IR como en la expresión de mRNA de nNOS, por la exposición perinatal al DE-79 en dosis baja y alta (**Fig. 11 y 12**, respectivamente).

Los resultados de la nNOS-IR en los núcleos hipotalámicos (**Fig. 11**) son consistentes con los resultados de la AVP-IR, donde en condiciones normosmóticas los grupos de dosis baja

mostraron niveles aumentados (significativos solo en SON); además, los grupos tratados no fueron capaces de presentar una respuesta al desafío hiperosmótico. Estos resultados también concuerdan con hallazgos anteriores con PCBs (Coburn et al., 2015), en donde se muestra que la actividad de la NOS está afectada en el SON de las ratas adultas jóvenes y mayores (3-5 y 14-16 meses de edad, respectivamente) expuestas prenatalmente a Aroclor 1254 (30 mg/kg/día de los 10 a los 19 días de gestación) y sometidas a un desafio hiperosmótico. Coburn et al (2015) observaron una disminución en la intensidad de tinción de la nicotinamida adenina dinucleótido diaforasa (NADPH-d) del grupo hiperosmótico expuesto a PCBs en comparación con el control hiperosmótico. Además, la disminución en los grupos expuestos hiperosmóticos fue similar a la de las ratas hiperosmóticas que se trataron con NG-nitro-L-arginina-metil éster (L-NAME), un inhibidor del NO (Coburn et al., 2015; Rees et al., 1990). Investigaciones previas demostraron que la NOS es blanco de los PCBs y PBDEs, y en algunas situaciones, como en estrés hiperosmótico, pueden actuar como inhibidores de la NOS (Coburn et al., 2015; Currás-Collazo, 2011; León-Olea et al., 2005; Sharma and Kodavanti, 2002;).

El análisis de la expresión de mRNA de nNOS en PVN y SON hipotalámicos (**Fig. 12**), mostró que los grupos normosmóticos expuestos a PBDEs aumentaron la expresión de mRNA en comparación con los controles, significativo sólo para el grupo de dosis baja en PVN. También mostró que todos los grupos expuestos al DE-79 tuvieron respuestas disminuidas al desafío hiperosmótico, más evidentemente en SON. Estos resultados confirman que la exposición perinatal al DE-79 atenúa la respuesta de nNOS después de una situación estresante como la hiperosmolaridad.

5.4 La alteración de la nNOS en el adulto como posible mecanismo de desregulación de AVP después de la exposición perinatal al DE-79

No se sabe con claridad el papel del NO en el sistema AVPérgico, los resultados más consistentes con respecto a su función sugieren que el NO aumenta la liberación somatodendrítica de AVP, esto restringe la liberación sistémica de AVP a través de sus autorreceptores, lo que convierte al NO en un modulador relevante de la función neuroendocrina magnocelular durante condiciones de alta demanda hormonal (Gillard et al., 2007; Kadekaro et al., 2006; Reid, 1994; Stern and Zhang, 2005). Otros estudios mostraron que la L-NAME (inhibidor de NO; inyección ICV) atenuó la liberación basal de AVP y suprimió las respuestas de AVP a la hipotensión inducida por nitroprusiato de sodio (IV; Cao et al., 1996). De manera similar, la S-nitroso-N-acetilpenicilamina (precursor de NO) causó un aumento en la concentración plasmática de AVP (Ota et al., 1993). Es muy probable que esta controversia esté relacionada con las diferencias en la estimulación fisiológica, los diseños experimentales, la ruta de administración, la dosis y el tiempo de respuesta a los diversos agonistas y antagonistas de NO utilizados.

El efecto de la exposición perinatal al DE-79 en la nNOS-IR y expresión de mRNA fue similar a lo observado en la AVP, donde la rata macho adulta expuesta perinatalmente no puede responder apropiadamente a la estimulación hiperosmótica. La exposición al DE-79 produce efectos que combinan las acciones de múltiples congéneres de PBDEs, que actúan como los PCBs sobre la PKC inhibiendo su translocación lo que produce fosforilación de la NOS y afecta la producción de NO (Currás-Collazo, 2011; Kodavanti and Curras-Collazo,

2010; Kodavanti and Ward, 2005). El NO interviene en la liberación somatodendrítica de AVP, que es una potente señal de retroalimentación por la cual la AVP liberada actua sobre las células neurosecretoras magnocelulares, autorregulando su propia actividad. Lo cual límita o inhibe la liberación sistémica de AVP (Gillard et al., 2007; Gouzènes et al., 1998; Hanoune 2009a; Ludwig and Leng, 2006). Por lo tanto, los PBDEs y PCBs actúan afectando la liberación somatodendrítica de AVP inducida por el NO, más evidentemente después de un estímulo hiperosmótico (Coburn et al., 2005, 2007).

El aumento en la liberación sistémica de AVP en respuesta a los cambios tempranos en la osmolaridad ocurre desde el comienzo del estímulo osmótico, sin embargo, los animales expuestos, principalmente los del grupo de dosis alta, no fueron capaces de presentar la respuesta a este desafío osmótico crónico como lo demuestra su incapacidad para mantener las reservas en los núcleos hipotalámicos (AVP-IR), la expresión de mRNA y la liberación de AVP, lo que conduce a altos niveles de osmolaridad al final de la prueba. Estas alteraciones podrían explicarse por una falla en la regulación de la liberación de AVP por la alteración del NO.

Futuras investigaciones deberían centrarse en dilucidar otros mecanismos y sitios en los que el sistema AVPérgico podría verse afectado por el DE-79, como la neurohipófisis y el riñón. Se reportó al estrés oxidativo como un efecto de los PBDEs y el incremento de especies reactivas de oxígeno está ligado a nefrotoxicidad, hipertensión e inactivación de NO (Albina et al., 2010; Milovanovic et al., 2018; Vaziri et al., 1999). Además, el NO tiene un rol importante en la regulación del flujo sanguíneo renal y de la excreción de sodio, pues la presencia de sustancias inhibidoras de NO como L-NAME disminuyen el flujo sanguíneo

medular y el filtrado glomerular, aumentan la retención de sodio y posteriormente se afecta la regulación de la presión arterial (Hall, 2015; Mattson et al., 1994).

Por otra parte, la AVP está modulada por diferentes neuropéptidos y neurotransmisores que son blancos de los PBDEs, por lo que podrían ser otros mecanismos de disfunción del sistema AVPérgico. Ejemplos de estos son: la angiotensina II que forma parte del sistema renina-angiotensina-aldosterona, es coactivada junto con la AVP por estímulos como la hiperosmolaridad y la hipovolemia, mejora la actividad de las neuronas magnocelulares productoras de AVP y proporciona un mecanismo regulador adicional para mantener la presión arterial y el equilibrio hídrico; el polipéptido activador de la adenilato ciclasa de la pituitaria, acetilcolina, noradrenalina, dopamina y glutamato que aumentan en situaciones de estrés (como hiperosmolaridad, hipovolemia, dolor, vómito, náusea) promoviendo la liberación de AVP; el péptido natriurético auricular y el péptido natriurético cerebral que se activan por hipervolemia e inhiben la liberación de AVP (Baylis, 1987; Gillard et al., 2006; Hall, 2015; Hanoune 2009a; Ohbuchi et al., 2015; Szczepanska-Sadowska et al., 2018). Por lo tanto, las nuevas líneas de investigación del DE-79 también deben realizarse con otros reguladores de AVP, modificaciones de los receptores de AVP, interacciones con otras hormonas/neurotransmisores y cambios epigenéticos. Esto último es especialmente interesante, pues se sabe que los PBDEs afectan los mecanismos reguladores epigenéticos en múltiples sistemas biológicos, incluido el neuroendocrino (Poston and Saha 2019).

6. CONCLUSIONES

En este estudio, mostramos que la exposición perinatal al DE-79, una mezcla comercial de retardantes de flama octabromados, produce una disrupción neuroendocrina crónica del sistema vasopresinérgico. Estos efectos se observaron en condiciones basales y más evidentemente cuando el sistema se sometió a un estímulo hiperosmótico crónico. La nNOS también se afectó en los núcleos productores de AVP. Dado que el NO es un regulador de la liberación de la AVP, y se afecta por la exposición perinatal al DE-79, proponemos al NO, como uno de los mecanismos por los cuales se afecta el sistema AVPérgico causando un desequilibrio en la osmorregulación. El DE-79 a las dosis utilizadas produjo efectos subletales persistentes sobre la regulación homeostática. Estos efectos deterioran la calidad de vida de los organismos expuestos ya que no están preparados para responder a desafíos fisiológicos.

Las funciones de la AVP van más allá de su papel en la homeostasis hídrica, pues participa en funciones cognoscitivas, de aprendizaje y en la regulación del comportamiento social entre otras (Bowers et al., 2015; Ji et al., 2019; Shou et al., 2017); así como el NO, que está implicado en casi todos los sistemas biológicos (Chachlaki and Prevot, 2019). Por lo tanto, es necesario realizar más estudios para dilucidar qué otras funciones de la AVP y el NO están afectadas por la exposición a los PBDEs. Además, debido a las concentraciones corporales altas de PBDEs que se han encontrado en los niños y los niveles altos existentes en el polvo doméstico, el estudio de los PBDEs debe abordarse como un problema de salud pública

(Drobná et al., 2019; Hudson-Hanley et al., 2018; Johnson-Restrepo and Kannan, 2009; Stapleton and Dodder, 2008).

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8. PUBLICACIONES DURANTE EL DOCTORADO

8.1 Publicación derivada de esta tesis

Perinatal exposure to octabromodiphenyl ether mixture, DE-79, alters the vasopressinergic system in adult rats. (2020) **Alvarez-Gonzalez Mhar Yovavyn**, Sánchez-Islas Eduardo, Mucio-Ramirez Samuel, de Gortari Patricia, Amaya María Isabel, Kodavanti Prasada Rao S, León-Olea Martha. Toxicol Appl Pharmacol. 15;391:114914. https://doi.org/10.1016/j.taap.2020.114914 (ver apéndice 1).

8.2 Publicación derivada de colaboración con otro proyecto

Perinatal exposure to organohalogen pollutants decreases vasopressin content and its mRNA expression in magnocellular neuroendocrine cells activated by osmotic stress in adult rats. (2017) Mucio-Ramírez Samuel, Sánchez-Islas Eduardo, Sánchez-Jaramillo Edith, Currás-Collazo Margarita, Juárez-González Victor R., **Álvarez-González Mhar Y,** Orser L.E., Hou Borin, Pellicer Francisco, Kodavanti Prasada Rao S., León-Olea Martha. Toxicol. Appl. Pharmacol. 329. https://doi.org/10.1016/j.taap.2017.05.039 (ver apéndice 1).

9. PARTICIPACIÓN EN CONGRESOS

9.1 Congresos nacionales

Efecto de la exposición perinatal al DE-79 sobre el sistema vasopresinérgico en ratas adultas (poster). **Álvarez-González Mhar Yovavyn**, Sánchez-Islas Eduardo, Mucio-Ramírez Samuel, de Gortari Patricia, Amaya María Isabel, León-Olea Martha. LIX Congreso Nacional de la Sociedad Mexicana de Ciencias Fisiológicas, Campeche, Campeche, México dek 14 al 18 de agosto de 2016. (ver apéndice 2)

La exposición perinatal al DE-79 afecta el contenido y la expresión de la vasopresina en los núcleos hipotalámicos de ratas adultas (poster). **Álvarez-González Mhar Yovavyn**, Sánchez-Islas Eduardo, Mucio-Ramírez Samuel, de Gortari Patricia, Amaya María Isabel, León-Olea Martha. XXXI Reunión Anual de Investigación del Instituto Nacional de Psiquatría Ramón de la Fuente Muñiz, CdMX, México del 12 al 14 de octubre de 2016. (ver apéndice 2)

9.2 Congresos internacionales

Perinatal exposure to commercial mixture of polybrominated diphenyl ethers DE79 affects vasopressin content and mRNA expression in hypothalamic nuclei of adult rats (poster). **Álvarez-González Mhar Yovavyn**, Sánchez-Islas Eduardo, Mucio-Ramírez Samuel, de Gortari Patricia, Amaya María Isabel, León-Olea Martha. Society for Neuroscience Annual Meeting 2016, San Diego, California, EUA, del 12 al 16 de nieviembre de 2016. (ver apéndice 2)

Perinatal exposure to commercial mixture of polybrominated diphenyl ethers, de-79, affects vasopressinergic system in adult male rats (poster). **Álvarez-González Mhar Yovavyn**, Sánchez-Islas Eduardo, Mucio-Ramírez Samuel, de Gortari Patricia, Amaya María Isabel, León-Olea Martha. 18th International Congress of Comparative Endocrinology (ICCE18), Banff National Park, Alberta, Canadá, del 4 al 9 de junio de 2017. (ver apéndice 2)

10. APÉNDICE

10.1 Artículos publicados durante el doctorado

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Perinatal exposure to octabromodiphenyl ether mixture, DE-79, alters the vasopressinergic system in adult rats

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are persistent environmental pollutants considered as neurotoxicants and endocrine disruptors with important biological effects ranging from alterations in growth, reproduction, and effects on the hypothalamus-pituitary-adrenal axis. The vasopressinergic (AVPergic) system is a known target for pentaBDEs mixture (DE-71) and the structurally similar chemicals, polychlorinated biphenyls. However, the potential adverse effects of mixtures containing octaBDE compounds, like DE-79, on the AVPergic system are still unknown. The present study aims to examine the effects of perinatal DE-79 exposure on the AVPergic system. Dams were dosed from gestational day 6 to postnatal day 21 at doses of 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day, and male offspring from all doses at 3-months-old were subjected to normosmotic and hyperosmotic challenge. Male offspring where later assessed for alterations in osmoregulation (*i.e.* serum osmolality and systemic vasopressin release), and both vasopressin immunoreactivity (AVP-IR) and gene expression in the hypothalamic paraventricular and supraoptic nuclei. Additionally, to elucidate a possible mechanism for the effects of DE-79 on the AVPergic system, both neuronal nitric oxide synthase immunoreactivity (nNOS-IR) and mRNA expression were investigated in the same hypothalamic nuclei. The results showed that perinatal DE-79 exposure AVP-IR, mRNA expression and systemic release in adulthood under normosmotic conditions and more evidently under hyperosmotic stimulation. nNOS-IR and mRNA expression were also affected in the same nuclei. Since NO is an AVP regulator, we propose that disturbances in NO could be a mechanism underlying the AVPergic system disruption following perinatal DE-79 exposure leading to osmoregulation deficits.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are chemical substances used as additive flame retardants in a wide range of products such as construction materials, coatings, plastics, textiles and in electronic equipment like mobile phones, computers, televisions, and electrical kitchen appliances (ATSDR, 2017; U.S. EPA, 2010; WHO, 1994). The lipophilic property and stability of PBDEs have resulted in worldwide environmental contamination leading to bioaccumulation in both sediments and biota, ultimately undergoing biomagnification throughout the food chain to expose humans (De Wit, 2002; Watanabe and Sakai, 2003). PBDEs have been found in indoor environments, airborne particles and house dust (Johnson-Restrepo and Kannan, 2009; Shoeib et al., 2004; WHO, 2010). Additionally, high levels of PBDEs have been found in fish, vegetables, meat, and human milk (Abdalla and Harrad, 2014; Boucher et al., 2018). Humans are exposed to PBDEs during development beginning in the fetal stage *via* the placental circulation, during the neonatal period *via* lactation, and throughout adulthood by direct ingestion/contact (Abdalla and Harrad, 2014; Cowell et al., 2018; Eskenazi et al., 2013; Li et al., 2013).

PBDEs contain 209 possible congeners; varying in both number and position of bromination. Congeners containing five bromines are dubbed penta-bromodiphenyl ethers (-BDEs), congers containing eight bromines are labeled octa-BDEs, and so on. Commercially, PBDEs are

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produced as mixtures of penta-, octa-, and deca-BDEs, which are named after the dominating homolog group (ATSDR, 2017; WHO, 1994). Based on their reported toxicity, penta- and octaBDE mixtures were banned worldwide in 2004, and decaBDE mixtures were only banned for use in Europe in 2008 (ATSDR, 2017; U.S. EPA, 2010). However, the PBDEs are still widespread and prevail through consumer products to which they were added. Besides, bioaccumulation is inversely related to the degree of bromination and some of the higher PBDE congeners as decaBDEs are metabolized into lower brominated BDE congeners as octaBDEs and pentaBDEs which are more toxic (Huwe and Smith, 2007; Kodavanti et al., 2017, 2018; Stapleton and Dodder, 2008; Watanabe and Sakai, 2003). OctaBDE mixture is the less studied of PBDEs despite its environmental presence and being classified as a possible risk factor for neuroendocrine disruptor such as infertility and teratogenicity (Kodavanti et al., 2017; U.S. EPA, 2010).

Toxicological studies have demonstrated that exposure to PBDEs result in adverse effects in mammals including carcinogenicity, neurotoxicity and endocrine disruption (ATSDR, 2017; Kodavanti et al., 2010; Kodavanti et al., 2017). As the polychlorinated biphenyls (PCBs), structurally similar chemicals, PBDEs affect intracellular signaling pathways including protein kinase C (PKC) translocation, calcium regulation and neurotransmitters in the brain (Fan et al., 2010; Kodavanti, 2005; Mariussen and Fonnum, 2003). Therefore, PBDEs exposure during critical periods of development (*i.e.* gestation and lactation) could have detrimental effects on the establishment of normal brain structure and subsequent function lasting into adulthood (Chen et al., 2014; Hudson-Hanley et al., 2018; Kodavanti, 2005; Viberg et al., 2006). There is evidence that PBDEs have effects on the neuroendocrine systems of mammals by affecting thyroid and reproductive hormones (Ji et al., 2019; Kodavanti et al., 2010; Kodavanti and Curras-Collazo, 2010; Kodavanti et al., 2017; León-Olea et al., 2014). Our group has previously reported that commercial mixtures of PCBs and PBDEs (Aroclor 1254 and DE-71, a pentaBDE mixture, respectively) affect the hypothalamic-neurohypophysial system, disturbing hormones such as arginine-vasopressin (AVP) and oxytocin (OXT; Coburn et al., 2005, 2007; Kodavanti and Curras-Collazo, 2010; Mucio-Ramírez et al., 2017).

The major biological action of AVP is to maintain the balance between absorption and excretion of water in the kidney (Hanoune, 2009; Verbalis, 2013). AVP is a 9-amino acid peptide with a 6-member disulfide ring and is synthesized mainly in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus where it is then transported by axonal processes to the posterior pituitary (Brownstein et al., 1980; Landgraf et al., 1988; Verbalis, 2013). There within the pituitary, the peptide is secreted into the systemic circulation, targeting the kidney, thus performing its antidiuretic function increasing water retention. AVP released into the bloodstream is stimulated in response to osmotic (*e.g.* dehydration) and non-osmotic (*e.g.* hypotension, hypovolemia, hypoglycemia) stimuli (Baylis, 1987; Murphy et al., 2016; Ohbuchi et al., 2015). AVP is also released within the PVN and SON, from the soma and dendrites of magnocellular neurons (somatodendritic release; Ludwig and Stern, 2015; Murphy et al., 2016). Somatodendritic AVP release acts as a short-loop feedback signal to modulate systemic AVP release (Ludwig and Leng, 2006). Of interest to this study is the effect of DE-79 (a commercial octaBDE mixture) on the vasopressinergic system (AVPergic) that controls osmoregulation.

On the other hand, nitric oxide (NO) is one of the molecules that regulates the activity of the magnocellular neuroendocrine system, and consequently regulates both systemic and somatodendritic AVP release (Gillard et al., 2007; Kadekaro et al., 2006; Kadowaki et al., 1994; Ota et al., 1993; Reid, 1994). NO is synthesized *via* calcium-calmodulin by nitric oxide synthase (NOS; Knowles and Moncada, 1994). Neuronal NOS (nNOS) is the most prominent isoform in the AVP neurons of the PVN and SON (Bredt et al., 1990; Eliasson et al., 1997; Rodrigo et al., 1994). Coexpression of nNOS and AVP in these nuclei have been described (Nylén et al., 2001a, 2001b; Sánchez et al., 1994). Furthermore, *in vitro* and *in vivo* studies reported that nNOS as well as AVP are targets for toxic chemicals like PCBs and PBDEs (Coburn et al., 2015; Currás-Collazo, 2011; Kodavanti and Curras-Collazo, 2010; Sharma and Kodavanti, 2002). It has been reported that PCB congeners impair the glutamate-NO-cGMP pathway and perturb intracellular calcium homeostasis (Kodavanti, 2005; Llansola et al., 2007). The AVP release is regulated indirectly by noradrenaline and glutamate (Glu) pathways which modulate the afferent activity of magnocellular neurons during stimuli like hyperosmolality, leading to an increase in free intracellular calcium, activating calmodulin and nNOS expression which increases the production of NO. Transcellular NO enhances excitatory glutamatergic drive *via* NMDA-type receptors, and in concert with the calcium gated ionotropic Glu receptors stimulates somatodendritic AVP release (Bains and Ferguson, 1997; Gillard 2007; Kadekaro, 2006; Komori et al., 2010). We have shown previously that NOS activity levels are reduced in both early and late adulthood rats exposed to PCBs in uterus and this effect may underlie altered neuroendocrine output of AVP from dendrites and axons of MNCs (Coburn et al., 2015). Our group has also reported that perinatal and postnatal exposure to penta-BDEs (DE-71), alters AVP functions related to cardiovascular regulation of adult rats (Shah et al., 2011); decreases AVP immunoreactivity and mRNA expression in the hypothalamus of adult rats during osmotic stress (Mucio-Ramírez et al., 2017); and reduces somatodendritic AVP release during hyperosmotic activation *in vitro* (Coburn et al., 2007). The mechanisms underlying PBDEs disruption of AVP activity are not well understood and the effects of octaBDE commercial mixtures on the AVPergic system are unknown. The aim of the present study is to characterize the effects of perinatal exposure to the commercial octaBDE mixture, DE-79, on the AVPergic system in adult rats, and determine if the nNOS is affected in the main nuclei producing AVP, the PVN and SON.

2. Materials and methods

2.1. Animals

Timed-pregnant Wistar rats on gestation day 2 (GD; n = 24) were obtained from the animal care facility of Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz (INPRFM), Mexico. Animals were housed individually in plastic cages and maintained under controlled laboratory conditions of temperature (21–23 °C), relative humidity (50–55%), and with a light-dark (12:12 h) cycle. Food (commercial pellets Lab Chow 5001 Purina USA) and tap water were provided *ad libitum*. The experiments were performed in accordance with National Institutes of Health guidelines for care and use of laboratory animals and with the approval of the Projects and Ethics Committee of the INPRFM.

2.2. Chemical

Commercial octaBDE mixture, DE-79 (technical grade octabromodiphenyl oxide; Lot #8525DG01A) was obtained from the Great Lakes Chemical Corporation, West Lafayette, IN, USA. The major congeners in DE-79 are: BDE-175/183 (42%), −197 (22.2%), −207 (11.5%), and − 196 (10.5%; La Guardia et al., 2006). The presence of impurities such as brominated biphenyls, dioxins, and furans in this mixture has been reported (Hanari et al., 2006).

2.3. Perinatal exposure to DE-79

Wistar pregnant dams were fed with popcorn infused with corn oil (vehicle) containing DE-79 at doses of 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day (n = 8/dose) from GD 6 to postnatal day (PND) 21 in addition to their normal diet (Fig. 1). The low and high doses concentrations were selected to match doses of the chemically similar PCB

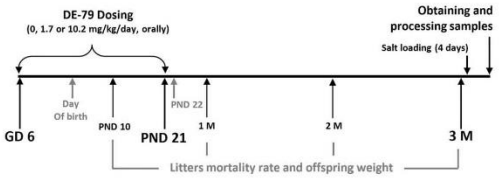

Fig. 1. Dosing and testing paradigm. Temporal paradigm for perinatal exposure to DE-79 at doses 0 (Control), 1.7 (low) or 10.2 (high) mg/kg/day. DE-79 exposure is from gestational day (GD) 6 to postnatal day (PND) 21. Male pups were weaned and separated at PND 22. Around 3 months (M) old, a group of male rats of all doses was subjected to salt loading, an osmotic challenge (hyperosmotic group; *ad libitum* NaCl 2% for the 4 days prior sacrifice), the other group had *ad libitum* access to tap water (normosmotic group). At 3 M of age, rats were randomly assigned to different groups for analysis (immunoreactivity, mRNA expression), samples were collected, and processed. Serum AVP and osmolality of all groups were also measured. Litters were analyzed for offspring mortality rate during the periods of PND 0–10, PND 10–1 M, 1–2 M, and 2–3 M, for offspring weight at PND 10, and only male offspring weights at 1, 2 and 3 M of age.

mixture, Aroclor 1254 (1 and 6 mg/kg/day) on a molar basis, which have extensive information from *in vitro* and *in vivo* studies (Fan et al., 2010; Kodavanti, 2005; Kodavanti et al., 2010; Kodavanti and Ward, 2005). The volume of each dosing mixture in corn oil was adjusted based on changes in the dam's weight. At PND 4, litters were culled to 8 pups per dam in all groups with an equal number of males and females. Pups exposed perinatally were weaned and separated by sex at PND 22. At 3 months (M) of age, a maximum of two male offspring of each litter were randomly assigned for studying different assays (Table 1). Litters, that include animals used in this article and in other experiments under similar conditions (males and females), were analyzed for: 1) offspring mortality rate during PND 0 to 10, PND 10 to 1 M, 1 to 2 M, and 2 to 3 M; and 2) offspring weight at PND 10, and only male offspring weight at 1, 2 and 3 M of age (see Fig. 1 for details).

2.4. Systemic osmotic challenge (salt loading)

Hyperosmotic challenge was utilized to determine if perinatal exposure to DE-79 alters AVP and nNOS immunoreactivity and mRNA expression in the PVN and SON of the hypothalamus, AVP systemic release, and serum osmolality. Therefore, two conditions were compared: A) normosmotic, where animals had *ad libitum* access to tap water; and B) hyperosmotic, where animals were subjected to salt loading (*ad libitum* access to 2% saline solution, 20 g NaCl/l, for 4 days) as previously described (Dai and Yao, 1995; Kadowaki et al., 1994; Mucio-Ramírez et al., 2017). Rats were weighed before and after osmotic challenge. Rats that lost about 10% of their body weight (data not shown) were considered dehydrated and included in the experimental groups.

2.5. Experimental groups

Animals of all groups were randomly assigned to four analysis groups: 1) serum osmolality to analyze the osmoregulation (n = 6–8/subgroup), 2) immunofluorescence for AVP and nNOS in order to evaluate the immunoreactivity of these molecules in the PVN and SON (n = 3–6 /subgroup), 3) endpoint reverse transcription polymerase chain reaction (PCR) for AVP and nNOS genes to determine mRNA expression in PVN and SON (n = 3–4/subgroup, and 4) enzyme-linked immunosorbent assay (ELISA) for serum AVP to examine release to the systemic circulation (n = 6–7/subgroup; see Table 1 for details).

2.6. Sample analysis

2.6.1. Serum osmolality

Blood collected at the time of sacrifice was centrifuged at 1500 xg for 18 min at 4 °C and the serum was separated, and quick frozen (−80 °C) for subsequent analysis. As an osmoregulation signal, serum osmolality was measured with a vapor pressure osmometer (Wescor 5500, Logan, UT). The osmometer was calibrated according to the manufacturer's instructions. Filter paper discs were placed in the instrument and saturated with 10 µl of serum, osmolality was measured in triplicate. Average measurements were obtained of each rat with respect to each experimental group. Results on osmolality were reported as mean ± s.e.m. in mOsm/kg and as percent of normosmotic control.

2.6.2. AVP and nNOS immunofluorescence in hypothalamic PVN and SON

Upon completion of the osmotic challenge (Table 1), rats were anesthetized with sodium pentobarbital (63 mg/kg) and cardiac blood was collected. Subsequently, rats were transcardially perfused with 150–200 ml of 0.9% saline containing 2500 IU/500 ml heparin (Pisa® Farmacéutica, México) followed by 250–350 ml of 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS (0.1 M phosphate-buffered saline, pH 7.4) as a fixative. Brains were removed and post-fixed in the same fixative at 4 °C for 2–4 h. Brains were then cryoprotected in 30% sucrose and stored at 4 °C until used. Coronal slices (30 µm) of PVN and SON (Bregma −0.80 to −2.12 mm; Paxinos and Watson, 2007) were cut on a sliding-freezing microtome (Leitz, Grand Rapids, MI) and collected in plastic wells containing PBS. Each well had a set (6 slices) of all representative rostrocaudal PVN and SON. Free-floating sections from 3 sets were processed for immunohistochemistry. Double-labeling immunofluorescence for AVP (n = 4–6/subgroup) and nNOS (n = 3–5/subgroup) was employed

Table 1
Experimental groups (control and DE-79 exposed, perinatally), osmotic conditions. and distribution of rats for analysis of different parameters.

Osmotic condition	DE-79 (mg/kg/day) GD 6 to PND 21	Analysis groups									
		AVP-IR (n)		nNOS-IR (n)		AVP-mRNA (n)		nNOS-mRNA (n)		Serum AVP (n)	Osmolality (n)
		PVN	SON	PVN	SON	PVN	SON	PVN	SON		
Normosmotic (Tap water)	0 (control)	5	5	4	4	3	4	3	4	7	8
	1.7 (low)	4	4	5	4	4	4	4	3	6	7
	10.2 (high)	6	5	5	5	4	3	4	3	7	8
Hyperosmotic (Salt loading)	0 (control)	4	4	3	3	4	4	4	3	6	8
	1.7 (low)	4	5	5	5	4	4	4	4	6	6
	10.2 (high)	5	5	6	5	4	3	4	3	6	8

Distribution of 3-month-old male rats (sample size -n-) exposed perinatally to DE-79 at doses: 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day dissolved in corn oil vehicle, from gestational day (GD) 6 to postnatal day (PND) 21. By osmotic condition (normosmotic rats –*ad libitum* tap water–; hyperosmotic rats –salt loading, *ad libitum* NaCl 2%, for 4 days–) and sample size (n) of analysis groups (vasopressin and neuronal nitric oxide synthase immunoreactivity –AVP-IR, nNOS-IR–; AVP and nNOS mRNA expression; serum AVP; and serum osmolality measurements).

(Mucio-Ramírez et al., 2017). Primary antibodies used for incubations were: AVP-neurophysin antibody (PS-41 monoclonal developed in mouse, gifted by H. Gainer, NIH; dilution 1:500; Ben-Barak et al., 1985; Whitnall and Gainer, 1985) and nNOS antibody (C-terminal polyclonal developed in rabbit, ImmunosStar, Inc. Hudson, WI; dilution 1:500; Eliasson et al., 1997; Sanchez-Islas et al., 2014). Secondary antibodies used were: Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 555 donkey anti-rabbit (Invitrogen Corp., Carlsbad, CA; dilution 1:250 for each). Some sections obtained from each group were used as methodological control sections by using a blocking solution without primary antibody (negative controls; Fig. S1). All sections were analyzed with a Zeiss 510 META laser scanning confocal microscope after a lambda stack to obtain the emission spectrum of each fluorophore. Images from each section (30 μm thickness) were acquired and analyzed bilaterally on the optimal focal plane, in single track mode, with the Ar laser/488 nm, pinhole diameter (1 airy unit) and detector gain (1) at 10× so the nuclei are complete in a single photo. All images were analyzed for integrated optical density (IOD) using computer-assisted densitometry software (Image Pro Plus 4.5, Media Cybernetics, MD, USA) as described in Mucio-Ramírez et al. (2017). Average IOD was obtained from bilateral PVN and SON of rats (6–8 slices of each rat that included representative rostrocaudal nuclei getting at least 3 images of anterior nuclei, 3 of middle nuclei and 3 of posterior nuclei) of all the experimental groups. The IOD was reported as mean ± s.e.m. in arbitrary units. The quantified IOD values for AVP-IR should not be taken as a linear index of the peptide's intracellular concentrations, even so, the observed AVP IOD values are likely to reflect changes in the AVP content of the PVN and SON.

2.6.3. AVP and nNOS mRNA semi-quantification in hypothalamic PVN and SON by endpoint PCR

Upon completion of the osmotic challenge (Table 1), rats were anesthetized with sodium pentobarbital (63 mg/kg). Brains were dissected and trunk blood was collected after quick decapitation with a guillotine. Brains were placed on dry ice and stored at −80 °C until use. Bilateral PVN and SON were dissected by the micropunch technique (Palkovits and Brownstein, 1988) and kept frozen in a tube. Total RNA was extracted as described elsewhere (Chomczynski and Sacchi, 1987). AVP (n = 3–4/subgroup) and nNOS (n = 3–4/subgroup) mRNA expression in the PVN and SON were semi-quantified by PCR with expression of ribosomal RNA, 18S, used as reference gene. The protocol used was as described in Jaimes-Hoy et al. (2008). cDNA was prepared from 1.5 μg RNA (reverse transcriptase, oligo dT, and specific oligonucleotides synthesized at the Biotechnology Institute facilities in the Universidad Nacional Autónoma de México). The number of cycles for each probe was optimized using 25 pmol of AVP (sense: 5′ CACCTCT GCCTGCTACTTCC 3′, antisense: 5′ GGGCAGGTAGTTCTCCTCCT 3′) or nNOS (sense: 5′ TGACTCTTGGGCTACGATGC 3′, antisense: 5′ GGTGG AAGGGGGCTTAAGTG 3′) or 18S (sense: 5′ ATGGCCGTTCTTAGTTG GTG 3′, antisense: 5′ CGCTGAGCCAGTTCAGTGTA 3′) and 0.5 μl Taq DNA polymerase (Biotecnologías Universitarias, UNAM). 4 μl of cDNA were used, final conditions were: 25 cycles for AVP, 29 cycles for nNOS and 18 cycles for 18S, each of 1 min at 94 °C, then 1 min at 64 °C and a final minute at 72 °C. All cDNA had a final extension time of 10 min at 72 °C. cDNA was semi-quantified from the same reverse transcriptase reaction. Products were separated by gel electrophoresis in 2% agarose -1× TBE, running buffer (0.5×), and stained with ethidium bromide; density was quantified with a Fluor-S MultiImager (BioRad Laboratories, Inc., Hercules, CA, USA). Relative amounts of cDNAs were calculated as the ratio of AVP or nNOS cDNA optical density (OD) over that of 18S. Care was taken to include samples of all groups in the same gel. The OD was reported as mean ± s.e.m. expressed as percent of normosmotic control.

2.6.4. Serum AVP analysis by ELISA

Blood samples from all animals were collected into chilled tubes and centrifuged at 1500 xg for 18 min at 4 °C. Serum samples were stored at −80 °C until processed as a single group. First, samples were delipidated by adding an equal volume of a mixture consisting of 40:60 butanol and ethyl ether, then vortexed. The samples were then centrifuged at 8000 xg for 5 min. The top organic layer was discarded, and aqueous layer was used for AVP extraction. To the aqueous layer, 2× the volume of ice-cold acetone was added, samples vortexed, and centrifuged at 12,000 xg for 20 min. The supernatants were combined with 5× the volume of ice-cold petroleum ether, vortexed, and centrifuged at 10,000 xg for 10 min. The top ether layer was discarded and then the aqueous protein layer was desiccated under vacuum. The final product was reconstituted with assay buffer. Lastly, AVP concentrations were measured in duplicate using the Arg8-Vasopressin ELISA kit, as reported previously (Enzo Life Sciences, Farmingdale, NY; Coburn et al., 2005) which has a sensitivity of 2.84 pg/ml. Results for serum AVP quantification were reported as mean ± s.e.m. in pg/ml and as percent of normosmotic control.

2.7. Statistical analysis

Statistical significance among groups was determined by two-way analysis of variance (ANOVA; factors tested: DE-79 dose and osmotic condition) whenever data met normal distribution/equal variance assumptions. When subsets of data did not satisfy these assumptions (serum AVP and nNOS-IR in both nuclei), the data was $\log_{10}$ transformed. Significant differences between groups were analyzed by the *post hoc* Holm-Sidak test. In all cases, an alpha level of $p \leq 0.05$ was considered statistically significant. Statistical analysis was done using SigmaPlot 12.3 software (Systat Software, Inc).

3. Results

3.1. Litter data

Perinatal exposure to DE-79 resulted in higher rates of pup mortality compared to controls from PND 0–10 and the increased rates persisted until 1 M of age in the high dose group (Fig. S2). There was a slight increase in the body weight of pups in low dose group compared to control only at PND 10 (Fig. S3). No abnormalities in rat behavior were observed following exposure to DE-79.

3.2. Perinatal DE-79 exposure affects adult osmoregulatory capacity

Osmolality was measured in the serum of rats under normosmotic and hyperosmotic conditions. The two-way ANOVA analysis (Table 2) showed no interaction between osmotic conditions and dose factors ($F_{2,39} = 0.329$, $p = 0.722$), but there was a statistically significant difference in the mean serum osmolality among osmotic condition factor groups ($F_{1,39} = 43.053$, $p < 0.001$). Multiple comparisons revealed a significant increase in serum osmolality in response to hyperosmotic stimulation across all doses ($p < 0.001$ for control and high dose and $p = 0.003$ for low dose). There was a DE-79 dose-related increase tendency among hyperosmotic groups, however, it was not significant. The high dose denoted a higher percentage of increase in serum osmolality after hyperosmotic challenge compared to the control group (12.23 ± 1.94% *vs* 9.79 ± 1.42% increase). Therefore, exposed animals showed the highest osmolality values among normosmotic and hyperosmotic groups.

3.3. Dysregulation of adult AVP system in rats perinatally exposed to DE-79

3.3.1. AVP immunofluorescence in hypothalamic PVN and SON

We analyzed AVP immunofluorescence images of coronal brain sections from the PVN (Fig. 2A-F) and SON (Fig. 2G-L) of perinatally DE-79 exposed male rats at doses 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day and under normosmotic conditions (Fig. 2A, B, C, G, H, I) or

Table 2
Effects of perinatal exposure to DE-79 on serum osmolality in 3-month-old male rats.

DE-79 (mg/kg/day)	Normosmotic (tap water)		Hyperosmotic (salt loading)	
	mOsm/kg	%	mOsm/kg	%
0 (control) (n = 8, n = 8)	311.5 ± 2.38	100 ± 0.72	342 ± 6.65***	109.79 ± 2.14***
1.7 (low) (n = 7, n = 6)	317.33 ± 3.5	101.87 ± 1.12	346.39 ± 8.89**	111.2 ± 2.86**
10.2 (high) (n = 8, n = 8)	316.5 ± 2.96	101.61 ± 0.95	354.63 ± 8.99***	113.84 ± 2.89***

Osmoregulatory capacity was measured in normosmotic (*ad libitum* tap water) and hyperosmotic conditions (salt loading; *ad libitum* 2% NaCl, for 4 days) in 3-month-old rats perinatally exposed to DE-79 at doses 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day. Numbers below doses denote sample size (n) corresponding to normosmotic and hyperosmotic groups respectively. Values are expressed as mean ± s.e.m. in mOsm/kg and as percent of normosmotic control (100%). Symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak *post-hoc* test. Hyperosmotic compared to normosmotic same dose; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

subjected to salt loading, a prolonged hyperosmotic challenge (hyperosmotic; Fig. 2D, E, F, J, K, L). Image analysis showed uniformly distributed AVP immunoreactivity (AVP-IR) in soma and fibers emanating from each nucleus under normosmotic conditions in the control groups (Fig. 2A, G) and a physiological increase in AVP-IR under hyperosmotic stress (Fig. 2D, J). We also observed an apparent increase in cell volume of the AVPergic neurons in both nuclei of hyperosmotic control groups as described previously (Johnson et al., 2015; Zhang et al., 2001). The low dose group under normosmotic conditions showed an increase of AVP-IR similar to the hyperosmotic control group (Fig. 2B, H, *vs* D, J). Interestingly, the hyperosmotic low dose group (Fig. 2E, K) did not show an increase compared to its own normosmotic AVP-IR. The high dose group had lower AVP-IR compared to control under normosmotic conditions and failed to present the physiological response to the hyperosmotic stimulus (Fig. 2F, L) compared to its normosmotic homolog (Fig. 2C, I). Mean integrated optical density (IOD) values of the images are shown in graphs (Fig. 2a, b). Statistical analysis using two-way ANOVA showed a significant interaction between osmotic condition and dose factors in PVN and SON ($F_{2,22} = 6.298$, $p = 0.007$ and $F_{2,22} = 6.623$, $p = 0.006$, respectively); and a statistically significant difference in the mean IOD values from AVP-IR between doses in both nuclei ($F_{2,22} = 14.912$, $p < 0.001$ for PVN and $F_{2,22} = 16.182$, $p < 0.001$ for SON). There were no statistically significant differences among osmotic condition factor groups ($F_{1,22} = 2.93$, $p = 0.101$ for PVN and $F_{1,22} = 0.814$, $p = 0.377$ for SON). Multiple comparisons showed that in normosmotic groups, the low dose had significantly higher AVP-IR compared to control in PVN and SON ($p = 0.033$ and $p = 0.028$, respectively) and compared to the high dose ($p < 0.003$ for PVN and SON). The hyperosmotic challenge led to a significant physiological AVP-IR increase relative to control groups of PVN and SON ($p < 0.001$ and $p = 0.002$, respectively). However, neither the low nor high dose groups showed the expected physiological increase in response to hyperosmotic activation. Conversely, the high dose group showed a significant decrease in AVP-IR across both nuclei ($p < 0.001$) compared to the hyperosmotic control. Therefore, AVP-IR among normosmotic groups was higher in low dose group and the physiological response to hyperosmotic stress in animals of both doses was blunted.

3.3.2. AVP mRNA expression in hypothalamic PVN and SON

AVP mRNA expression from PVN and SON punches were normalized with 18S, rRNA reference gene, and analyzed as percent of normosmotic controls (Fig. 3). Measurements were made in normosmotic conditions and under a hyperosmotic challenge. The two-way ANOVA analysis of the AVP mRNA expression showed an interaction between osmotic condition and dose factors in both nuclei ($F_{2,17} = 49.452$, $p < 0.001$ for PVN and $F_{2,16} = 11.55$, $p < 0.001$ for SON). There was a statistically significant difference in the mean OD of mRNA expression among osmotic condition factor groups in PVN ($F_{1,17} = 29.798$, $p < 0.001$). Also, there was a significant difference between doses in SON ($F_{2,16} = 5.834$, $p = 0.013$). Multiple comparisons showed a significantly increased mRNA expression with the high dose compared to control and to the low dose in normosmotic groups in PVN ($p < 0.001$ for both) and in SON ($p = 0.001$ high dose *vs* control; $p = 0.023$ high *vs* low dose). Hyperosmotic activation increased mRNA expression in control compared to its respective normosmotic group in PVN only ($p < 0.001$), and in low dose compared to its normosmotic homologous in both nuclei ($p < 0.001$ for PVN; $p = 0.040$ for SON). In contrast, the high dose group blunted the hyperosmotic activation response resulting in a significant mRNA expression decrease in PVN and SON ($p < 0.001$ for both nuclei) compared to its respective normosmotic group. Among hyperosmotic groups, the high dose presented a significant decrease compared to control in PVN ($p < 0.001$) and compared to low dose in both nuclei ($p < 0.001$ for PVN and $p = 0.006$ for SON).

3.3.3. Serum AVP levels

Blood collected from animals of all groups was analyzed for serum AVP levels under normosmotic and hyperosmotic conditions. The two-way ANOVA analysis on log-transformed data (Fig. 4) showed a significant interaction between osmotic condition and dose factors ($F_{2,32} = 4.082$, $p = 0.026$). Besides, there was a statistically significant difference among osmotic condition factor groups ($F_{1,32} = 11.371$, $p = 0.002$). A *post hoc* multiple comparison test showed a physiologically significant increase in hyperosmotic control animals compared to its respective normosmotic group ($p < 0.001$), corresponding to approximately 2.5-fold increase of AVP serum levels. Among normosmotic groups, there was a statistically significant increase of the low and high dose groups compared to control ($p = 0.029$ for low *vs* control and $p = 0.008$ for high *vs* control), this was of about a 2-fold AVP serum increase. Meanwhile, hyperosmotic activation showed a blunted response in serum AVP levels of low and high dose groups, since they are no statistically different from their normosmotic counterpart.

3.4. Perinatal DE-79 exposure affects nNOS in hypothalamic nuclei

3.4.1. nNOS immunofluorescence in PVN and SON

We analyzed nNOS immunofluorescence images, in PVN (Fig. 5A-F) and SON (Fig. 5G-L) of perinatally exposed to DE-79 male rats at doses of 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day, and under normosmotic conditions (Fig. 5A, B, C, G, H, I) or subjected to a hyperosmotic challenge (hyperosmotic; Fig. 5D, E, F, J, K, L). Image analysis showed that normosmotic nNOS immunoreactivity (nNOS-IR) in soma and fibers in control group (Fig. 5A, G) physiologically increased during hyperosmotic stress (Fig. 5D, J). The normosmotic low dose group showed an apparent increase in nNOS-IR in SON (Fig. 5H *vs* G), while the high dose group showed an apparent decrease in PVN, compared to normosmotic controls (Fig. 5C *vs* A). Among hyperosmotic groups, the

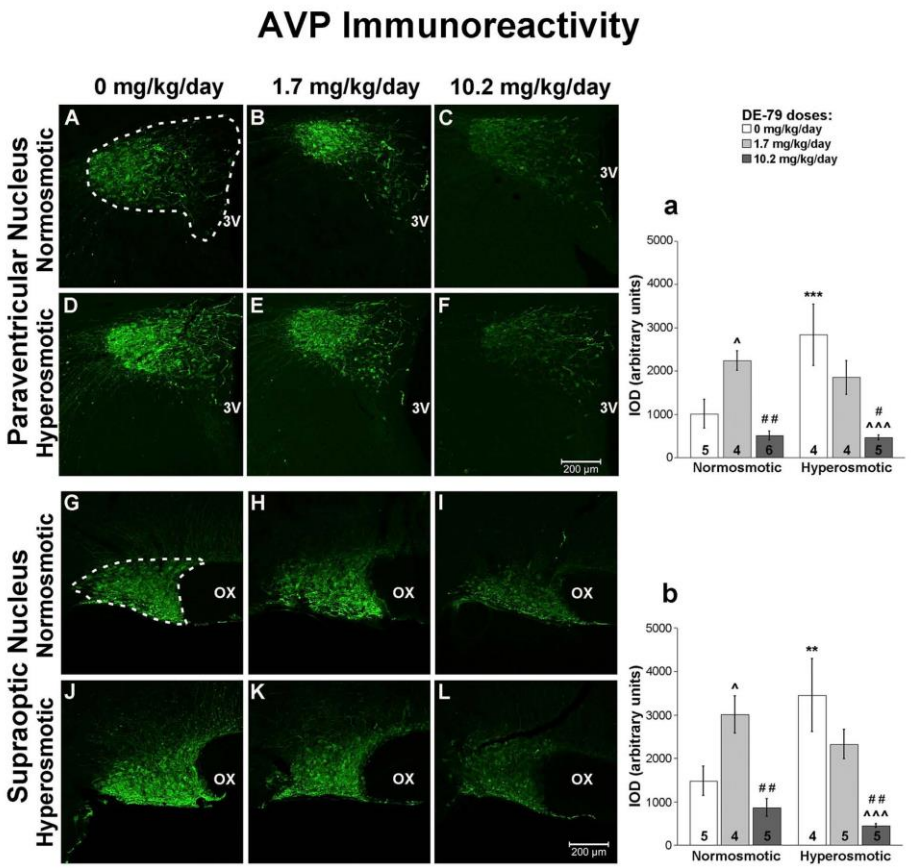

Fig. 2. Effects of perinatal exposure to DE-79 on AVP immunoreactivity (AVP-IR) in 3-month-old male rats. Left: Panel of representative confocal images of coronal sections of PVN (top; images A to F) and SON (down; images G to L) with AVP-IR. DE-79 doses 0 (control; A, D, G, J); 1.7 (low; B, E, H, K) or 10.2 mg/kg/day (high; C, F, I, L); normosmotic (A, B, C, G, H, I) or hyperosmotic rats (salt-loaded; D, E, F, J, K, L). There was a physiological increase in AVP-IR of hyperosmotic control rats (D, J) compared to normosmotic control groups (A, G). In contrast, there was no increase in hyperosmotic rats of low and high doses compared to its normosmotic counterparts. Right: Graphs display the effects of perinatally DE-79 exposure on AVP-IR integrated optical density (IOD) in PVN (a) and SON (b) from male adult rats. The dotted lines show the region of interest (ROI) used to perform IOD measurements. Bars represent mean values ± s.e.m. and the numbers inside them denote sample size (number of rats, from which 6 bilateral cuts, on average, per rat where analyzed). Symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak *post-hoc* test. (*) hyperosmotic compared to normosmotic same dose; (^) low/high dose compared to control same group (normo−/hyperosmotic); (#) high dose compared to low dose same group (normo−/hyperosmotic); ^, # = $p \leq 0.05$; **, # # = $p \leq 0.01$; ***,^^^ = $p \leq 0.001$. Abbreviations: third ventricle (3 V); optic chiasm (OX). Bar = 200 µm.

low and high doses did not show the physiological increase of nNOS-IR (Fig. 5E, F, K, L) compared to their normosmotic counterparts (Fig. 5B, C, H, I). Statistical analysis (Fig. 5a, b) using two-way ANOVA on log-transformed data showed a significant interaction between osmotic condition and dose factors in PVN and SON ($F_{2,22} = 5.844$, $p = 0.009$ and $F_{2,20} = 9.655$, $p = 0.001$, respectively). There was also a significant difference in the mean IOD values from nNOS-IR between doses in both nuclei ($F_{2,22} = 8.471$, $p = 0.002$ for PVN and $F_{2,20} = 9.326$, $p = 0.001$ for SON); and among osmotic condition factor groups only in SON ($F_{1,20} = 23.324$, $p < 0.001$). Multiple comparisons revealed differences between the low dose and the control in the normosmotic groups, statistically significant only in SON ($p = 0.008$). Hyperosmotic stimulation showed a physiological increase of nNOS-IR relative to control groups in PVN and SON ($p = 0.006$ and $p < 0.001$, respectively). Among hyperosmotic groups there was a significant decreased nNOS-IR of low ($p = 0.024$ in PVN and $p = 0.038$ in SON) and high dose ($p < 0.001$ in PVN and SON), both compared to the hyperosmotic control group. Therefore, nNOS-IR failed to increase in response to the hyperosmotic activation in the low and high dose groups compared to its normosmotic counterparts.

3.4.2. nNOS mRNA expression in PVN and SON

nNOS mRNA expression was normalized with 18S, rRNA reference gene, and analyzed as percent of normosmotic control (Fig. 6). Measurements were made in normosmotic conditions and with a hyperosmotic activation. Two-way ANOVA analysis indicated a significant

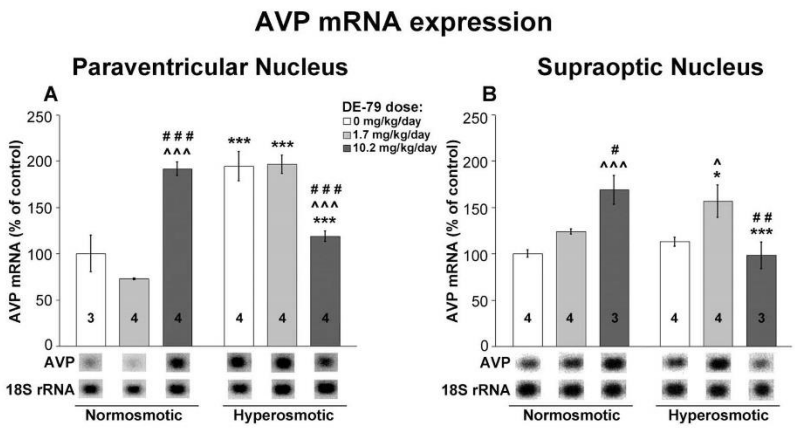

Fig. 3. Effects of perinatal exposure to DE-79 on AVP mRNA expression by semiquantitative endpoint PCR in 3-month-old male rats. Analyses of PVN (A) and SON (B) micropunches were carried out in rats perinatally exposed to DE-79 on doses 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day and normosmotic or hyperosmotically stimulated adult rats. In A, we observed a physiological increase in AVP mRNA expression of hyperosmotic rats compared to normosmotic ones in the control and low dose. In contrast, there was no increase in the hyperosmotic groups of high dose. In B, we observed a lack of AVP mRNA expression increase in hyperosmotic high dose, an increase of control and low dose, significant only in the latter, compared to its respective normosmotic. Bars are the mean values ± s.e.m. expressed as percent of normosmotic control (100%), and the numbers inside them denote sample size. Below each bar is one example of the electrophoresis bands from AVP and 18S rRNA. A statistical analysis of densitometry (optical density) for AVP mRNA normalized to the 18S rRNA reference gene was performed. Symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak *post-hoc* test. (*) hyperosmotic compared to normosmotic same dose; (^) low/high dose compared to control same group (normo−/hyperosmotic); (#) high dose compared to low dose same group (normo−/hyperosmotic); *, ^, # − $p \leq 0.05$; # # − $p < 0.01$; ***, ^^^, # # # − $p < 0.001$.

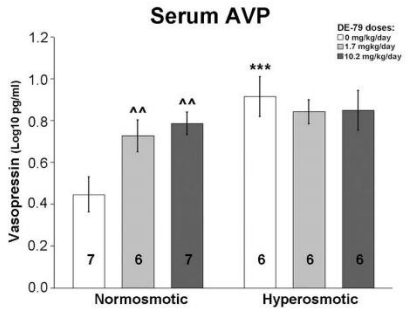

Fig. 4. Effects of perinatal exposure to DE-79 on serum vasopressin (AVP) in 3-month-old male rats. Serum AVP was analyzed in adult rats perinatally exposed to DE-79 at doses 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day under normosmotic or hyperosmotic conditions. Bars represent mean values ± s.e.m. of log-transformed data in pg/ml. The numbers inside the bars denote sample size. Symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak *post-hoc* test. (*) hyperosmotic compared to normosmotic same dose; (^) low/high dose compared to control same group (normo−/hyperosmotic); ^^ = $p \leq 0.01$; *** = $p \leq 0.001$.

interaction between osmotic condition and dose factors in both nuclei ($F2,17 = 34.99$, $p < 0.001$ for PVN and $F2,14 = 4.392$, $p = 0.033$ or SON). There were also significant differences among osmotic condition factor groups in both nuclei ($F1,17 = 92.157$, $p < 0.001$ for PVN and $F1,14 = 12.557$, $p = 0.003$ for SON); as well as between doses ($F2,17 = 14.014$, $p < 0.001$ for PVN and $F2,14 = 5.03$, $p = 0.023$ for SON). Multiple comparisons showed a significant increase in nNOS mRNA expression in the low dose compared to control in normosmotic groups in PVN ($p = 0.004$). Hyperosmotic activation increased nNOS mRNA expression in control compared to its respective normosmotic group in both nuclei ($p < 0.001$); while the low dose group also showed an increase with the hyperosmotic activation, statistically significant only in PVN ($p = 0.015$). Interestingly, hyperosmotic activation did not increase the nNOS mRNA expression of high dose in any nuclei. Among hyperosmotic groups, the low and high doses produced decreased nNOS mRNA expression compared to hyperosmotic control, significant in PVN ($p < 0.001$ *vs* low and high doses) and in SON ($p = 0.01$ *vs* low dose; $p = 0.048$ *vs* high dose). Moreover, also in hyperosmotic groups of PVN, the nNOS mRNA expression in the high dose was significantly lower compared to the low dose ($p = 0.004$). Once more, the low and high dose groups did not show the expected physiological mRNA expression increase as the control group.

4. Discussion

In the present study, we demonstrated that perinatal exposure to a commercial octaBDE mixture, the DE-79, disrupts both the AVP and nNOS systems in the hypothalamic PVN and SON in adulthood. These disruptions affect the regulation of electrolyte and bodily fluids balance, thus representing an adverse challenge for maintaining homeostasis.

The adverse effects produced by brominated flame retardants are related to the congeners exposed to, latency from exposure and age at exposure (Diamanti-Kandarakis et al., 2009). It has been established that PBDEs promote endocrine disruption when the exposure is during the early stages of development more than if the exposure is during adulthood (Chen et al., 2014; Coburn et al., 2015; Eriksson et al., 2002; Kodavanti, 2005; León-Olea et al., 2014). The DE-79 exposure in this study was carried out perinatally (GD 6-PND 21) and testing was conducted at 3 months of age. In our litter data, we observed a higher mortality rate of DE-79-treated animals compared to controls (until 1 month of age; Fig. S2) therefore we are analyzing the less affected surviving offspring. Despite this, our results show that adult rats exposed perinatally to DE-79 present dysregulation of AVP system under

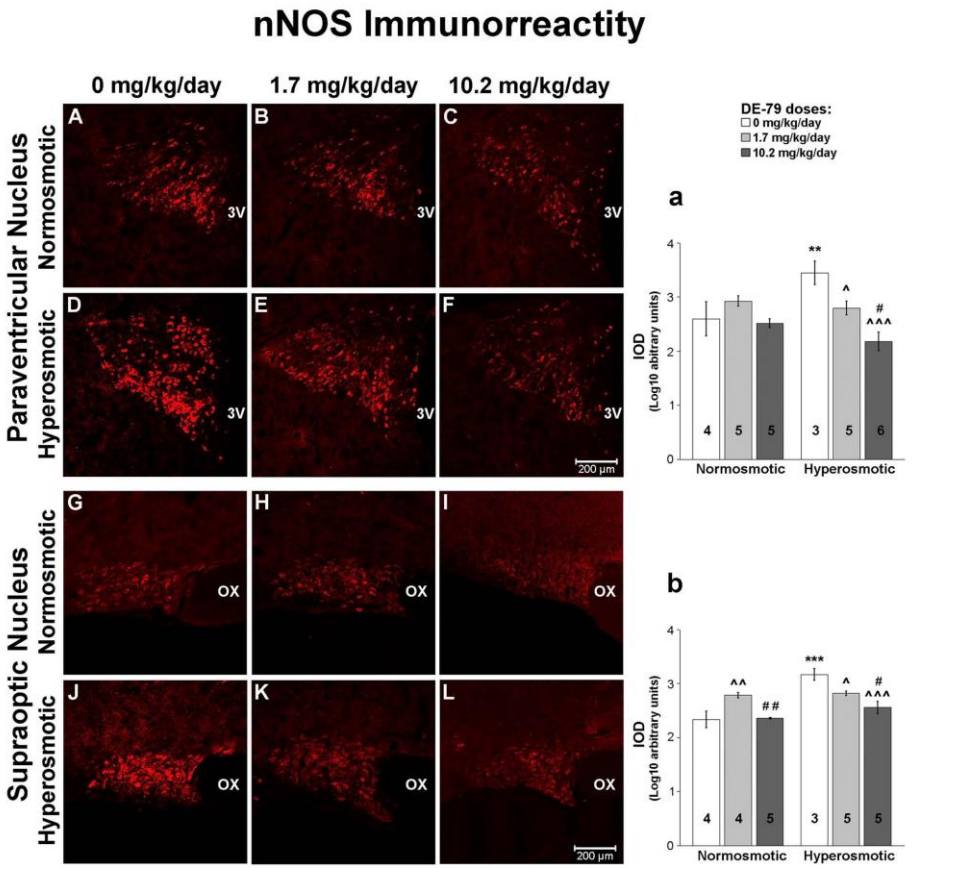

Fig. 5. Effects of perinatal exposure to DE-79 on nNOS immunoreactivity (nNOS-IR) in 3-month-old male rats. Left: Panel of representative confocal images of coronal sections of PVN (top; images A to F) and SON (down; images G to L) with nNOS-IR. DE-79 doses 0 (control; A, D, G, J); 1.7 (low; B, E, H, K) or 10.2 mg/kg/day (high; C, F, I, L); normosmotic (A, B, C, G, H, I) or hyperosmotic rats (salt-loaded; D, E, F, J, K, L).There was a physiological increase in nNOS-IR in hyperosmotic control rats (D, J) compared to normosmotic control groups (A, G). In contrast, there was no physiological nNOS-IR increase of hyperosmotic rats of low and high doses compared to its normosmotic counterparts. Right: Graphs represent the effects of perinatally DE-79 exposure on nNOS-IR integrated optical density (IOD) data in PVN (a) and SON (b) of male adult rats. Bars represent mean values ± s.e.m. of log-transformed data and the numbers inside them denote sample size (number of rats, from which 6 bilateral cuts, on average, per rat where analyzed). Symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak *post-hoc* test. (*) hyperosmotic compared to normosmotic same dose; (^) low/high dose compared to control same group (normo−/hyperosmotic); (#) high dose compared to low dose same group (normo−/hyperosmotic); ^, # = $p \leq 0.05$; ^^, ##,** = $p \leq 0.01$; ***,^^^ = $p \leq 0.001$. Abbreviations: third ventricle (3 V); optic chiasm (OX). Bar = 200 µm.

normosmotic conditions and they are not capable of mounting the physiological response to a hyperosmotic stimulus.

We do not know the reason of the increased mortality but it could be due to multiple causes, from mother's effects, to offspring endocrine disruption like thyroid system that is essential for development and other hormones, including the AVP that is also involved in early stages of development (Boer, 1985; Ji et al., 2019; Kodavanti and Curras-Collazo, 2010; Moog et al., 2017; Zelena et al., 2009).

4.1. Perinatal DE-79 exposure affects adult osmoregulation

Perinatal exposure to DE-79, low and high doses, resulted in higher serum osmolality values under normosmotic conditions, compared to control. As expected, the hyperosmotic challenge (salt loading) resulted in a physiological rise in serum osmolality of about 30 mOsm/kg (9.8%) of the control groups (Table 2). However, hyperosmotic low and high dose groups showed even higher serum osmolality compared to hyperosmotic control. Although these differences were not statistically significant, they are biologically significant since, under physiological conditions, small increases of as little as 3 mOsm/kg (about 1%) are sufficient to provoke measurable increases in the concentration of AVP in serum, and to enhance water reabsorption in the kidney, as well as the sensation of thirst (Baylis, 1987; Verbalis, 2013). In this study, the effects of perinatal exposure to DE-79 on osmoregulation were similar to those we have reported with exposure to both PCBs and pentaBDEs (Coburn et al., 2015; Mucio et al., 2017; Shah et al., 2011).

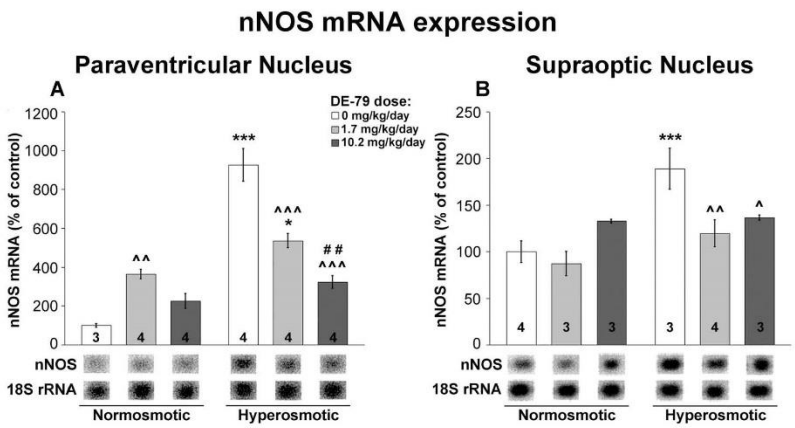

Fig. 6. Effects of perinatal exposure to DE-79 on nNOS mRNA expression by semiquantitative endpoint PCR in 3-month-old male rats. Analyses were conducted in PVN (A) and SON (B) micropunches obtained from adult rats perinatally exposed to DE-79 on doses 0 (control), 1.7 (low) or 10.2 (high) mg/kg/day and normosmotic or hyperosmotically stimulation. We observed a physiological increase in nNOS mRNA expression in hyperosmotic rats compared to normosmotic ones in the control group of PVN and SON. In contrast, there was no marked increase in hyperosmotic rats of low and high doses. Bars are the mean values ± s.e.m. expressed as percent of normosmotic control (= 100%), and the numbers inside them denote sample size. Below each bar is one example of the electrophoresis bands from nNOS and 18S rRNA. A statistical analysis of densitometry (optical density) for nNOS mRNA normalized to the 18S rRNA reference gene was performed. Symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak *post-hoc* test. (*) hyperosmotic compared to normosmotic same dose; (^) low/high dose compared to control same group (normo−/hyperosmotic); (#) high dose compared to low dose same group (normo−/hyperosmotic); *, ^ − $p \leq 0.05$; ^^, # # − $p \leq 0.01$; ***, ^^^ = $p \leq 0.001$.

The alterations on osmoregulation suggest dysregulation of the AVP system and so we analyzed the AVP-IR and mRNA expression of hypothalamic AVP producing nuclei as well as systemic AVP release.

4.2. Perinatal DE-79 exposure affects adult AVP regulation

We found, as others reported (Dai and Yao, 1995; Johnson et al., 2015; Landgraf et al., 1988; Mucio-Ramírez et al., 2017), that concomitant with the physiological rise in serum osmolality under hyperosmotic challenge, the control group showed a marked increase of AVP-IR (Fig. 2) and AVP mRNA expression (Fig. 3) in hypothalamic PVN and SON in addition to the increase of circulating AVP levels (Fig. 4).

Among normosmotic groups, the low dose exhibited a significant increase in AVP-IR in both nuclei compared to control group (Fig. 2B, H, a, b). This increase was in the range of the hyperosmotic control group, so it would appear that low dose acted as a stressor similar to hyperosmotic stimulation under normosmotic conditions. However, with the hyperosmotic stimulus there were no added changes in AVP-IR (Fig. 2B, E, H, K, a, b). Among the normosmotic groups, the high dose group, showed the lower density levels of AVR-IR and the response to hyperosmotic challenge was blunted (Fig. 2C, F, I, L, a, b). These responses are similar to the ones obtained on adult rats with perinatal exposure to a commercial PCB mixture or pentaBDE mixture (30 mg/kg/day of Aroclor 1254 and DE-71, respectively; Mucio-Ramírez et al., 2017) where both pollutants presented a blunted response to hyperosmotic stimulation. To determine if the abnormalities in AVP-IR of PBDE-treated animals may be due to dysregulation of AVP synthesis, we studied the AVP mRNA expression.

Concerning the AVP mRNA expression of the low dose groups presented a similar tendency as the control groups, increasing significantly with the hyperosmotic challenge compared to its normosmotic counterpart (Fig. 3A). The high dose showed an elevated AVP mRNA expression under normosmotic conditions, compared to the control, but presented a clear blockage to the hyperosmotic response (Fig. 3A, B). This suggest that perinatal exposure acts as stressor under normosmotic conditions but the system is unable to respond to a second stimulus such as hyperosmotic challenge. The high dose effects are different to the ones found on adult rats with perinatal Aroclor 1254 exposure (30 mg/kg/day), where treated rats in normosmotic conditions did not show an increase in mRNA expression. However, they also presented a blockage of the physiological response to the hyperosmotic challenge (Mucio-Ramírez et al., 2017). Neither of the two treatments (Aroclor 1254 or DE-79 high dose) presented an adequate physiological response to the hyperosmotic stimulus, suggesting a common target and possibly similar adverse outcome pathway for PCBs and PBDEs.

Regarding the systemic AVP release (Fig. 4), normosmotic low and high dose animals displayed a statistically significant increase in serum AVP compared to control, which suggests that the AVPergic system is overworking. Then, after the hyperosmotic stimulus, both doses were not different compared to their respective normosmotic groups, meaning the system was not capable of responding the same way as the hyperosmotic control group. The high osmolality values found in hyperosmotic exposed groups may be due to the restricted systemic AVP release in these animals. The systemic release findings contrast to that described by Coburn et al. (2005), where hyperosmotic challenge increased systemic AVP release in PCB-fed rats relative to control rats. These discrepancies may be due to differences in experimental design such as 1) their acute (intraperitoneally 3.5 M NaCl) *versus* our chronic hyperosmotic stimulus in exposed animals, where the AVPergic system could respond after an acute challenge but not after a prolonged stimulus as salt loading; 2) adult PCB exposure (30 mg/kg/day Aroclor 1254 for 15 days during adulthood) *versus* perinatal DE-79 exposure, where the developing organisms are more susceptible to toxic disruption (Coburn et al., 2015; Kodavanti, 2005).

Our results showed that the magnitude of the effects depends on the DE-79 dose. Under normosmotic conditions, the low dose showed high AVP-IR in spite of the high AVP serum levels and of maintaining the normal AVP mRNA expression, the reason of the latter is not clear, but in our study normal mRNA expression apparently is enough to increase the AVP stores and systemic release. Meanwhile the high dose

presented an increased AVP mRNA expression and systemic release at the expense of diminished stores. Hence, both doses produced alterations from normosmotic conditions, acting as stressors. Nevertheless, after a hyperosmotic stimulus the low dose groups were still able to respond, albeit minimally, increasing the mRNA expression, therefore maintaining the high levels of serum AVP and AVP-IR, but without the expected physiological increase compared to their respective normosmotic groups. Conversely, the high dose was not able to respond to additional stressors like the hyperosmotic challenge, the mRNA expression was not increased probably due to system exhaustion and the AVP-IR was depleted perhaps due to avoid the drop of the serum AVP levels, in spite of the latter this group presented the highest osmolality levels. These results maybe are explained by a disruption in others AVPergic system regulators, one of them is the NO system. Consequently, we observed the nNOS-IR and mRNA expression.

4.3. Perinatal exposure to DE-79 compromises the physiological activation of nNOS during hyperosmotic challenge

In this study, we observed, as others that hyperosmotic challenge produced an increase in nNOS immunoreactivity (nNOS-IR) and nNOS mRNA expression of control groups in hypothalamic PVN and SON (Kadowaki et al., 1994; Ueta et al., 1995; Villar et al., 1994). Also, we reported here a disruption of the hyperosmotic response by perinatal exposure to DE-79 low or high doses in both, nNOS-IR and -mRNA expression (Figs. 5 and 6, respectively).

The nNOS-IR results in hypothalamic nuclei (Fig. 5) are consistent with the AVP-IR results, where under normosmotic conditions the low dose groups showed increased levels (significant only in SON), and the high dose groups displayed no differences than control; then, the treated groups presented a blunted response to the hyperosmotic challenge. This also parallels our previous findings with PCBs (Coburn et al., 2015) where SON nicotinamide adenine dinucleotide diaphorase (NAPDH-d; assessing NOS activity) was disturbed in hyperosmotic young and late adult rats (3–5 and 14–16-month-old, respectively) prenatally exposed to Aroclor 1254 (30 mg/kg/day from 10 to 19 gestation day). Coburn et al. (2015) observed a decrease in NADPH-d staining intensity of hyperosmotic PCB-exposed animals as compared to hyperosmotic control. Furthermore, the decrease in hyperosmotic exposed groups was similar to the hyperosmotic rats with acute NOS blockers (by injection with NG-nitro-L-arginine-methyl ester; L-NAME; Coburn et al., 2015; Rees et al., 1990). Previous research has demonstrated that NOS is a target for PCBs and PBDEs, and in some cases, like hyperosmotic stress, can act like NOS inhibitors (Coburn et al., 2015; Currás-Collazo, 2011; León-Olea et al., 2005; Sharma and Kodavanti, 2002).

The analysis of the nNOS mRNA expression in hypothalamic PVN and SON (Fig. 6), showed that the normosmotic PBDE-exposed groups increased nNOS expression compared to control but was significant only for the low dose group in PVN. Also, all DE-79 exposed groups had diminished responses to hyperosmotic challenge, more evidently in SON. These results confirm that perinatal exposure to DE-79 attenuates the nNOS response after a stressful situation such as hyperosmolality.

4.4. Adult nNOS disruption as a possible mechanism of AVP dysregulation after perinatal exposure to DE-79

There is controversy about the precise role of NO in the AVPergic system. The most consistent results regarding its role suggests that NO increases release of somatodendritic AVP, which restrains systemic AVP release *via* autoreceptors, thus making NO a relevant modulator of magnocellular neuroendocrine function during conditions of high hormonal demand (Gillard et al., 2007; Kadekaro et al., 2006; Reid, 1994; Stern and Zhang, 2005). Conversely other studies showed that nNOS inhibitors (i.c.v. injection) attenuated the basal release of AVP and suppresses the AVP responses to hypotension induced by sodium nitroprusside (i.v.; Cao et al., 1996). Similarly, the S-nitroso-*N*-acetylpenicillamine (NO precursor) caused an increase in the plasma AVP concentration (Ota et al., 1993). This controversy is most likely related to differences in physiological stimulation, experimental designs, administration route, dose and time responses to the diverse NO agonists and antagonists used.

The effect of perinatal DE-79 exposure in nNOS-IR and -mRNA expression was like that observed in AVP-IR and -mRNA expression, where the adult system perinatally exposed is unable to mount the appropriate response to hyperosmotic stimulation. DE-79 exposure produces effects combining the actions of multiple PBDE congeners, that act like PCBs on the PKC, which facilitates NO production by NOS phosphorylation and on the associated calcium-binding protein calmodulin, a required protein for calcium-dependent as well as NO dependent signaling and somatodendritic release of AVP (Currás-Collazo, 2011; Kodavanti and Curras-Collazo, 2010; Kodavanti and Ward, 2005). AVP somatodendritic release is a modulator of AVP systemic release, acting as a powerful feedback signal by which magnocellular neurosecretory cells autoregulate their own activity (Gillard et al., 2007; Gouzènes et al., 1998; Hanoune, 2009; Ludwig and Leng, 2006); in this matter, studies confirm the somatodendritic AVP release as directly affected by PBDEs and PCBs, more evidently after a hyperosmotic stimulus (Coburn et al., 2005, 2007). The increase in systemic AVP release in response to early changes in osmolality occurs from the beginning of salt loading, however the exposed animals, mainly the high dose group, were not able to maintain the response to a chronic osmotic challenge as evidenced by their inability to maintain the nuclei reserves (AVP-IR), mRNA expression, and release of AVP, thus leading to high osmolality levels at the end of the test. These alterations could be explained by a fault in regulation of AVP release by disruption of NO.

Further work needs to be done to elucidate other mechanisms and sites in which AVPergic system could be affected by DE-79, such as neurohypophysis and kidney. It is known that the kidney is a target for PBDEs (Albina et al., 2010; Milovanovic et al., 2018), these toxics interfere with mechanisms involved in renal water absorption *via* aquaporin 1 water channels, which are regulated by V2-AVP receptors. Also, NO is involved in renal blood flow and glomerular filtration rate (Ma et al., 1998; Mattson et al., 1994; Tewari et al., 2009).

Besides, AVP is modulated by different neuropeptides and neurotransmitters that might be PBDEs targets, hence they could be other mechanisms of AVPergic system dysfunction, such as: angiotensin II which is coactivated with AVP as a part of the renin-angiotensin-aldosterone system by stimuli like hypovolemia and hyperosmolality, improves the activity of AVP magnocellular neurons and provides an additional regulatory mechanism for maintain blood pressure and body fluid balance; the pituitary adenylate cyclase activating polypeptide, acetylcholine, noradrenaline, dopamine and glutamate that increase in hyperosmolality and in stress situations (*e.g.* hypovolemia, pain, vomiting, nausea) promoting AVP release; atrial natriuretic peptide and brain natriuretic peptide which are activated by hypervolemia and inhibit AVP release (Baylis, 1987; Gillard et al., 2006; Hanoune, 2009; Ohbuchi et al., 2015; Szczepanska-Sadowska et al., 2018). Therefore, new lines of research of DE-79 should also be performed on other AVP regulators, AVP receptor modifications, missinteractions with other hormones and neurotransmitters and epigenetic changes. The latter is especially interesting, since it is known that PBDEs affect epigenetic regulatory mechanisms across multiple biological systems including the neuroendocrine system (Poston and Saha, 2019).

5. Conclusions

In the current study, the results show that perinatal exposure to DE-79, an octabrominated commercial PBDE mixture, promotes chronic neuroendocrine disruption by altering AVP system regulation. These effects were observed under basal conditions and more evident when the system was challenged by a chronic hyperosmotic stimulus. nNOS

was also affected in the same nuclei. Since NO is a regulator of AVP, we propose NO disturbance, by perinatal DE-79 exposure, as one of the mechanisms of AVPergic system disruption causing an imbalance in osmoregulation.

The DE-79 effects were sublethal causing homeostatic dysregulation, therefore the organisms were not prepared to respond to physiological challenges. AVP is involved in other systems, participating in cognitive functions, learning and social behavior regulation (Bowers et al., 2015; Ji et al., 2019; Shou et al., 2017), as well as the NO, which is implicated in almost every biological system (Chachlaki and Prevot, 2019). Thus, continued efforts are needed to better understand the disruption of other AVP and NO functions by the PBDEs. And because the high body burdens of this toxics in children and its high existent levels in house dust (Drobná et al., 2019; Hudson-Hanley et al., 2018; Johnson-Restrepo and Kannan, 2009; Liang et al., 2019; Stapleton and Dodder, 2008) must be addressed as a public health issue.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2020.114914.

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Author contributions were as follow:

Mhar Y. Alvarez-Gonzalez: PhD scholarship, perform the experiments, writing original draft, formal analysis, review and editing. Eduardo Sánchez-Islas: perform and validation the experiments and formal analysis. Samuel Mucio-Ramirez: perform the experiments, implementation of computer program, formal analysis, writing, review and editing. Patricia de Gortari: methodology and final review. María I. Amaya: methodology. Prasada Rao S. Kodavanti: provided some resources, review and editing. Martha León-Olea: Conceptualization, methodology, writing, review and editing, supervision, funding acquisition and project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Perinatal exposure to organohalogen pollutants decreases vasopressin content and its mRNA expression in magnocellular neuroendocrine cells activated by osmotic stress in adult rats

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ABSTRACT

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are environmental pollutants that produce neurotoxicity and neuroendocrine disruption. They affect the vasopressinergic system but their disruptive mechanisms are not well understood. Our group reported that rats perinatally exposed to Aroclor-1254 (A1254) and DE-71 (commercial mixtures of PCBs and PBDEs) decrease somatodendritic vasopressin (AVP) release while increasing plasma AVP responses to osmotic activation, potentially emptying AVP reserves required for body-water balance. The aim of this research was to evaluate the effects of perinatal exposure to A1254 or DE-71 (30 mg kg/day) on AVP transcription and protein content in the paraventricular and supraoptic hypothalamic nuclei, of male and female rats, by in situ hybridization and immunohistochemistry. cFOS mRNA expression was evaluated in order to determine neuroendocrine cells activation due to osmotic stimulation. Animal groups were: vehicle (control); exposed to either A1254 or DE-71; both, control and exposed, subjected to osmotic challenge. The results confirmed a physiological increase in AVP-immunoreactivity (AVP-IR) and gene expression in response to osmotic challenge as reported elsewhere. In contrast, the exposed groups did not show this response to osmotic activation, they showed significant reduction in AVP-IR neurons, and AVP mRNA expression as compared to the hyperosmotic controls. cFOS mRNA expression increased in A1254 dehydrated groups, suggesting that the AVP-IR decrease was not due to a lack of the response to the osmotic activation. Therefore, A1254 may interfere with the activation of AVP mRNA transcript levels and protein, causing a central dysfunction of vasopressinergic system.

1. Introduction

The maintenance of water homeostasis is vital for living beings. The neuropeptides secreted by the hypothalamic-neurohypophysial system, vasopressin (AVP) and oxytocin (OXT), play key roles in homeostasis and fine-tuning of osmotic regulation (Bourque, 2008; Schrier and Martin, 1998; Weitzman and Kleeman, 1979). These neuropeptides exert a wide range of actions and control not only upon water and sodium excretion by the kidneys, they also influence cardiovascular

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responses, learning and memory, thermoregulation, social, sexual, reproductive behavior and stress-related responses, among others (Cunningham and Sawchenko, 1991; Pittman and Bagdan, 1992; Riphagen and Pittman, 1986; Neumann and Landgraf, 2012; Witt, 1995). Osmotic and volume stimuli, such as dehydration and hemorrhage, activate magnocellular neuroendocrine cells (MNCs) in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus. In response to this activation, MNCs produce both AVP and OXT that are released into the systemic circulation from axon terminals located in the neurohypophysis and within the PVN and the SON from soma and dendrites (somatodendritic release) (Hatton et al., 1978; Ludwig and Leng, 1998; Ludwig et al., 1994). Somatodendritic AVP exerts autoinhibitory control over plasma AVP hormonal output. Therefore, the central and peripheral AVP are essential to maintain osmoregulation (Ludwig and Leng, 1998; Riphagen and Pittman, 1986; Wotjak et al., 1994).

It has been well established that a wide variety of environmental pollutants have specific effects on the neuroendocrine systems of mammals, therefore, the concept of neuroendocrine disruptors was developed (Leon-Olea et al., 2014; Currás-Collazo, 2011; Waye and Trudeau, 2011). Industrial organohalogens such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), widely used as flame retardants, are neurotoxic agents and neuroendocrine disruptors that pollute outdoor and indoor environments (de Wit et al., 2012; Birnbaum and Staskal, 2004). They are lipophilic and bioaccumulate in wildlife and humans (de Wit, 2002; Kodavanti et al., 1998; Tilson et al., 1998). After two accidental contamination incidents that lead to death and several adverse health effects in humans, PCBs were banned from production in 1977 but still linger in human tissues and the environment (Lang, 1992; Mariussen and Fonnum, 2006; Safe, 1993; Wu et al., 2011). PBDEs are similar in structure to PCBs but the bromide radical substitutes for chloride (Covaci et al., 2011; Wiseman et al., 2011). The current commercial formulation of PBDEs is decaBDE (10 bromines). PentaBDE and octaBDE (5, 8 bromines respectively) were voluntarily withdrawn from the market in 2004 (U.S. Environmental Protection Agency, USEPA, 2010). These pollutants, as the PCBs, are added to household appliances, furniture, textiles, plastics, electronic devices and packaging materials, thus it is a major source of the indoor pollution that affects humans, especially infants and toddlers (Birnbaum and Staskal, 2004; de Wit et al., 2012). PCBs and PBDEs have been detected in human blood, adipose tissue, and they cross through the placental barrier and in to breast milk (Jacobson et al., 1984; Kodavanti et al., 1998; Takagi et al., 1986). They disrupt brain development, learning and memory, and the reproductive and endocrine systems via alterations in thyroid, growth, and reproductive hormones (Chung et al., 2001; Hamers et al., 2006; Kodavanti et al., 2010; Kodavanti and Curras-Collazo, 2010; Ness et al., 1993; Steinberg et al., 2007; Viberg et al., 2006). The effects of these organohalogens depend on factors such as dosage, sex, susceptibility of each organism, type of congener, duration of exposure, physiological activation, and life stage at which the organism is exposed (Faroon et al., 2001; McKinney and Waller, 1994; Viberg et al., 2006). PCBs and PBDEs produce neurotoxicity by disrupting protein kinase C (PKC) and perturbing intracellular calcium homeostasis, nitric oxide signaling, neurotransmitter release, synaptic plasticity, gene expression, oxidative stress, and energy metabolism in the brain (Coburn et al., 2008, 2015; Kodavanti et al., 2011; Royland and Kodavanti, 2008; Tilson et al., 1998; Westerink, 2014; Wong et al., 1997).

Organohalogens also disrupt the vasopressinergic system (Coburn et al., 2007; Coburn et al., 2005; Kodavanti and Curras-Collazo, 2010). We have previously reported that exposure to Aroclor 1254 (A1254) and DE-71, commercial mixtures of PCBs and PBDEs respectively, increases plasma vasopressin release, and decrease somatodendritic AVP release during hyperosmotic stimulation in adult rats, suggesting that PCBs and PBDEs could disrupt signaling mechanisms required for AVP release in response to an osmotic stimulus (Coburn et al., 2007; Coburn et al., 2005). Moreover, perinatal exposure to PBDEs alters AVP-related functions, producing exaggerated cardiovascular reactivity and compromised osmoregulatory capacity in late-adulthood rats (Shah et al., 2011). The mechanisms by which PCBs and PBDEs disrupt the vasopressinergic system remain unknown. Based on our previous report showing elevated plasma and reduced somatodendritic vasopressin in response to these pollutants, we hypothesize that these organohalogens may disrupt the AVP transcription and/or protein content in the PVN and SON cells populations in rats with prolonged or acute hyperosmotic challenge. Therefore, the aim of this research was to elucidate the effects of perinatal exposure to either A1254 or DE-71 (30 mg kg/day) on AVP gene and protein responses to osmotic stimulation in the rat PVN and SON hypothalamic nuclei.

2. Materials and methods

2.1. Animals

The experimental animals were maintained under controlled laboratory conditions with a light-dark (12:12 h) cycle and ad libitum access to water and a regular diet (commercial pellets Lab Chow 5001 Purina USA). A group of time-pregnant Wistar rats on gestational day (GD) two (based on a GD 0, as the day of insemination, indicated by copulatory plug) was obtained from the animal care facility of INPRFM, México (cohort I and cohort III). In parallel, a group of timed-pregnant Sprague-Dawley (SD) rats (cohort II), a subspecies of Wistar with similar physiology and behavior (Andersen and Tufik, 2016), was reared at the animal care facility of the University of California, Riverside (UCR). The experiments were performed in accordance with NIH guidelines for care and use of laboratory animals and with the approval of the Projects and Ethics Committee of the INPRFM and the IACUC of the UCR.

2.2. Chemicals

Commercial PCB mixture, Aroclor 1254 (purity > 99%; Lot: 124-191-B), was obtained from Accustandard, New Haven, CT. The constituent PCB congeners (pg/ng) based on ortho-substitutions in this PCB mixture were: non-ortho 0.02%; mono-ortho 24.1%; di-ortho 53.8%; tri-ortho 21.2%; tetra-ortho 0.85% (Kodavanti et al., 2001). Commercial PBDE mixture, DE-71 (technical pentabromodiphenyl oxide; CAS no. 32534-81-9; Lot 2550OA30A) was obtained from Great Lakes Chemical Corporation, West Lafayette, IN. The composition of individual congeners in DE-71 was: PBDE-47 (36%); PBDE-99 (42%); PBDE-100 (10%); PBDE-153 (3%); PBDE-154 (4%); and PBDE-85 (2%). The remaining 3% consists of several identified tri-heptaBDEs and some unidentified PBDEs (Dunnick et al., 2012).

2.3. Perinatal exposure to A1254 and DE-71

Perinatal exposure was accomplished by feeding pregnant dams (n = 25, Wistar and n = 8, SD) with snacks (popcorn or Cheetos) infused with A1254 (30 mg/kg/day) dissolved in corn oil vehicle or infused with vehicle only (controls), for 10 days during gestation (GD 10-19) (cohort I, II). Although A1254 was administered prenatally, the offspring was still exposed via breast milk. Several studies have shown that PCBs have a longer half-life (Tanabe et al., 1981) and slowly decrease in breast milk throughout lactation (LaKind et al., 2009; Takagi et al., 1986). Due to this, A1254 exposure was considered as perinatal exposure. Cohort III: Wistar pregnant dams (n = 10), received snacks dosed with the commercial penta-PBDE mixture DE-71 (30 mg/kg/d) dissolved in corn oil vehicle or with vehicle only (control), from GD 6 to postnatal day (PND) 21. The volume of each dosing mixture in corn oil was adjusted based on changes in the dam's weight. The pups were weaned and separated by sex at PND-22 and were allowed to mature until 3–5 months old.

The dosing and experimental paradigm used in this study match the experimental conditions in our previous reports (Coburn et al., 2015; Kodavanti et al., 2010; Shah et al., 2011). Kodavanti et al. (1998) where we showed that adult rats dosed orally with A1254 (30 mg/kg/day for 20 days), have brain tissue concentrations of 8.2–15.1 ppm or 20–50 μM. Such tissue concentrations have been shown to disrupt neuronal signaling without producing overt cell death (Kodavanti and Tilson, 2000). For reference, brain PCB levels of up to 29.5 ppm (w/w) and until 80 ppm (fat weight basis) have been found in wildlife exhibiting overt neurological symptoms (Gabrielsen et al., 1995; Skaare et al., 2000; Kodavanti, 2005). For PBDEs in a regimen of 50 ppm in food pellets from gestational day 8 to postnatal day 14, brain tissue concentration was 1.2 μg/g weight tissue (Rickert et al., 1978). We used this high dose and the same lot numbers of A1254 or DE-71 that were proven to produce neurotoxicity in previous studies (Kodavanti et al., 2010; 2001; Kodavanti and Ward, 2005). Delivery of toxicants via dietary snacks has consistently proven to be an effective method of oral exposure in our lab and others using different snacks or mixed in pellets (Coburn et al., 2015; Coburn et al., 2005; Kodavanti et al., 2010; Rickert et al., 1978). A1254 at 30 mg/kg/day of exposure could reduce litter size (Brezner et al., 1984). Therefore, at PND 4 the offspring number was adjusted to eight pups per dam. The pup ratio was 60% females and 40% males (4–5 females/l).

At 3–5 months of age (250–450 g), the pups of the litters were randomly assigned for the different groups of analysis, so pups of the same litter were included in different groups of analysis, (maximum two female or male pups were chosen) (Table 1). Cohort I, male and female Wistar rats, controls and A1254 exposed, were assigned to three groups of analysis: brain tissues were analyzed for immunofluorescence for AVP (n = 15 males, 13 females); endpoint RT-PCR analysis of cFOS, (n = 12 males, 12 females), and in situ hybridization for AVP (n = 14 males, 20 females). All groups included four treatment conditions: euhydrated rats (Control) and exposed to A1254; salt-loaded control rats (Hyper) and exposed (Hyper + A1254). Special care was taken to make sure that all female rats in the different groups were in the same phase of the estrous cycle. Cohort II, adult male SD rats (n = 18) were processed with immunoperoxidase to stain AVP as previous report (Khan et al., 2000). This group included four treatment conditions: euhydrated rats (Control) and exposed to A1254; with acute hyperosmotic challenge control (Hyper) and exposed (Hyper + A1254) rats. Cohort III, Wistar male rats (n = 15), were processed for immunofluorescence to stain AVP. This group included rats euhydrated control and exposed to DE-71; salt-loaded control (Hyper) and exposed (Hyper + DE-71), (see Table 1).

2.4. Prolonged osmoregulatory challenge (salt loading)

To determine if perinatal exposure to A1254 or DE-71 alters AVP content and synthesis in the PVN and SON of euhydrated and physiologically activated animals, a subset of A1254 and DE-71-treated or control rats (from each cohort) were exposed to a chronic hyperosmotic challenge. It was carried out by replacing their drinking water with 2% saline solution (20 g NaCl/l; ad libitum access) for 5 days, as previously described (Curras-Collazo and Dao, 1999; Amaya et al., 1999). Rats were weighed before and after the hyperosmotic challenge. They were considered dehydrated when they lost about 10% of their body weight (data not shown). These were labeled as hyperosmotic rats (Hyper). Euhydrated (normosmotic) rats (control and treated) had ad libitum access to tap water.

2.5. Acute osmoregulatory challenge

To determine whether perinatal A1254 exposure had effects on AVP immunoreactivity after acute hyperosmotic challenge, a group of SD male offspring 3–5 months old (n = 18) was used. Male offspring were injected intraperitoneally (ip; 0.6 ml NaCl/100 g b.w.) with either 3.5 M NaCl (Hyperosmotic solution) or 0.154 M NaCl (0.9% NaCl isotonic solution; Control) and animals were sacrificed 4–4.5 h later as described (Ludwig et al., 1994; Coburn et al., 2015). This manipulation has been shown to produce marked stimulation of somatodendritic AVP release (Coburn et al., 2015; Ludwig et al., 1994). Tail blood was collected before sacrifice and plasma osmolality was measured to confirm increased plasma osmolality in Hyper rats of 5.0% or greater.

2.6. Tissue processing

Upon completion of the osmotic challenge, all rats were anesthetized with sodium pentobarbital (63 mg/kg). They were transcardially perfused with a clearing solution (150–200 ml of 0.9% saline containing 2500 IU/500 ml heparin (PISA Farmacéutica Mexicana Mex.) followed by 350–400 ml of 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS (0.1 M phosphate-buffered saline, pH 7.4). Brains were removed and postfixed in the same fixative at 4 °C for 2 h. They were then cryoprotected in 30% sucrose and stored at 4 °C until used. Coronal slices (30 μm) through the hypothalamic PVN and SON (Bregma: −0.80

Table 1
Cohort distribution, rat strains used, dosing pattern pups used, and testing paradigm.

Cohort	Dams/pups (used)	Chemical and doses	Osmotic challenge	Treatment groups	Osmolality	AVP-IF or AVP-IP	AVP-mRNA ISHH	cFOS mRNA RT-PCR
I	Wistar 25/86	A1254 30 mg/kg/day orally GD: 10–19	Euhydrated (Tap water)	Control	8 M, 4 F	15 M,13 F	14 M, 20 F	12 M,12 F
				A1254	8 M, 4 F	Section 2.8, Section 2.11	Section 2.12	Section 2.13
			Prolonged (Salt Loading) Section 2.4	Hyper	10 M, 3 F	Stat: 2-way ANOVA	Stat: 2-way ANOVA	Stat: 2-way ANOVA
				Hyper + A1254	9 M, 4 F			
II	SD 8/18	A1254 30 mg/kg/day orally GD: 10–19	Euhydrated (0.154 M NaCl sol. i.p.)	Control	5 M	18 M		
				A1254	4 M (No data)	Section 2.9		
			Acute (3.5 M NaCl sol. i.p.) Section 2.5	Hyper	4 M	Stat: 1-way ANOVA		
				Hyper + A1254	5 M			
III	Wistar 10/15	DE-71 30 mg/kg/day orally GD: 6 to PND 21	Euhydrated (Tap water)	Control	4 M, 4 F	15 M		
				DE-71	3 M, 7 F	Section 2.8, Section 2.11		
			Prolonged (salt loading) Section 2.4	Hyper	4 M, 3 F	Stat: 1-way ANOVA		
				Hyper + DE-71	4 M, 6 F			

Table shows cohort distribution, strain of rats, dosing pattern, number of offspring used, treatment groups, and testing paradigm for the perinatal exposure to commercial Aroclor 1254 (A1254) or PBDE (DE-71) mixtures (30 mg/kg/day, orally). For A1254, the exposure was from gestational day (GD) 10 to 19. For DE-71, the exposure started at GD 6 and continued through postnatal day (PND) 21. At 3–5 months of age (250–450 g), before sacrifice, some male and female rats from control and A1254 or DE-71 exposed were subjected to a prolonged (NaCl 2% drinking solution during 5 days ad libitum) or acute (ip; 3.5 M NaCl) osmotic challenge. Pups were randomly assigned for the different groups of analysis. For cohort I: vasopressin immunofluorescence (AVP-IF), AVP-mRNA in situ hybridization histochemistry (ISHH) and cFOS-mRNA final point reverse transcription polymerase chain reaction (RT-PCR). For cohort II: AVP immunoperoxidase (AVP-IP). For cohort III: AVP-IF. The paradigms included four treatments groups: euhydrated (Control) and exposed (A1254 or DE-71) rats, salt-loaded control (Hyper) and exposed (Hyper+A1254 or DE-71) rats. All groups included plasma osmolality measurements.

to −2.12 mm) (Paxinos and Watson, 1998) were cut on a sliding-freezing microtome (Leitz, Grand Rapids, MI) and collected in plastic wells containing PBS. Serial sections were placed alternately in six wells containing eight cuts per well approximately. Each well had a set (eight slices) of all representative rostrocaudal PVN and SON. Free-floating sections from three sets were processed for immunohistochemistry.

2.7. Osmolality

Under anesthesia and prior to perfusion, 2 ml cardiac blood samples were collected from each rat and centrifuged at 8000 rpm for 5 min. Osmolality was measured in triplicate with a vapor pressure osmometer (Wescor 5500, Logan, UT). Results for osmolality are reported as mean ± s.e.m. in mOsm/kg units.

2.8. Immunofluorescence for AVP

Immunofluorescence was carried out in Wistar rats from cohorts I and III that included four treatment groups for A1254: Control (n = 4 males, 3 females), A1254, (n = 3 males, 3 females), Hyper (n = 5 males, 4 females), and Hyper + A1254 (n = 3 males, 3 females). For DE-71 (male rats): Control (n = 4), DE-71 (n = 3), Hyper (n = 4), and Hyper + DE-71 (n = 4) (see Table 1). The sections containing anterior, middle, and posterior hypothalamic PVN and SON regions were processed simultaneously for all groups of each A1254 or DE-71 studies. They were incubated in blocking solution containing 5% normal donkey serum, 5% BSA, and 0.3% Triton X-100, for 60 min at room temperature to minimize nonspecific staining. Then, the sections were incubated with vasopressin-neurophysin antibody (PS-41 monoclonal, gifted by H. Gainer, NIH) (Ben-Barak et al., 1985; Whitnall et al., 1985) at a 1:200 dilution in blocking buffer containing 1% teleostean gelatin (Sigma Chemical Co., St. Louis, MO), for 48 h at 4 °C in free flotation with continuous shaking. The sections were then washed for three times for 10 min each in PBS-T (PBS with 0.3% Triton X-100) followed by a 1.5 h incubation with Alexa Fluor 488 donkey anti-mouse secondary antibody (Invitrogen Corp., Carlsbad, CA) at 1:200 dilution in blocking solution, in a humidified chamber at 37 °C. Afterward, the sections were washed (3 × 10 min) in PBS and mounted onto glass slides with an anti-fade mounting medium (ProLong Antifade Kit, Molecular Probes; Eugene, Oregon USA). For several sections obtained from each group, the primary antibody was replaced by blocking solution representing methodological control sections. Staining observed in these sections was used in background correction as part of computer-assisted densitometry (see below). The structures in the immunohistochemical control sections in which the fluorescence was not observed were considered immunoreactive to AVP. The sections were analyzed with a Zeiss 510 META laser scanning confocal microscope, equipped with a 488-nm argon-ion laser (Alexa Fluor 488 dye) attached to an Axiovert 200 M microscope. They were analyzed with a 10× Plan-Neofluar NA = 0.3 and a 20× Plan-Neofluar NA = 0.5 Objective (Carl Zeiss). Before analyzing the sections, we conducted a lambda stack to obtain the emission spectrum of this fluorophore. Barrier filters were placed to obtain only the peak fluorescence emission. Images from each section (30 μm thickness) were acquired and analyzed bilaterally on the optimal focal plane, in single track mode, with the Ar laser/488 nm, pinhole diameter (1 airy unit) and detector gain (1). Laser power was adjusted to provide an optimal dynamic range for the measurements (the same in all slides). Confocal images were converted to an 8-bit TIF format with a Zeiss LSM image browser (v 3.5).

2.9. Immunoperoxidase for AVP

After acute hyperosmotic challenge, animals were fixed as above (4% paraformaldehyde). Coronal brain sections (40 μm) of perinatal A1254 exposed male Sprague-Dawley rats (n = 18), were processed with immunoperoxidase to stain AVP. The technique was carried out as previously reported (Khan et al., 2000). Briefly, brain sections were mounted on gelatin-subbed glass slides. In each experiment control sections received all solutions except the primary antibody (methodological control). Sections on slides were washed in PBS and treated with blocking/permeabilization solution (0.2% gelatin, 0.3% Triton-X, 0.4% BSA) for 30 min. Tissue was incubated with an AVP antibody PS41, dilution 1:100. After wash, the sections were incubated with an anti-mouse secondary antibody (Dako; K4001). AVP immunoreactivity was developed in nickel intensified 3,3′-Diaminobenzidine solution. Sections were dehydrated in an alcohol series and coverslipped using DPX mounting medium (Electron Microscopy Sciences), later analyzed using bright field microscopy and images were taken of PVN and SON (3–7 images per nucleus per rat) using a digital camera (Spot Insight Meyer).

2.10. Image analysis

All images were analyzed for integrated optical density (IOD) using computer-assisted densitometry software (Image Pro Plus 4.5, Media Cybernetics, MD, USA). Images were converted to a gray scale (0–255) and their background was subtracted. Subsequently, the IOD of the immunoreactivity was quantified for the area of interest. The PVN and the SON areas were manually outlined in at least six sections per nucleus per rat. Integrated density values (density × area) were determined per section for each nucleus. The IOD was reported as arbitrary units. Average IOD was obtained for each regions of interest (bilateral PVN and bilateral SON) by pooling IOD values of rats in respective experimental groups.

2.11. Cell count analysis of AVP-IR neurons

To determine if A1254 or DE-71 exposure affected the number of vasopressinergic neurons or only the intensity of AVP immunoreactivity (measured with an IOD analysis), we counted the AVP immunoreactive (AVP-IR) neurons in PVN and SON for all male groups (cohort I, III). We selected 3–5 immunofluorescent micrographs, of PVN and SON—only the medial portions—for each animal in each group, for IOD quantification (Bregma: −1.80 mm to −1.88 mm for PVN and −1.30 mm to −1.40 mm for SON) (Paxinos and Watson, 1998). Neurons with evident nuclei and AVP immunoreactivity were considered. To avoid counting the same neurons more than once, we overlaid a grid with square sections (24.5 × 24.5 μm) on the images and counted the cells that were marked with a X. For this analysis, we used Image-J software with the plugin analyzer-cell counter (v 1.44p, National Institute of Health, available at http://imagej.nih.gov/ij).

2.11.1. Cell count analysis of Nissl staining. In addition to analyze the possible changes in the overall cell density we counted the total number of cells in the above-mentioned medial portions of the PVN and the SON in Nissl-stained sections of normosmotic control and A1254- and DE-71-exposed adult male rats. The brains of four control and three A1254- or DE-71-exposed rats were fixed and collected as previously described. For Nissl staining, coronal brain slices (30 μm thick) were mounted on gelatin-coated slides and dried overnight. The sections were immersed in a solution (100 ml) containing cresyl-violet (0.5%), sodium acetate (2.7%), and glacial acetic acid (0.92%), for 2 min. Then, sections were dehydrated through a series of graded ethyl alcohols (70%, 80%, 96%, each for two minutes; 100% twice for two minutes), cleared in xylene twice (5 min), and cover-slipped with Entellan resin (Merck, Germany). Cells were counted with the aforementioned procedures.

2.12. In situ hybridization histochemistry (ISHH)

We conducted in situ hybridization to determine the anatomical distribution of AVP mRNA and to determine the effect of perinatal

exposure to A1254 on the synthesis of AVP in euhydration or in response to hyperosmotic activation. Male and female rats perinatally exposed to A1254 or vehicle, were allowed to mature to 3 months of age, and randomly assigned to four experimental groups: 1) control (n = 4 males, 5 females), 2) A1254 (n = 3 males, 5 females), 3) Hyper (n = 3 males, 4 females) and 4) Hyper + A1254 (n = 4 males, 6 females). Both control and exposed rats were randomly selected to be dehydrated. The fixed tissue (4% paraformaldehyde) was processed in the same way as for immunohistochemistry (see above), except that materials and solutions were RNAse free. A series of 18-μm thick coronal sections were cut through the rostrocaudal extent of the PVN and the SON on a cryostat (Microm HM525, GmbH, Germany). Every fourth section cut through the PVN or the SON was collected and mounted onto Superfrost/Plus glass slides to obtain four sets for each PVN and SON. Sections were desiccated overnight at 42 °C and stored at −80 °C until processed for in situ hybridization histochemistry.

2.12.1. AVP probe for ISHH. The 200 bp-DNA fragment containing AVP was amplified by PCR using male or female rat cDNA from the PVN as a template. Two oligonucleotides were chosen using the Primer3 (v. 0.4.0) software [http://bioinfo.ut.ee/primer3-0.4.0/primer3/] with an introduction of a restriction enzyme site for posterior steps of subcloning. Sense SHindIII 5′- TCA GTC **AAG CTT** CAC CTC TGC CTG CTA CTT C -3′, which introduced a *Hind*III restriction site (bold), and Antisense *Eco*RI 5′- TAC TAT **GAA TTC** GGG GTA CAG GTT CTC CTC C -3′, which introduced an *Eco*RI site (bold). The PCR conditions were 27 cycles at 94 °C - 1 min, 64 °C − 1 min, 72 °C − 1.15 min, and a final cycle at 72 °C − 10 min.

The resulting PCR product was extracted and purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). The purified fragment was cloned into the pJET1.2/blunt vector from CloneJET™ PCR Cloning Kit (Fermentas) as specified by the manufacturer, with the ligation reaction at 22 °C for 20 min. The ligation mixture was electro-transformed using 50 μl of electrocompetent DH5α cells and was incubated at 37 °C for 1 h. Subsequently, the electroporated cells were poured into Petri dishes containing 2xYT medium and ampicillin (200 mg/ml final concentration) and were incubated at 37 °C for 12 h.

To select positive colonies containing the vector and vasopressin fragment, we performed a colony PCR using SHindIII and AsEcoRI and the above-described PCR conditions. Plasmids of selected colonies were isolated according to a standard alkaline lysis protocol, and single-pass sequencing of the 5′-termini was conducted with the pJET1.2 Forward Sequencing Primer (5′-CGA CTC ACT ATA GGG AGA GCG GC-3′) and an automated analyzer (Model 3100, Applied Biosystems, Foster City, CA), as specified by the manufacturer.

The pJET plasmid containing vasopressin was digested with the *Hind*III and *Eco*RI restriction enzymes, and the released fragment was extracted from agarose gel and subsequently purified using the High Pure PCR Product Purification Kit. The purified fragment was ligated into a pSPT18 vector (Roche, Cat. 10,999,644,001), previously digested with *Hind*III and *Eco*RI enzymes, using T4 DNA ligase, as per the instructions of the manufacturer. The ligation was electroporated into electrocompetent DH5α cells, and plasma DNA from four colonies was sequenced using SHindIII and AsEcoRI to confirm the presence of the insert containing AVP. Transcripts obtained from the four clones gave the expected mRNA distribution pattern for AVP in the CNS when used as a probe for ISHH (not shown).

2.12.2. ISHH technique. Every fourth section of the PVN and the SON was hybridized with a 200-bp single-stranded α [^{35}S] UTP-labeled RNA probe, complementary to the coding region of the rat AVP gene (47 to 247 nucleotides of the cDNA; GenBank: M25646.1). Hybridization was performed as previously described (Sanchez et al., 2009). The tissues were pre-treated in 50% formamide/2× SSC. The hybridizations were performed in a buffer containing 50% formamide, 2× SCC, 10% dextran sulfate, 1× Denhardt's [0.25% BSA, 0.25% Ficoll 400, 0.25% polyvinylpyrrolidone], 0.25% 1 M Tris-HCl pH 8.0, 5% sodium dodecyl sulfate, 250 μg/ml denatured salmon sperm DNA, and a 5 × 10^5 cpm radiolabeled probe at 54 °C for 16 h. Slides with hybridized brain sections were dipped into Kodak NTB autoradiography emulsion (Eastman Kodak) diluted 1:1 in distilled water. These slides were developed after 4 days of exposure at 4 °C. Developed silver grains analyzed from the hybridized slides were visualized under dark-field illumination (Olympus BX51, 10×/0.3 NA objective). Images were obtained with a SPOT II digital camera (Diagnostic Instruments, Inc.), the areas of interest (PVN and SON) were outlined. IOD values were estimated in each section with background correction (as background the adjacent tissue without AVP mRNA signal was taken). The specificity of the signal was demonstrated in hybridized tissue using the sense probes. Blind quantitative analysis was performed independently by two observers, without major differences among them. The average of the IOD values of up to six rostrocaudal slices/animal was calculated, treated as one determination, and was used to estimate the mean and s.e.m./group. Two different repetitions were performed and analyzed twice by independent ISHH trials on different sets of sections, giving similar results.

2.13. Endpoint reverse transcription polymerase chain reaction (RT-PCR)

Endpoint RT-PCR to cFOS gene expression was performed to determine if the PVN and SON of A1254 perinatally exposed rats are activated in response to hyperosmotic challenge. We used three-month-old rats, 12 females and 12 males (Control, A1254, Hyper, Hyper + A1254; n = 3 rats per sex per group). Upon completion of the osmotic challenge, the rats were killed by rapid decapitation with a guillotine, and trunk blood was collected to determine plasma osmolality. The brains were rapidly removed from the skull, placed on dry ice, and stored at −80 °C until use. Brains were sectioned on a cryostat until reaching the beginning of the regions of interest, taken out on dry ice and cut frozen into a thick coronal section in a range of −1.30 to −2.12 mm for the PVN and −0.80 to −1.80 mm for the SON caudal to Bregma (Paxinos and Watson, 1998). The PVN and the SON were punched out of the tissue using the micropunch technique (Palkovits, 1988) and kept frozen in a tube on dry ice. Total RNA was extracted as described (Chomczynski and Sacchi, 1987), and RNA concentration was determined by absorbance at 260 nm. Only samples with a 260/280 nm ratio of >1.8 and >2.0 28S/18S ratio, verified by gel electrophoresis in 1% agarose -1× TBE, were used. One microgram of RNA was transcribed with M-MLV reverse transcriptase and oligo-dT. The 18S was used as reference gene. PCR reactions were performed as follows, 1336–1553 of 18S ribosomal RNA (rRNA) cDNA (GenBank: X01117.1); final product: 218 bp; sense: 5′- ATGGCCGTTCTTAGTTGGTG -3′, antisense: 5′- CGCTGAGCCAGTTCAGTGTA -3′. For cFOS cDNA, sense: 5′ -CAATACACTCCATGCGGTTG -3′, antisense: 5′ – CCCGTAGACCTAGGGAGG AC-3′. To ensure adequate conditions for the semi-quantification of mRNA expression for each probe, cDNAs prepared from 1.0 μg of RNA in the PVN and SON were subjected to PCR amplification cycles (Mastercycler, Ependorff, Hamburg, Germany). The number of cycles for each cDNA was obtained from the ascending part of the curve that plots optical density versus number of PCR cycles. 18S rRNA: 2 μl of cDNA per 25 μl PCR [94 °C 1′15″, 65 °C 1′, 72 °C 1′] × 30 cycles + 15 min at 72 °C for a final extension. cFOS: 2 μl of cDNA per 25 μl PCR [94 °C 1′15″, 60 °C 1′, 72 °C 1′] × 32 cycles + 15 min at 72 °C for a final extension. Products were separated by gel electrophoresis in 2% agarose -1× TBE, running buffer (0.5×), and stained with ethidium bromide; density was quantified with a Fluor-S MultiImager (BioRad). Samples of control and experimental animals from the same group were included in the same gel. The relative values for cFOS mRNA were estimated as the ratios of mRNA signal to 18S rRNA signal.

2.14. Statistical analysis

Statistical significance between groups was determined by one or two-way ANOVA depending on the factors tested. For two-way ANOVA the factors tested were sex and treatment, while data from males was assessed with one-way ANOVA (treatment). Where overall significance was obtained ($p < 0.05$), post-hoc comparisons were made using Holm-Sidak method. Results for osmolality are reported as mean ± s.e.m. in mOsm/kg units. Average values from each cohort were statistically analyzed in an independent way using two-way ANOVA for cohort I and III and one-way ANOVA for cohort II.

For AVP immunofluorescence and immunoperoxidase, the mean IOD values were tested with two-way ANOVA (cohort I), and one-way ANOVA (cohort II, III). For cell counting, averages of immunoreactive neurons from cohort I and III were evaluated with one-way ANOVA. Nissl-stained neurons were analyzed using one-way ANOVA. For ISHH, the mean IOD values were tested with two-way ANOVA (cohort I). For endpoint reverse transcription polymerase chain reaction (RT-PCR), the statistical significance between groups was determined by two-way ANOVA (cohort I). Statistical analysis was performed using SigmaPlot 12.3 (Systat Sofware, Inc). ANOVA was performed where data met normality and homogeneity of variance assumptions. Statistical significance was acknowledged at an alpha level of 0.05.

3. Results

3.1. Perinatal A1254 and DE-71 treatment affect osmoregulatory capacity during hyperosmotic challenge in adulthood

Cardiac blood was collected and analyzed for plasma osmolality (Table 2) from the three cohorts. Plasma responses were measured in normosmotic conditions: corn-oil vehicle (Control); A1254 (A1254) or DE-71 (DE-71) exposed, and in rats subjected to prolonged or acute hyperosmotic challenge: corn-oil vehicle (Hyper); exposed to A1254 (Hyper + A1254), or DE-71 (Hyper + DE-71). In all cohorts plasma osmolality data showed an expected elevation in response to hyperosmotic stimulation. Cohort I (males and females perinatal exposed to A1254 and subjected to prolonged hyperosmotic challenge) was evaluated with two-way ANOVA (sex and treatments). Plasma osmolality values were not significant differences between sexes ($F_{1,147} = 1.56$, $p = 0.21$); there was no interaction between sex and treatment ($F_{3,147} = 0.77$ $p = 0.51$). There was a statistically significant difference in the mean omoslality values between the treated groups ($F_{3,147} = 9.17$, $p < 0.001$). Post-hoc multiple comparison testing showed that Hyper and Hyper + A1254 group had a significant increase in plasma osmolality relative to normosmotic controls ($p = 0.002$, $p < 0.001$ respectively). Hyper + A1254 group showed a significant increase in plasma osmolality relative to A1254 group ($p = 0.035$).

Cohort II (males perinatally exposed to A1254 and subjected to acute hyperosmotic challenge) was evaluated using one-way ANOVA. It showed significant differences between treatment groups ($F_{2,13} = 12.6$, $p = 0.001$). Multiple comparison revealed that both Hyper ($p = 0.012$) and Hyper + A1254 ($p = 0.001$) groups had a significant increase in plasma osmolality relative to control.

Cohort III (DE-71 perinatal exposure and subjected to prolonged hyperosmotic challenge) was evaluated with two-way ANOVA. Plasma osmolality values were not significant different between sexes ($F_{1,94} = 1.04$, $p = 0.31$), but there was a significant sex and treatment interaction ($F_{3,94} = 4.31$ $p = 0.007$). There were statistically significant differences in the mean osmolality values between the treated groups ($F_{3,94} = 54.4$, $p < 0.001$). Post-hoc multiple comparison testing showed that Hyper males and females had a significant increase in plasma osmolality relative to normosmotic controls ($p < 0.001$). Female Hyper + DE-71 rats showed a significant increase in plasma osmolality relative to DE-71 normosmotic control ($p < 0.001$). A similar tendency was seen in males but the effect was not statistically significant ($p = 0.11$). Additionally, in both sex groups, Hyper + DE-71 rats showed significant lower plasma osmolality relative to Hyper group ($p < 0.001$). There were not differences between males and females within control, Hyper and Hyper + DE-71 groups ($p > 0.05$), but there was a significant sex difference within the DE-71 treatment ($p = 0.011$). In combination these findings may indicate aberrant osmotic activation and/or dysregulation induced by developmental exposure to PBDEs in male and female adult Wistar and SD rats.

3.2. Perinatal exposure to A1254 prevents the AVP physiological response to hyperosmotic challenge

3.2.1. AVP immunofluorescence. Image analysis of coronal brain sections from the PVN and SON of male and female rats perinatally exposed to A1254 and subjected to prolonged hyperosmotic challenge (cohort I) shows that in control groups (vehicle) AVP-IR is mainly in MNCs. Basal AVP-IR was abundant and uniformly distributed in soma and fibers emanating from these nuclei (Fig. 1A, E). Perinatal exposure to A1254 leads to increase the AVP-IR in PVN compared to control group (Fig. 1B, F). Prolonged hyperosmotic stimulation (Hyper) showed an expected physiological increase of AVP-IR relative to control groups. These differences are notable in MNCs cytoplasm and in some parvocellular cell

Table 2
Plasma osmolality (mOsm/kg) in adult rats perinatally exposed to A1254 or DE-71 and in response to prolonged or acute hyperosmotic challenge.

Cohort	Experimental conditions			Hyperosmotic challenge			
				Prolonged		Acute	
	PCBs	Control	A1254	Hyper	Hyper + A1254	Hyper	Hyper + A1254
I	W. Males + Females	303.3 ± 4 n = M8, F4	324.8 ± 4.8 n = M8, F4	331.8 ± 5** n = M10, F3	346.4 ± 4.9^*** n = M9, F4		
II	SD. Males	297.3 ± 1.7 (n = 5)				329.1 ± 4.4** (n = 4)	340.4 ± 9.6*** (n = 5)
	PBDEs	Control	DE-71	Hyper	Hyper + DE-71		
III	W. Males	307.2 ± 2.0 (n = 4)	306.3 ± 2.3 (n = 3)	333.7 ± 4.7*** (n = 4)	314.8 ± 1.9### (n = 4)		
	W. Females	313.3 ± 1.9 (n = 4)	299 ± 2.0† (n = 7)	336.3 ± 2.7*** (n = 3)	320.9 ± 1.9^^^### (n = 6)		

Table shows osmoregulatory capacity measured during normosmotic and hyperosmotic conditions in Wistar (W) or Sprague Dawley (SD) adult rats (3–5 months old) perinatally exposed to A1254 or DE-71 (30 mg/kg/day orally) or corn-oil vehicle (Control). Rats from these groups were subjected to prolonged hyperosmotic challenge (NaCl 2% drinking solution during 5 days ad libitum) or acute osmotic challenge (NaCl 3.5 M *i.p.*), (Hyper; Hyper + A1254 or Hyper + DE71groups). Under anesthesia, blood was collected at sacrifice. Plasma osmolality was measured and the values are expressed as mean ± s.e.m. in mOsm/kg. Average values from each cohort were statistically analyzed independently using two-way ANOVA for cohort I and III and one-way ANOVA for cohort II, followed by post-hoc comparisons using the Holm-Sidak method. For the plasma osmolality values from cohort I, there were no significant interactions between sexes and between sex and treatment. This is the reason that it is depicted as a single group. Symbols indicates significant differences as determined by one or two-way ANOVA. Asterisks (*) compared to control, number symbols (#) compared to Hyper, carets (^) compared to A1254 or DE-71 treatment and daggers (†) compared to the other sex ($p < 0.05$ = ^†, $p < 0.01$ = **, ##, $p < 0.001$ = ***, ###, ^^^).

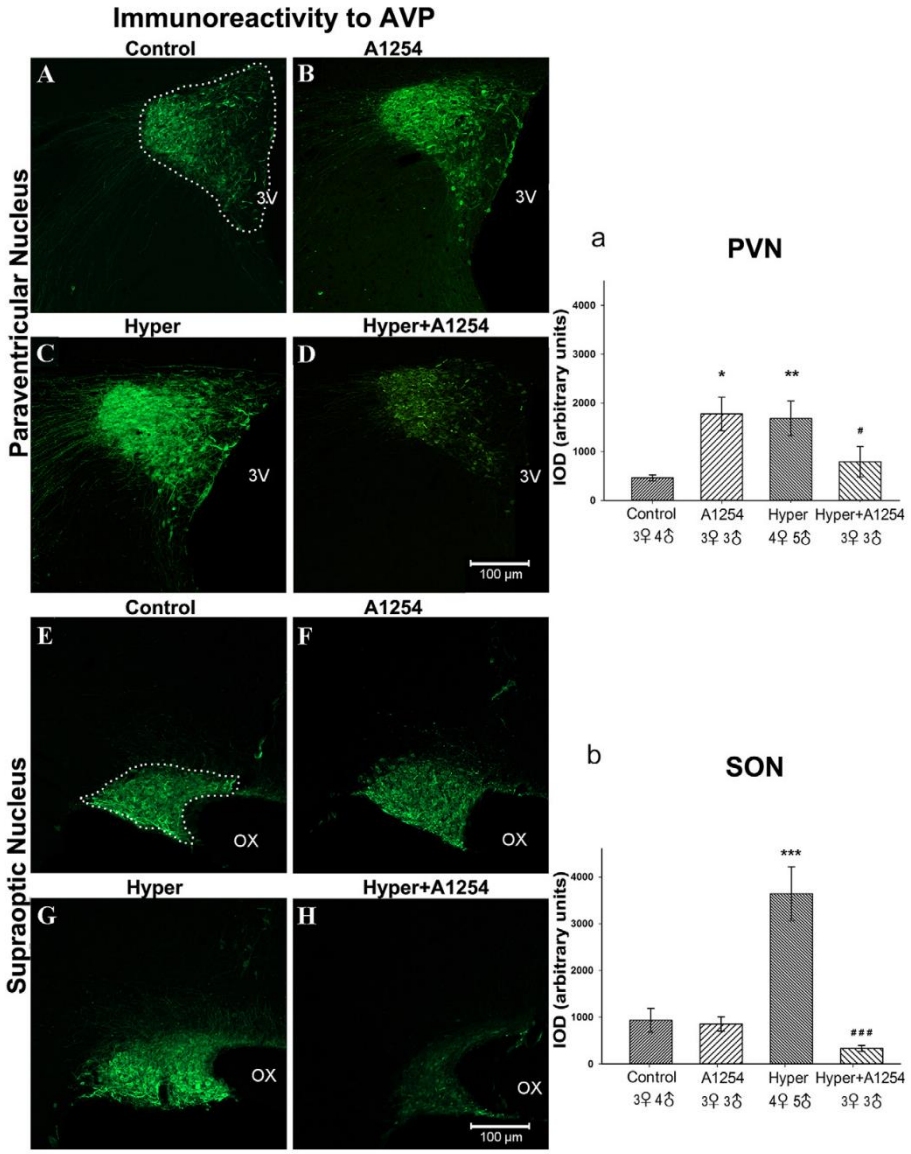

Fig. 1. Effects of perinatal exposure to Aroclor 1254 (A1254) on AVP immunoreactivity (AVP-IR) in 3-month-old Wistar rats. LEFT: Panel of representative confocal images from male rats of PVN and SON coronal sections with AVP-IR: control rats (images A and E); A1254-treated rats (30 mg/kg/day) (images B and F); hyperosmotic salt-loaded rats (Hyper) (images C and G); and hyperosmotic A1254-treated rats (Hyper + A1254; D and H). There was an increase in AVP-IR in Hyper rats (C and G). In contrast, there was an evident decrease in immunoreactive intensity and number of AVP-IR neurons and fibers after A1254 exposure (Hyper + A1254; D and H). RIGHT: The graphs show the effects of A1254 on AVP-IR integrated optical density (IOD) in the PVN (a) and the SON (b) from males and females. There were no significant differences between sexes and no interactions between sex and treatment. This is the reason that it is depicted as a single group. The dotted line shows the ROI used to perform IOD measurements. The bars represent mean values ± s.e.m. The symbols represent statistical significance as determined by two-way ANOVA and Holm-Sidak post-hoc tests. Asterisks (*) compared to control and number symbols (#) compared to Hyper ($^{*,\#} = p < 0.05$; $^{**} = p < 0.01$; $^{***,\#\#\#} = p < 0.001$). Abbreviations: third ventricle (3V); optic chiasm (OX); Bar = 100 µm.

bodies and their long vesiculated fibers (Fig. 1C, G). In contrast, the intensity of AVP-IR and the amount of the AVP-IR cells were lower in the PVN and SON from the Hyper + A1254 group as compared to the A1254 and to the Hyper groups (Fig. 1D, H). These observations were corroborated with semiquantitative analysis of the AVP-IR density (Fig. 1a, b), evaluated with two-way ANOVA (sex and treatment).

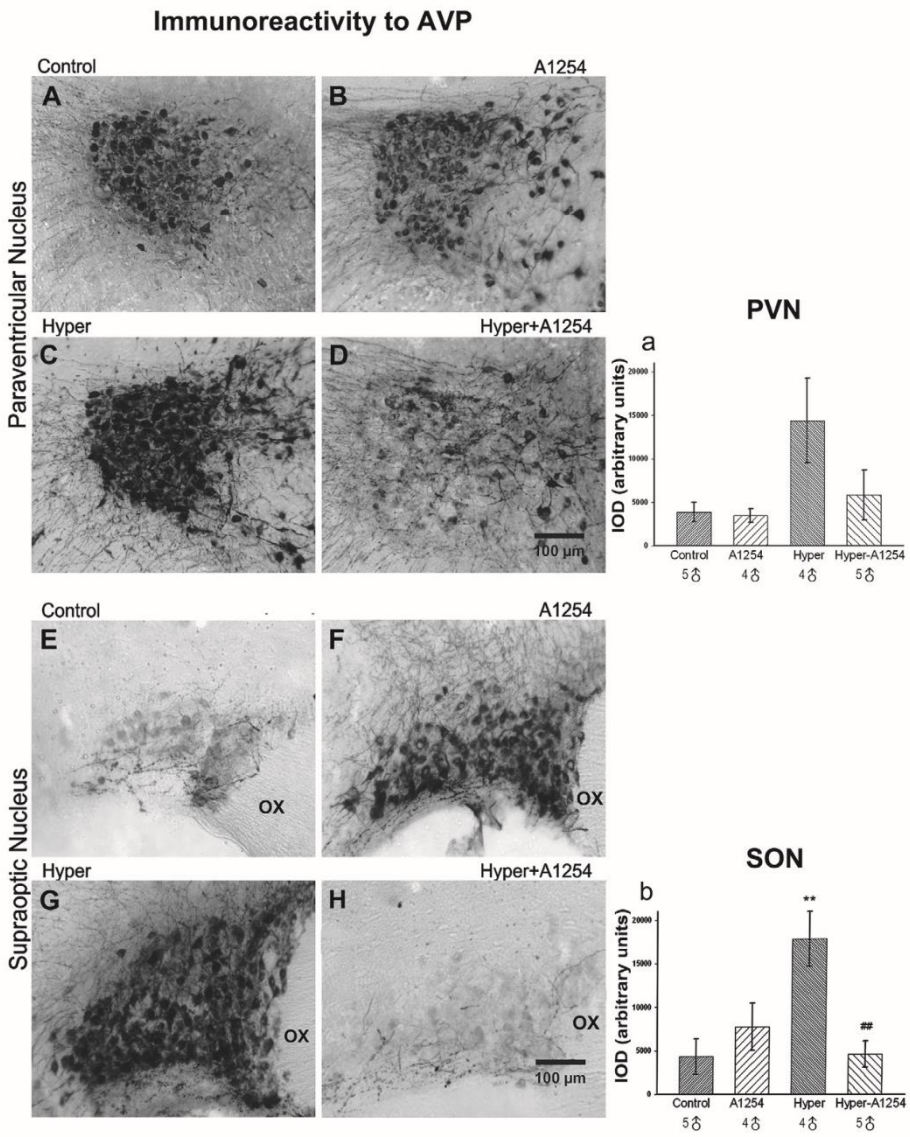

Fig. 2. A1254 prevents upregulation of AVP-IR in response to acute osmotic activation in the SON and PVN of male Sprague-Dawley rats perinatally exposed to Aroclor 1254 (A1254; 30 mg/kg/day). LEFT: Representative micrographs were generated using AVP immunoperoxidase in PVN and SON sections. Control (vehicle) images A and E; A1254-treated rats B and F; rats subjected to an acute hyperosmotic challenge (Hyper; 3.5 M NaCl; 0.6 ml/100 g body weight, *i.p.*) images C and G; and hyperosmotic A1254-treated rats (Hyper + A1254; D and H). There was an increase in AVP-IR in Hyper rats (C and G). In contrast, there was a marked reduction in upregulation of immunoreactive intensity and distribution of AVP-IR neurons and fibers in Hyper + A1254 group (D and H). RIGHT: Bars show mean ± s.e.m. values of AVP-IR IOD in PVN (a) and SON (b). Symbols represent statistical significance as determined by one-way ANOVA and Holm-Sidak post-hoc tests. Asterisks (*) compared to control, number symbols (#) compared to Hyper (**, $^{\#\#} = p < 0.01$). Abbreviations: optic chiasm (OX); Calibration bar = 100 µm.

Mean IOD values were not significantly different between sexes in the PVN ($F_{2,63} = 3.18$, $p = 0.08$) nor in the SON ($F_{1,60} = 3.75$, $p = 0.05$). Neither was significant interaction between sex and treatments in the PVN ($F_{3,63} = 2.08$, $p = 0.11$) or SON ($F_{3,60} = 1.38$, $p = 0.25$). As expected, there were significant differences between the treatments groups, both in the PVN ($F_{3,63} = 6.05$, $p = 0.001$) and SON ($F_{3,60} = 18.56$, $p < 0.001$). Multiple comparison indicated significant differences when comparing A1254 group with control group from PVN ($p = 0.012$). Mean IOD values from the Hyper groups showed statistically significant increases compared to controls in PVN ($p = 0.004$) and in the SON ($p < 0.001$). In contrast, the mean IOD for the Hyper + A1254 groups was not different from the controls. When comparing Hyper + A1254 to the Hyper groups they have a significant IOD decrease, (PVN $p = 0.047$; SON $p = 0.001$; Fig. 1a, b). In the PVN, the comparison of Hyper group versus Hyper + A1254, shows a reduction in the mean IOD values of 74.7% in males and 67.08% in females. In the SON this comparison showed a reduction in mean IOD values of the 95% in males and 86.24% in females.

3.2.2. AVP immunoperoxidase. AVP immunostaining in PVN and SON sections of cohort II (adult males perinatally exposed to A1254 and subjected to acute dehydration) showed similarly decreased AVP-IR responses to acute hyperosmotic stimulation when compared to rats that were chronically activated (Fig. 2). First, AVP-IR was uniformly distributed in soma and fibers of MNCs in PVN and SON. A1254 treated rats had similar AVP-IR to controls in both nuclei. As in Cohort I Hyper rats showed more robust AVP-IR as compared to the control group. Upregulated AVP-IR responses in the Hyper + A1254 group were not observed when compared to A1254 and Hyper groups both in PVN and SON. Mean IOD values for AVP-IR were evaluated using one-way ANOVA. In the PVN there were not significant differences between groups ($F_{3,14} = 3.10$, $p = 0.061$). In the SON there were significant effects between treatment groups ($F_{3,14} = 6.29$, $p = 0.006$). Multiple comparison showed that mean IOD values from Hyper group were significantly greater compared to control ($p = 0.011$). When comparing Hyper versus Hyper + A1254, IOD values decreased significantly ($p = 0.011$; Fig. 2a and b).

3.2.3. AVP-IR in DE-71-exposed male rats. Brain sections of control normosmotic groups have a medium intensity (basal) AVP-IR in the PVN and the SON, mainly in MNC cell bodies (Fig. 3A, E). Sections from DE-71-treated rats showed an increase in basal AVP-IR in PVN and SON (Fig. 3B, F). Sections from Hyper rats showed an evident physiological AVP-IR increase in MNCs and parvocellular neurons in the PVN and in MNCs of the SON (Fig. 3C, G). Hyper + DE-71-treated rats did not show this increase; rather, they had weak AVP-IR in the PVN and the SON (Fig. 3D, H). Also the number of AVP-IR neurons was reduced, finding confirmed by cell count analysis (Fig. 4C, D). Mean IOD values for AVP-IR are shown in the graphs of Fig. 3(a, b). The differences of the mean IOD values for AVP-IR between the treatment groups were evaluated using one-way ANOVA, Indicating significant differences both in the PVN and SON ($F_{3,40} = 10.9$, $p < 0.001$ and $F_{3,45} = 14.54$, $p < 0.001$ respectively). Multiple comparison showed, in the SON, that the IOD values from DE-71 treated group were significantly different compared to the control group ($p = 0.021$). The Hyper groups from PVN and SON showed statistically significant increases compared to controls ($p < 0.001$ both). In contrast, the mean IODs for the Hyper + DE-71 groups were not different from the controls. The Hyper + DE-71 group showed a significant decrease in the SON ($p = 0.026$), when compared with its own control, the DE-71 group. When compared Hyper with Hyper + DE-71 groups, the mean IOD decreased significantly in both PVN and SON ($p < 0.001$ both; Fig. 3a, b). These comparisons showed a reduction in mean AVP-IR IOD values of the 88.24% in the PVN, and 77.03% in the SON.

3.3. Cell count analysis of AVP-IR neurons

A cell count analysis was performed with same immunofluorescence images acquired from cohorts I and III male rats. Only the photographs from the middle part of the PVN and the SON were used (Bregma: −1.80 mm to −1.88 mm for the PVN, and −1.30 mm to −1.40 mm for the SON) (Paxinos and Watson, 1998). Cell counts included all AVP-IR MNCs and parvocellular neurons. One-way ANOVA indicated significant differences between treatment groups in the number of AVP-IR neurons by A1254 treatment in PVN ($F_{3,70} = 5.35$, $p = 0.002$) and SON ($F_{3,45} = 22.14$, $p < 0.001$). Multiple comparisons showed that in A1254-treated rats, the number of the AVP-IR neurons in the PVN and SON were not significantly different compared to its control (Fig. 4A, B). The Hyper groups had a significantly increased number of AVP-IR neurons compared to control both in the PVN and in the SON ($p = 0.002$, $p < 0.001$ respectively). In the Hyper + A1254 group, the number of AVP-IR neurons was similar to that of the control group, but significantly lower compared to the Hyper groups in PVN and SON ($p = 0.026$, $p < 0.001$ respectively). The Hyper + A1254 group showed a significant decrease in the number of neurons in the SON when it was compared with its own control, the A1254 group ($p = 0.002$) (Fig. 4B).

In the case of DE-71 exposure, the one-way ANOVA showed significant differences between treatment groups in PVN ($F_{3,57} = 12.49$, $p < 0.001$) and SON ($F_{3,32} = 25.96$, $p < 0.001$) (Fig. 4C, D). Pos-hoc multiple comparisons showed that the number of AVP-IR neurons in the SON increased significantly in DE-71 group ($p < 0.001$) compared to the control. Hyper groups from PVN and SON had a significantly increased number of AVP-IR neurons compared to the control ($p = 0.003$, $p < 0.001$ respectively). When compared Hyper + DE-71 with its own control, the DE-71 group, there was a significant decrease in the number of neurons in the SON ($p < 0.001$) (Fig. 4D). The Hyper + DE-71 groups showed a significant decrease in the number of AVP-IR neurons compared to the Hyper groups in both nuclei ($p < 0.001$ both). These results were similar to those observed for the A1254 exposed group (Fig. 4B, D).

3.3.1. Cell count analysis of Nissl staining. We used alternating brain sections containing medial PVN and SON that were not processed for AVP immunohistochemistry. Normosmotic control and A1254- and DE-71-treated rats were randomly chosen from three different litters. There was no statistically significant difference in cell counts between groups in PVN ($F_{2,29} = 1.48$, $p = 0.243$) and SON ($F_{2,34} = 0.09$, $p = 0.914$). The mean values ± s.e.m. from PVN and SON were graphed (Fig. 4E, F). Further, Nissl-stained neurons seemed to have normal morphological characteristics in all groups.

3.4. cFOS mRNA expression after perinatal exposure to A1254

The cFOS mRNA expression was assessed to demonstrate if neurons of PVN and SON were activated. cFOS genes were evaluated together with the reference gene 18S ribosomal RNA. Males and females perinatally exposed to A1254 and subjected to prolonged osmotic activation were evaluated by two-way ANOVA. The IOD data indicated no statistically significant differences between sexes, for the PVN ($F_{1,15} = 2.04$, $p = 0.174$) or SON ($F_{1,16} = 3.32$, $p = 0.087$), nor did it show any interaction between sex and treatment in the PVN ($F_{3,15} = 1.18$, $p = 0.35$) or SON ($F_{3,16} = 0.26$, $p = 0.857$). As expected, there was a significant differences between treatment groups in PVN ($F_{3,15} = 15.2$, $p < 0.001$) or in SON ($F_{3,16} = 22.79$, $p < 0.001$). Post-hoc multiple comparisons indicated that Hyper groups from PVN and SON showed no significant increase of cFOS mRNA compared to control groups ($p = 0.086$, $p = 0.366$ respectively). However Hyper + A1254 group displayed the highest cFOS mRNA expression in PVN and SON with a statistically significant increase as compared to control ($p < 0.001$ both); as compared to A1254 groups (PVN $p = 0.002$; SON $p < 0.001$) and as compared to Hyper ($p = 0.002$, $p < 0.001$ respectively), (Fig. 5A, B).

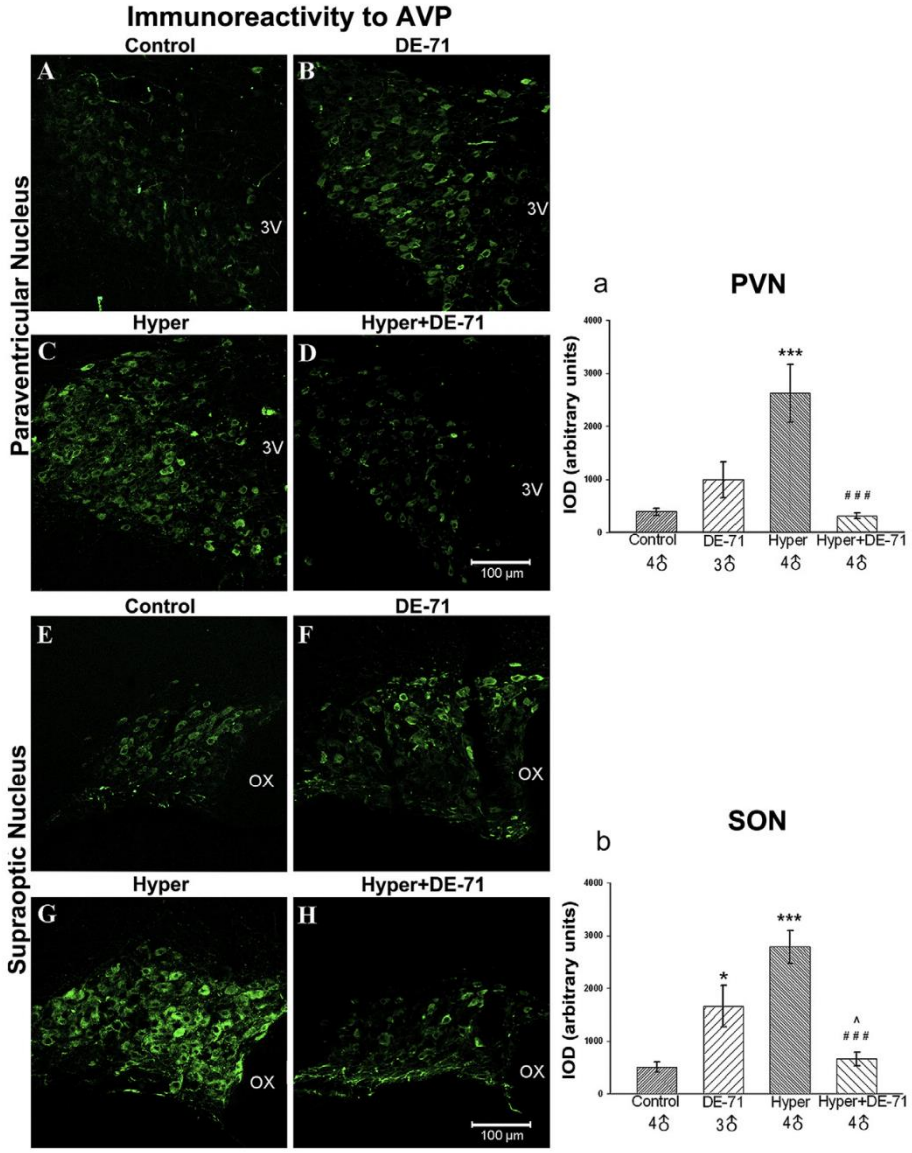

Fig. 3. Effects of perinatal exposure to DE-71 on AVP immunoreactivity (IR) in 3-month-old Wistar males rats. LEFT: Panel of representative confocal images of PVN and SON coronal sections with AVP-IR: control rats (images A and E); DE-71-treated rats (30 mg/kg/day) (B and F); hyperosmotic salt-loaded rats (Hyper) (C and G); and hyperosmotic DE-71-treated rats (Hyper + DE-71; D and H). AVP-IR increased in DE-71-treated and Hyper rats. In contrast, there was not physiological increase in staining intensity and number of AVP-IR neurons in Hyper + DE-71 rats. These results were confirmed with the IOD evaluation as showed in the graphs. RIGHT: The graphs show the effects of DE-71 on AVP-IR IOD in the PVN (a) and the SON (b). The bars represent mean values ± s.e.m. The symbols represent statistical significance as determined by one-way ANOVA and Holm-Sidak post-hoc. Asterisks (*) compared to control, number symbols (#) compared to Hyper and carets (^) compared to DE-71 treatment ($^{*,\wedge} = p < 0.05$; $^{***,\#\#\#} = p < 0.001$). Abbreviations: third ventricle (3V); optic chiasm (OX); Bar = 100 µm.

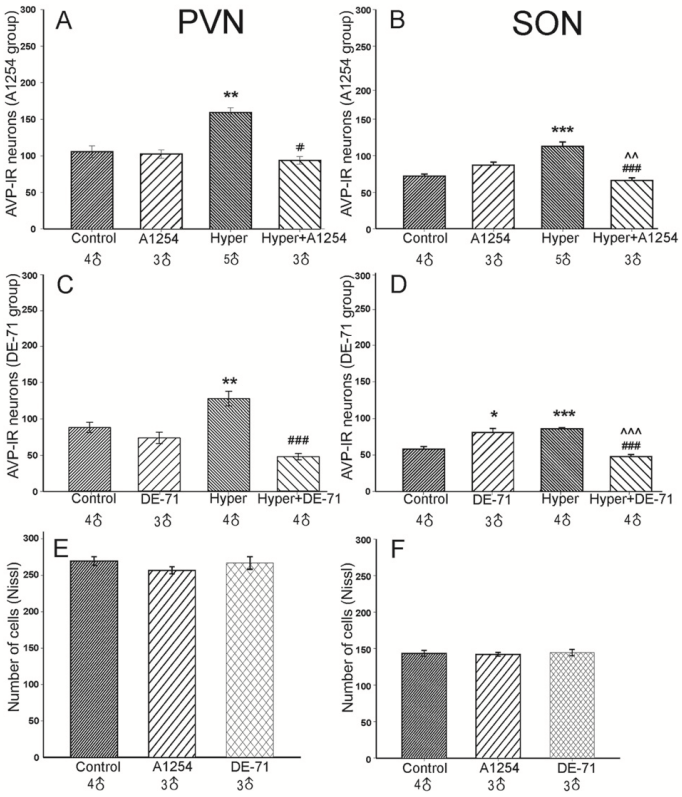

Fig. 4. Effects of perinatal exposure to A1254 or DE-71 on the number of AVP-IR neurons and Nissl-stained cells, from the PVN and SON (A, B, C, D in 3-month-old male rats during normosmotic and hyperosmotic conditions). Mean values for AVP-IR neurons increased significantly after salt loading in vehicle-treated rats (Hyper) but not in salt loaded A1254- and DE-71-treated rats (Hyper + A1254; A and B; Hyper + DE-71; C and D).These groups showed a significant decrement when compared with Hyper groups. Graphs E and F showed no change in the number of PVN and SON Nissl-stained cells of adult male rats perinatally exposed to A1254 or DE-71 (30 mg/kg). The bars represent mean ± s.e.m. values of AVP-IR neurons, and Nissl-stained cells in control, A1254- and DE-71-treated rats. The symbols represent statistical significance as determined by one-way ANOVA and Holm-Sidak post-hoc. Asterisks (*) compared to control, number symbols (#) compared to Hyper and carets (^) compared to A1254 or DE-71 treatment ($^{*,\#}p < 0.05$; **, $^{\wedge\wedge}p < 0.01$; ***, ###, $^{\wedge\wedge\wedge}p < 0.001$).

3.5. In situ hybridization histochemistry

In situ hybridization allowed us to identify and localize AVP mRNA expression within the PVN and SON. Developed silver grains of the film, denoting AVP mRNA, were widely distributed in all MNCs and some parvocellular neurons in male and female PVN and SON brain sections (Fig. 6). IOD data from males and females were evaluated with two-way ANOVA. The test indicated no statistically significant differences between sexes in the PVN ($F_{1,320} = 2.11$, $p = 0.147$) and SON ($F_{1,249} < 0.001$, $p = 0.98$). There was statistically significant differences in the mean IOD values between the treated groups (PVN; $F_{3,320} = 33.75$, $p < 0.001$, and SON; $F_{3,249} = 24.35$, $p < 0.001$). In addition, there was an interaction between sex and treatment in the PVN ($F_{3,20} = 3.46$, $p = 0.017$). Post-hoc multiple comparisons indicated a significant increase in IOD values of AVP mRNA in the Hyper groups when compared to the control in the PVN for males and females ($p < 0.001$, $p < 0.004$ respectively) and from SON in both sexes ($p < 0.001$; Fig. 6a, b). Comparison of control and A1254-exposed rats revealed no significant differences. However, A1254 treatment blunted AVP mRNA levels in animals subjected to prolonged hyperosmotic challenge, although some clusters with high signal intensity remained in the PVN and SON (Fig. 6D, H). When compared with the Hyper group, the AVP mRNA values for Hyper + A1254 decreased significantly in the PVN ($p < 0.001$ for both males and females) and SON ($p < 0.001$). The percentage decrease of Hyper + A1254 compared to the Hyper group was 68.12% in the males and 63.86% in the females in the PVN, and an average of 54.57% for both males and females in the SON. There were not differences between males and females within control, A1254 and Hyper + A1254 groups ($p > 0.05$) in the PVN, but there was a significant sex difference within the Hyper groups ($p = 0.005$).

4. Discussion

4.1. Perinatal exposure to A1254 and DE-71 disrupts AVP responses to hyperosmotic challenge in the PVN and SON of adult rats

This study demonstrates that perinatal exposure to organohalogen pollutants such as PCBs and PBDEs adversely affects the vasopressinergic system in adult rats. This effect was evident when the MNC system was stimulated by physiological demand such as

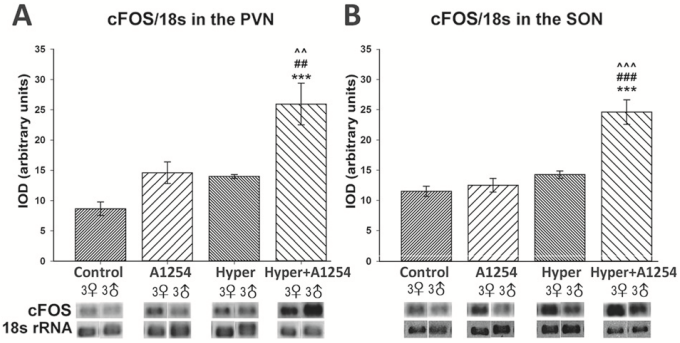

Fig. 5. Effects of perinatal exposure to A1254 on semiquantitative endpoint RT-PCR analysis of cFOS mRNA in 3-month-old males and females Wistar rats. Analyses of micropunches taken from the PVN (A) and the SON (B) were carried out in control rats (oil vehicle and normosmotic), rats perinatally exposed to A1254, rats hyperosmotically stimulated (Hyper), and rats treated with A1254 and prolonged salt-loading (Hyper + A1254). Densitometric analyses (arbitrary units) for cFOS mRNA normalized to the 18S rRNA reference gene revealed that there were no significant differences between sexes and no iteractions between sex and treatment. This is the reason that it is depicted as a single group. The A1254 and Hyper groups increased lightly but did not were significantly different compared to control group. The Hyper + A1254 groups for both PVN and the SON showed the greatest increase when compared to control, A1254 and Hyper groups. The bars represent mean IOD ± s.e.m. Below each bar there is one example from each sex of the electrophoresis bands from cFos and 18S rRNA. Statistical significance was determined by two-way ANOVA and Holm-Sidak post-hoc test. The symbols represent statistical significance, asterisks (*) compared to control, number symbols (#) compared to Hyper and carets (^) compared to A1254 or DE-71 treatment (***, ###, ^^^$p < 0.001$).

after acute or chronic hyperosmotic challenges. As previously reported, hyperosmotic stimulus promotes an increase in AVP content in the hypothalamic PVN and SON and plasma AVP release. Circulating AVP leads to water retention by the kidneys and maintains plasma volume and osmolality (Ludwig et al., 1994; Weitzman and Kleeman, 1979). Also, hyperosmotic stimulation promotes release of AVP from somatodendritic components of MNCs (central AVP release) which autoregulates plasma AVP release to prevent excessive axonal AVP release during physiological demand (Liu et al., 1996; Ludwig et al., 1994). The rise in AVP content within MNC nuclei is a normal neuroendocrine response by the hypothalamo-neurohypophysial system to an increased physiological demand (Amaya et al., 1999; Bourque, 2008; Johnson and Thunhorst, 1997). This is also accomplished by increased hydration via water drinking, in the salt-loaded model and in acute hyperosmotic challenge the animals were not allowed to drink water, thereby they were forcing the system to maintain osmotic balance (Leng et al., 2001, 1999; Ludwig et al., 1996). The expected physiological increase in AVP-IR in salt-loaded rats (Hyper group) (Fig. 1C, G; Fig. 3C, G) was found in cell bodies in both the PVN and the SON, mainly in MNCs, but also in parvocellular neurons as reported by our lab and others (Amaya et al., 1999; Elgot et al., 2012; Gamrani et al., 2011; Whitnall and Gainer, 1985). The large increase in immunoreactivity to AVP-neurophysin antibody is representative of the AVP peptide and has been shown to be associated with secretory granules in MNCs (Ben-Barak et al., 1985; Whitnall and Gainer, 1985). The quantified values of IOD should not be taken as a linear index of the intracellular concentrations of the peptide, as the size of the sample was not large (3–5 rats per treatment, over six slides per nucleus -two nuclei for brain). In spite of these limitations, the observed AVP IOD values likely reflect changes in AVP content within both the PVN and the SON. Our results showed that adult rats (3 months old) perinatally exposed to commercial PCB and PBDE mixtures (A1254 and DE-71, respectively) and subjected to hyperosmotic challenge suppress the upregulation of AVP content in MNCs in the hypothalamic PVN and SON. Densitometry results suggested uniform depletion of AVP content in the neuroendocrine cells of these nuclei compared to Hyper groups (Fig. 1D, H; Fig. 3D, H), since almost all neurons in MNCs of the PVN and the SON of Hyper + A1254 and Hyper + DE-71 animals showed poor immunoreactive intensity. The reduced upregulation of AVP content may occur as a result of exaggerated axonal AVP release secondary to a decrease in central AVP release caused by perinatal exposure to A1254 and DE-71 (Coburn et al., 2007, 2005). This could lead to deplete AVP stores in the hypothalamic PVN and SON and activate the synthetic machinery in the hypothalamic MNC nuclei. These stores are needed during osmotic stress and the carrying storage capacity of the system affects osmoregulation (Amaya et al., 1999; Shoji et al., 1994). SD rats perinatally exposed to A1254 displayed similar physiology to Wistar rats. In this sense there was no surprise to find similar results even when using an acute hyperosmotic challenge (Fig. 2). This suggest a fast and more direct effect on transcription and/or translation of AVP and a permanent deficit in the neuroendocrine functions of MNCs both in Wistar and Sprague-Dawley rats.

In the control normosmotic group, AVP-IR in fibers and cell bodies showed basal staining similar to that found by other authors using the same antibody (Ben-Barak et al., 1985; Whitnall and Gainer, 1985; Whitnall et al., 1985). However, normosmotic rats exposed to either A1254 or DE-71 showed increased AVP-IR, which was statistically significant in the PVN of A1254-exposed and the SON of DE-71-exposed males (Figs. 1B, a, and 3F, b). The normosmotic rats perinatally exposed to A1254 showed a non-significant increase (7%) in the osmolality values (Table 2), a significant increase in immunoreactivity (Fig. 1Ba), and a similar trend in the number of AVP-IR neurons (Fig. 4B). Similarly, under basal normosmotic conditions, DE-71-treated male rats showed elevated AVP-IR in the SON (Fig. 3F, b; Fig. 4D) in conjunction with normal plasma osmolality (Table 2). However, normosmotic A1254-treated male rats displayed elevated plasma osmolality without showing reduced AVP-IR, a result that may be explained by negative effects of A1254 on kidney function that can impact osmoregulation (Esteban et al., 2014). In combination, these results indicate a disrupted AVP system under basal normosmotic conditions.

In the groups of exposed and subjected to the hyperosmotic challenge rats (Hyper + A1254 and Hyper + DE-71) the osmolality values were the greatest, indicating that body water balance was compromised and, hence, osmoregulation was affected (Table 2). These groups also showed the lowest AVP-IR densities indicating that their deficient osmoregulation may be due to disregulated AVP signaling (Coburn et al., 2005; Shah et al., 2011). The effects of organohalogen contamination may have significant consequences for physiological homeostasis dependent on AVP. We and others have previously shown that PCBs and PBDEs affect thyroid hormones, alter osmoregulation and cardiovascular parameters and damage kidney function (Albina et al., 2010; Brouwer et al., 1998; Kodavanti and Curras-Collazo, 2010; Li et al.,

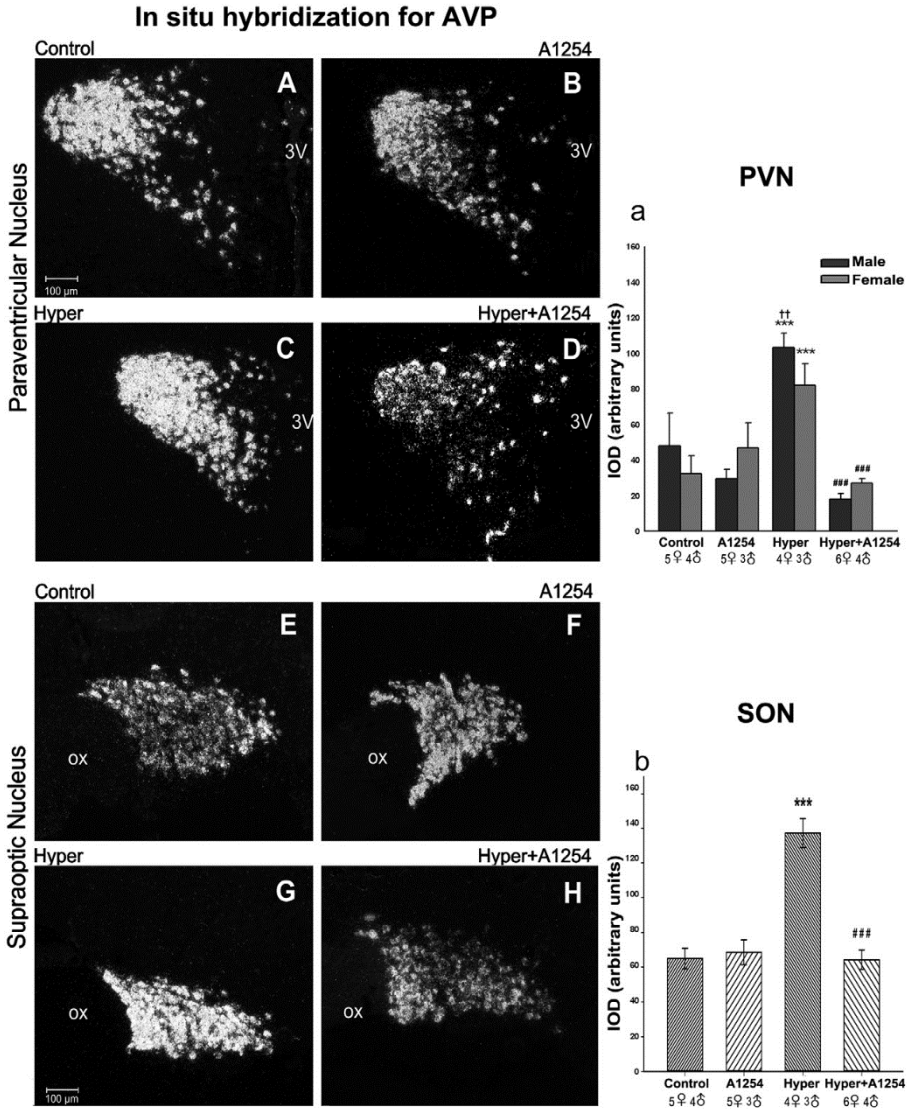

Fig. 6. Effects of perinatal exposure to A1254 on AVP mRNA expression in PVN and SON of male and female Wistar rats. mRNAs were detected using in situ hybridization with [^{35}S]-labeled probes. LEFT. Dark-field representative images from male rats of PVN and SON coronal sections show AVP mRNA in the PVN and SON in control, A1254, Hyper, and Hyper + A1254 groups. In the Hyper group, AVP mRNA increased both in the PVN and the SON as compared to control (C and G). In contrast, a decrease in the AVP mRNA of neurons occurred after hyperosmotic stimulation in the Hyper + A1254 as compared to Hyper. RIGHT: The graphs show the IOD of ISHH of AVP mRNA in the PVN (a) and the SON (b). The ANOVA from PVN indicated no statistically significant differences between sexes but there was interaction between sex and treatment groups. In the SON there were no significant differences between sexes and no interactions between sex and treatment. This is the reason that it is depicted as a single group. In the PVN and SON the comparison of control and A1254-exposed rats revealed no significant differences. However, A1254 treatment blunted AVP mRNA levels in animals subjected to prolonged hyperosmotic challenge, although some clusters with high signal intensity remained in the PVN and SON. When compared with the Hyper group, the AVP mRNA values for Hyper + A1254 decreased significantly in the PVN and SON. The bars represent mean ± s.e.m. values. Statistical significance was determined by two-way ANOVA and Holm-Sidak post-hoc test. The symbols represent statistical significance, asterisks (*) compared to control, number symbols (#) compared to Hyper, daggers (†) compared to the other sex ($^{\dagger\dagger}p < 0.01$, ***, $^{\#\#\#}p < 0.001$). Abbreviations: third ventricle (3V); optic chiasm (OX). Calibration bar = 100 µm.

2014; Shah et al., 2011). Our collaborative group has previously shown that perinatal exposure to DE-71 leads to hyperactive cardiovascular responses to osmotic stress, which may be associated with disrupted AVP-mediated processes and/or kidney function (Shah et al., 2011). Hypothyroid agents like PCBs or PBDEs may alter AVP secretion and osmoregulation indirectly by dysregulating thyroid hormone status (Kodavanti and Curras-Collazo, 2010). Ali et al. (1985) showed that in salt-loaded rats with induced hypothyroidism, antidiuresis is affected by reducing AVP-binding sites in the kidneys (Ali et al., 1987, 1985). In the PVN and SON hypothyroidism prevents osmotically-stimulated upregulation of transcripts for nitric oxide synthase (NOS), which is required for AVP functions (Ueta et al., 1995). Related to this our group has shown persistent reduction of NOS activity in the osmotically activated MNC system after similar developmental exposure to A1254 (Coburn et al., 2015; Leon-Olea et al., 2004; Mucio-Ramírez et al., 2008). Moreover, A1254 affects the expression of aquaporin 1 (AQP1) water channels (Tewari et al., 2009), which are regulated and linked to AVP V2 receptors and nitric oxide synthase (NOS) involved in water reabsorption (Klokkers et al., 2009; Swenson et al., 1997); deficiencies in the latter has been shown to impair water retention (Ma et al., 1998).

4.2. Cell count analysis of AVP-IR and Nissl-stained neurons does not indicate MNC cell death of AVP neurons by A1254

In accordance with significant reduction of mean AVP-IR IOD in hyperosmotic groups treated with organohalogens, cell counts of AVP-IR neurons showed significant decreases in the number of positive neurons compared to the Hyper group in PVN and SON ($p < 0.001$ both; Fig. 4A, B, C, D). Exposure to PCBs and PBDEs has been reported to induce a concentration-dependent loss of cell viability and apoptosis (Bradner et al., 2013; Costa et al., 2015; Johansson et al., 2006; Sanchez-Alonso et al., 2004). Therefore, to determine whether diminished upregulation of AVP-IR in Hyper + A1254 and Hyper + DE-71 was possibly linked to MNC cell death, we counted and the total number of cells in Nissl stained neurons in the medial part of PVN and the SON of all groups. A comparison of the total number of Nissl-stained cells in control and A1254-exposed rats did not show a significant difference in adult rats (Fig. 4E, F). Nor did cresyl violet staining show apparent morphological damage in the PVN and the SON. This suggests that the neurotoxic effect of A1254 on AVP-IR was not due to cell death at these ages. This does not preclude that organohalogens may produce adverse morphological changes early in development that may be reversed and undetected in adulthood. For example, our group has demonstrated neuronal shrinkage, nuclear pyknosis, and cellular death in nitrergic neurons within MNC nuclei from A1254-treated male rats at PN10 (Coburn et al., 2015; Leon-Olea et al., 2004). Cell death was likely to have occurred at early development stages or during treatment. These changes were short-lived and not observed in adult rats under basal conditions. Transient effects on the number of mature neurons, in the hippocampal dentate gyrus, have been reported after developmental exposure to decabrominated PBDEs in rats on PN20 (Saegusa et al., 2012). As expected, hyperosmotic (Hyper) groups showed a physiological increase in AVP-IR neurons in PVN magnocellular and parvocellular regions and SON (Amaya et al., 1999). Interestingly, exposure to DE-71 alone led to a significant increase in the number of AVP-IR neurons in the SON of adult male rats (Fig. 4D). In combination, these data suggest that the decrease in AVP-IR in the hyperosmotic/treated adult groups were not due to cell death but due to depletion in AVP content. More refined studies would be required to know which mechanisms are involved in these changes.

4.3. Hyperosmotic challenge and A1254 exposure activates cFOS gene in PVN and SON neurons

To explore if reduced AVP content during prolonged hyperosmotic stress in A1254-exposed rats may be due to vasopressinergic cells not receiving sufficient activation signals from osmotic stimuli, we performed end point RT-PCR for cFOS mRNA. The results showed that Hyper + A1254 group had a marked increase in cFOS mRNA in the PVN and SON of males and females (Fig. 5). Therefore, upregulated cFOS mRNA transcript levels correlated well with the rise in plasma osmolality. However, AVP-IR did not parallel the upregulation seen in cFOS mRNA levels. Although the micropunches of PVN and SON have other types of neurosecretory cells, not only vasopressinergic neurons, the results suggest that in general the osmotic challenge induced an increase in cFOS reaction that is positive in the activation of the system. Nevertheless this activation was not enough to maintain the AVP protein levels in the nuclei. This could be due to disrupted AVP synthesis which may result, in part, from reduced somatodendritic AVP (Coburn et al., 2007, 2005), that provides local autoregulatory signals (Wotjak et al., 1994). As expected, the salt-loading induced an increase in cFOS mRNA levels in the PVN and SON of male and female control groups (Fig. 5; Leng et al., 1999). This correlates with AVP immunoreactivity and plasma osmolality increase, as discussed above. Interestingly, the euhydrated rats exposed to A1254 also showed increase cFOS tendency in the PVN but not in the SON. Under these experimentally unstimulated conditions, plasma osmolality was abnormally elevated as compared to euhydrated rats (Table 2) and the upregulated c-FOS mRNA levels were consistent with elevated AVP-IR. The specific effect on PVN and the abnormal rise in plasma osmolality under "basal" conditions could suggest the involvement of altered renal signals mediated by autonomic nerves or kidney damage. Alternatively, A1254 may have a more focused effect on AVP transcription (but see below), translation or degradation that is only effective in the absence of hyperosmotic activation. For these experiments we normalized the values with the 18S rRNA gene since it was the most stable gene (Fig. 5A, B). We also probed several reference genes that were tested to normalize the results: actin, G3PDH and cyclophilin. These genes showed changes in their levels under chronic activation (data not shown), which suggested a general systemic damage due to exposure to these organohalogens.

4.4. Perinatal exposure to A1254 suppresses the physiological increase of AVP mRNA levels in the PVN and SON during chronic hyperosmotic stress

Previous studies have shown that repeated osmotic stimulation induces a compensatory increase in AVP mRNA levels and peptide production within the PVN and SON (Amaya et al., 1999; Meister et al., 1990). To explore if reduced AVP content during chronic hyperosmotic stress in A1254-exposed rats may be due to disrupted gene expression, we performed in situ hybridization histochemistry for AVP mRNA. Measurement of AVP mRNA levels (expressed as the density of developed silver grains) showed a physiologically activated increase in the PVN and the SON of salt-loaded males and females, compared to their controls (Fig. 6C, G), a finding that has been previously reported by several authors (Amaya et al., 1999; Meister et al., 1990). In contrast, in the Hyper + A1254-exposed groups, there were no expected physiological increases in AVP mRNA levels—hence, no differences from their control groups for nucleus or sex (Fig. 6D, Ha, b). Instead, there was a significant decrease in mean AVP mRNA compared to the Hyper groups, correlating with the lack of upregulated AVP-IR on this group. For the A1254-exposed normosmotic groups there were no significant differences in mean AVP mRNA IOD values as compared to the control groups, indicating a lack of induction of AVP mRNA in response to elevated cFOS and plasma osmolality seen in this group (especially males). It is unclear how the disconnection between c-FOS and AVP mRNA occurs in the A1254 groups but it may involve changes in NOS. For example, NOS isoforms such as inducible NOS may negatively regulate AVP gene expression (Oliveira-Pelegrin et al., 2010). However, A1254 may still induce the markedly elevated AVP-IR discussed above through downstream effects on neuronal NOS. For example, a recent study by our group showed that in utero exposure to A1254 alone produces abnormally elevated neuronal NOS activity in adult rats as measured by NADPH-diaphorase staining in fixed brain tissue (Coburn et al., 2015). Our lab

and others showed that neuronal NOS is required for AVP secretion (Gillard et al., 2007; Kadowaki et al., 1994).

In conclusion, both PCBs and PBDEs disrupt basal and stimulated AVP responses in MNC nuclei. Although PVN and SON in exposed groups were activated in response to the hyperosmotic stimulus (increased cFOS), neither the rapid translation of cFOS nor AVP mRNA was enough to restore AVP content in the PVN and SON, during hyperosmotic conditions. Nor was it enough to regulate plasma osmolality, as evidenced by the fact that the highest values ($p < 0.001$) were in the Hyper treated groups especially in Hyper + A1254 group (Table 2). This could lead to serious disturbances in homeostasis. A1254 treatment alone resulted in activation of c-FOS mRNA in PVN and AVP-IR without upregulation of AVP mRNA. The mechanisms underlying these effects are unclear but may be related to the effects of PCBs on NOS which can affect AVP system at transcription and secretion levels. Other mechanisms that may have intervened to decrease AVP content during chronic osmotic stress in A1254- and DE-71-exposed rats may be disrupted downstream synthetic mechanisms. A possible mechanism may have been that dioxin-like organohalogens activate the aryl hydrocarbon receptor (AhR; Denison and Nagy, 2003; Kodavanti and Curras-Collazo, 2010). After ligand binding, a heterodimer is formed which translocates into the nucleus and links to specific DNA regions. This, in turn, regulates transcription velocity of specific genes and produces genetic alterations that modify processes and functions in the cell (Denison and Nagy, 2003; Safe et al., 1985; Tilson et al., 1998), which could partially explain the unbalanced effects on AVP mRNA and content reported here.

Further research is warranted to identify the mechanisms involved in the disruption of the AVP system by organohalogens, since the AVP system is one of the main systems necessary to maintain hydromineral homeostasis that can impact osmoregulation, cardiovascular function and central AVP-regulated behavior.

5. Conclusions

Perinatal exposure to either A1254 or DE-71 affects the AVP system similarly in males and females; this effect was more evident during the hyperosmotic challenge although significant effects were detected under basal conditions. Exposure dramatically reduces hyperosmotically stimulated responses in AVP content and mRNA expression of MNCs in the PVN and the SON. These PCBs and PBDEs seem to produce similar disruption of neuroendocrine processes. The physiological outcome of the lack of response to increment AVP content in MNC during hyperosmotic stress in A1254- or DE-71-exposed rats led to a rise in plasma osmolality, an indication of disrupted water and electrolyte balance.

Vasopressin not only participates in endocrine regulation of body fluid and cardiovascular function, but also regions such as the hypothalamus, amygdala, and hippocampus are known to contain either AVP neurons or terminals and play a role in cognitive functions and regulating complex social behaviors (Shou et al., 2017; Stoop, 2012). Our findings of AVP system disruption highlight the possibility that these organohalogens may affect those other AVP brain functions (Messer, 2010; Shou et al., 2017). More refined studies would be required to assess impacts on these functions. In addition, the high body burdens of PCBs and PBDEs in children are of particular concern. Environmental organohalogens should, therefore, be considered a public health threat that must be addressed.

Conflict of interest

All authors declare no conflict of interest.

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