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SÍNDROME DEL OVARIO POLIQUÍSTICO, UNA PATOLOGÍA REGULADA POR LA INERVACIÓN EXTRÍNSECA DEL OVARIO

TESIS

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PRESENTA:

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Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 28 de septiembre de 2015, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** del (la) alumno (a) **ROSA LINARES CULEBRO** con número de cuenta **97261687** con la tesis titulada **"Síndrome del ovario poliquístico, una patología regulada por la inervación extrínseca del ovario"** realizada bajo la dirección del **DR. ROBERTO DOMÍNGUEZ CASALÁ:**

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"Sólo aquellos que se arriesgan a ir muy lejos,

pueden llegar a saber lo lejos que pueden ir"

ESTRUCTURA DE LA TESIS

En esta tesis se analiza, de manera experimental, la participación del nervio vago en la regulación de la ovulación y la secreción hormonal en ratas a las que se les indujo el modelo "Síndrome de Ovario Poliquístico" (SOPQ) por la inyección de valerato de estradiol (VE). La información se agrupó en secciones generales y en capítulos con temas específicos. La primera parte corresponde a la introducción general de esta tesis mientras que los siguientes capítulos contienen los manuscritos donde se describen y analizan los resultados experimentales obtenidos y que han sido publicados o enviados a revistas indexadas internacionales para su publicación. Los manuscritos atienden las normas editoriales de cada revista.

El **capítulo I** aborda las bases teóricas que sustentan esta investigación, así como el planteamiento, la hipótesis y objetivos del estudio. En el **capítulo II** se analiza la participación del nervio vago en la regulación del desarrollo del SOPQ inducido por la inyección de VE en la rata juvenil. Los resultados de este estudio nos llevaron a sugerir que el papel que juega el nervio vago en la regulación del desarrollo del SOPQ es mediado por la regulación que ejerce sobre la actividad monoaminérgica del ganglio celíaco mesentérico superior (GCMS). Para probar esta hipótesis en el **capítulo III** se analizaron los efectos de la vagotomía uni o bilateral realizada a ratas prepúberes inyectadas con VE sobre la actividad monoaminérgica del GCMS. En el **capítulo IV** se analizó la participación del nervio vago sobre la regulación de las funciones del ovario y la actividad monoaminérgica del GCMS en ratas prepúberes y adultas. Los resultados indican que el papel del nervio vago varía en función la edad del animal. Con base en los resultados obtenidos, en el **capítulo V** se analiza y discute cual es la participación del nervio vago en la modulación de la ovulación y la esteroidogénesis ovárica y su vinculación con el sistema monoaminérgico del GCMS en la rata adulta con o sin inyección de VE.

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ABREVIATURAS

SOPQ	Síndrome del Ovario Poliquístico
NIH	Institutos Nacionales de la Salud
ESHRE	Sociedad Europea de Reproducción Humana y Embriología
ASRM	Sociedad Americana de Medicina Reproductiva
LH	Hormona Luteinizante
FSH	Hormona Folículo Estimulante
GnRH	Hormona Liberadora de las Gonadotropinas
IGF-1	Factor de Crecimiento Semejante a la Insulina tipo 1
PT	Propionato de Testosterona
VE	Valerato de Estradiol
NA	Noradrenalina
NGF	Factor de Crecimiento Neural
NPY	Neuropéptido Y
hCG	Gonadotropina Coriónica Humana
DHEA	Dehidroepiandrosterona
DHT	5α-dihidrotestosterona
LC	Locus Coeruleus
NOS	Nervio Ovárico Superior
GCMS	Ganglio Celíaco Mesentérico Superior

RESUMEN. La hiperactividad del sistema nervioso simpático es uno de los mecanismos propuestos para explicar el desarrollo del síndrome del ovario poliquístico (SOPQ). En ratas con SOPQ inducido por la inyección de valerato de estradiol (VE), la sección unilateral del nervio ovárico superior (NOS) restaura la ovulación en el ovario inervado, mientras que la sección uni o bilateral del nervio vago lo hace en ambos ovarios. Los somas de las neuronas que originan al NOS están localizados en el ganglio celíaco mesentérico superior (GCMS), el cual recibe inervación por el nervio vago. En este estudio analizamos si el sistema monoaminérgico del GCMS es regulado por la inervación vagal en ratas sin y con SOPQ inducido por la inyección de VE. Para ello, ratas de 10 días de edad fueron inyectadas con 2 mg de VE disuelto en 0.1 ml de aceite de maíz. A los 24 (prepúberes) ó 76 (adultas) días de edad a ratas sin o con inyección de VE se les realizó una laparotomía ventral seguida por la vagotomía unilateral o bilateral y fueron sacrificadas entre los 80-82 días de edad, cuando presentaron el estro vaginal. Los resultados se compararon con grupos de animales a los cuales sólo se les realizó la laparotomía ventral. Las ratas prepúberes o adultas inyectadas con VE no ovularon, pero sí lo hicieron aquellos animales sometidos a la vagotomía unilateral o bilateral. En ratas prepúberes inyectadas con VE: La vagotomía bilateral resultó en la mayor concentración de progesterona, testosterona y estradiol. La concentración de noradrenalina (NA) en el GCMS fue menor en ratas con vagotomía derecha o bilateral. La vagotomía uni o bilateral no modificó la concentración de dopamina (DA). La concentración de serotonina (5-HT) en el GCMS fue menor en los animales con vagotomía bilateral. En ratas adultas inyectadas con VE: La vagotomía derecha resultó en altas concentraciones de progesterona y estradiol, mientras que la vagotomía uni o bilateral disminuyó la concentración de testosterona. La concentración de NA en el GCMS no se modificó por efecto de la vagotomía uni o bilateral. En los animales con vagotomía izquierda la concentración de DA en el GCMS fue menor y mayor en los animales

con vagotomía derecha. La concentración de 5-HT en el GCMS fue mayor en los animales con vagotomía derecha. En ratas prepúberes sin inyección de VE: La vagotomía uni o bilateral no modificó la respuesta ovulatoria. La concentración de testosterona y estradiol fue mayor en los animales con vagotomía izquierda, mientras que la vagotomía bilateral resultó en la menor concentración de progesterona y testosterona. La concentración de NA en el GCMS fue menor en ratas con vagotomía bilateral. La vagotomía uni o bilateral no modificó la concentración de DA y 5-HT en el GCMS. En ratas adultas sin inyección de VE: La vagotomía unilateral resultó en la disminución en el número de ovocitos liberados. La vagotomía uni o bilateral no modificó la concentración de progesterona. La concentración de testosterona fue menor en ratas con vagotomía derecha o bilateral. La concentración de estradiol fue mayor en ratas con vagotomía bilateral. La vagotomía uni o bilateral resultó en la mayor concentración de NA en el GCMS. La vagotomía uni o bilateral no modificó la concentración de DA. La concentración de 5-HT en el GCMS fue mayor en ratas con vagotomía derecha o bilateral. A partir de estos resultados sugerimos que en ratas con SOPQ inducido por VE, el GCMS sirve como un centro de comunicación nerviosa entre el nervio vago y la inervación monoaminérgica de los ovarios. Además, proveen la primera evidencia de que el nervio vago regula el sistema monoaminérgico del GCMS y que esta regulación varía con la edad y el estado endócrino del animal.

Palabras Clave: Síndrome de ovario poliquístico inducido, nervio vago, ganglio celíaco mesentérico superior, monoaminas.

ABSTRACT. The hyperactivity of the sympathetic nervous system is one of the mechanisms involved in the development of polycystic ovary syndrome (PCOS). In PCOS rats induced by injection of estradiol valerate (EV), the unilateral section of superior ovarian nerve (SON) restores ovulation in ovarian innervated while unilateral or bilateral section of the vagus nerve does in both ovaries. The cell bodies of neurons originating the SON are located in the superior mesenteric celiac ganglion (GCMS), which receives innervation of the vagus nerve. We analyzed whether the vagal innervation of the GCMS regulates the participation of the SON in rats with and without PCOS. Then, 10 days old rats were injected with 2 mg of EV dissolved in 0.1 ml corn oil. At 24 (prepuberal) or 76 (adults) day-old rats with or without injection EV underwent unilateral or bilateral vagotomy and were sacrificed between 80-82 days of age, when they presented the vaginal estrus. Ovulation did not occur in prepubertal and adult rats with PCOS, while unilateral or bilateral vagotomy restored ovulation in both ovaries. In prepubertal rats injected with EV: The bilateral vagotomy resulted in the highest concentration of progesterone, testosterone and estradiol. The concentration of noradrenaline (NA) in the GCMS was lower in rats with bilateral or right vagotomy. The unilateral or bilateral vagotomy did not change the concentration of dopamine (DA). The concentration of serotonin (5-HT) in the GCMS was lower in animals with bilateral vagotomy. In adult rats injected with EV: The right vagotomy resulted in higher concentrations of progesterone and estradiol, whereas unilateral or bilateral vagotomy decreased testosterone levels. NA concentration in the GCMS did not modify by effects to unilateral or bilateral vagotomy. In animals with left vagotomy the DA concentration in the GCMS was lower and higher in animals with right vagotomy. The concentration of 5-HT in the GCMS was higher in animals with right vagotomy. In prepubertal rats without injection VE: The unilateral or bilateral vagotomy did not change the ovulatory response. The concentration of testosterone and estradiol were higher in animals with left vagotomy, while

bilateral vagotomy resulted in lower concentrations of progesterone and testosterone. The NA concentration in the GCMS was lower in rats with bilateral vagotomy. The unilateral or bilateral vagotomy did not change the concentration of DA and 5-HT in the GCMS. In adult rats without injection of VE: Unilateral vagotomy resulted in the decrease in the number of oocytes released. The unilateral or bilateral vagotomy did not change the progesterone levels. The testosterone level was lower in rats with right or bilateral vagotomy. The estradiol levels was higher in rats with bilateral vagotomy. The unilateral or bilateral vagotomy resulted in higher NA levels in GCMS. The unilateral or bilateral vagotomy did not change the DA levels. The 5-HT level in the GCMS was higher in rats with right or bilateral vagotomy. From these results suggest that PCOS rats induced by VE, the GCMS serve as a nerve center for communication between the vagus nerve and monoaminergic innervation of the ovaries. They also provide the first evidence that the vagus nerve regulates of the GCMS monoaminergic system and that this regulation depends on the age and endocrine status of the animal.

Keywords: Induced polycystic ovarian syndrome, vagus nerve, celiac superior mesenteric ganglia, monoamines.

C A P Í T U L O I

Introducción General

Criterios de Diagnóstico del Síndrome del Ovario Poliquístico

Según Franks y col. (2008) aproximadamente el 22% de las mujeres presentan quistes en los ovarios, pero sólo algunas de ellas presentan las características del síndrome del ovario poliquístico (SOPQ), el cual se asocia principalmente con incremento en la concentración de andrógenos, obesidad e infertilidad. El SOPQ es una endocrinopatía que afecta del 4 al 18% de la población femenina en etapa reproductiva (Lim y col. 2013) y su prevalencia depende de los criterios de diagnóstico utilizados. De acuerdo a los institutos Nacionales de la Salud de los EE. UU (NIH, por sus siglas en inglés) se necesita la presencia simultánea de hiperandrogenismo (clínico o bioquímico) y anovulación. Con base en estos criterios el SOPQ se presenta del 4 al 7% de las mujeres en etapa reproductiva (Zawadzki y col. 1992; Knochenhauer y col. 1998; Diamanti-Kandarakis y col. 1999; Asunción y col. 2000; Azziz y col. 2004). En el consenso realizado en Rotterdam por la Sociedad Europea de Reproducción Humana y Embriología y la Sociedad Americana de Medicina Reproductiva (ESHRE/ASRM, por sus siglas en inglés) se concluyó que las mujeres diagnosticadas con SOPQ deben presentar dos de las tres características siguientes: anovulación, hiperandrogenismo (clínico o bioquímico) y ovarios poliquísticos. Con base en estos criterios, el SOPQ se presenta entre el 15 y 18% de la población femenina (March y col. 2010; Mehrabian y col. 2011). En el año 2006, la Sociedad del Síndrome de Ovario Poliquístico y el Exceso de Andrógenos (AE-PCOS, por sus siglas en inglés), definieron a una paciente con SOPQ cuando está presente el hiperandrogenismo (clínico o bioquímico) como característica fundamental, acompañado de anovulación o presencia de ovarios poliquísticos; así como la exclusión de otras fuentes de hiperandrogenismo. Con base en estas características, la incidencia del SOPQ es aproximadamente del 9% (Azziz y col. 2009).

Etiología del Síndrome del Ovario Poliquístico

El SOPQ es un desorden multifactorial en el que participan señales del medio ambiente, el estilo de vida y la alimentación. Estos factores pueden modificar la expresión génica que controla las vías metabólicas y hormonales que regulan nuestro organismo (Bremer, 2010). Las teorías sobre la etiología del SOPQ se centran en el papel estimulante que la LH y la insulina tienen sobre la producción de andrógenos, lo que provoca un estado de hiperandrogenismo (Poretsky, 1994), que es la característica fundamental en el diagnóstico del SOPQ.

El origen del hiperandrogenismo se explica por la participación de diversos factores que regulan la síntesis de andrógenos.

1. Alteración en los pulsos de secreción de la GnRH: En mujeres adultas y adolescentes diagnosticadas con el SOPQ, la frecuencia de los pulsos de la hormona liberadora de las gonadotropinas (GnRH) es mayor que en las mujeres sin el síndrome (Waldstreicher y col. 1988; Haisenleder y col. 1991); aumenta la expresión del RNAm de la subunidad β de la hormona LH, y provoca un incremento en la proporción de LH/FSH (Taylor y col. 1997); las células de la teca aumentan la secreción de andrógenos, por la activación del complejo citocromo P450c17 (Miller, 2008); las células de la granulosa presentan una menor actividad de las aromatasas por lo que no pueden aromatizar los andrógenos a estrógenos y en consecuencia se presenta una condición de hiperandrogenismo y anovulación (Nicandri y Hoeger, 2012).

En mujeres con SOPQ el incremento en la concentración de LH se correlaciona con la alta concentración de testosterona libre (Lobo y col. 1983). El tratamiento con estrógenos reduce la concentración de LH y ello favorece la disminución de andrógenos circulantes y el crecimiento del vello facial (Chang y col. 1983). Dado que algunas mujeres con SOPQ tienen concentraciones

normales de LH, se sugiere que otros factores pueden contribuir a la producción excesiva de andrógenos (Chang, 2007).

2. Resistencia a la insulina: Aproximadamente del 50 al 70% de las mujeres con SOPQ tienen algún grado de resistencia a la insulina (Legro y col. 2004). La mayoría de los estudios apoyan la hipótesis de que la resistencia a la insulina es el factor primario responsable del aumento de la producción de andrógenos (Bremer y Miller, 2007). La insulina modula la producción de andrógenos por mecanismos directos e indirectos. La insulina reduce la secreción de la globulina transportadora de hormonas sexuales por parte del hígado, lo que resulta en el incremento de andrógenos disponibles y activos (Pfeifer y Kives, 2009). También se propone que en las células de la teca interna la insulina incrementa la expresión de los genes que codifican el ARNm del complejo P450c17, el cual hidroxila a la pregnenolona y la progesterona que son precursores de andrógenos (Baptiste y col. 2010).

En las pacientes con SOPQ la acción de la insulina sobre el transporte y las vías metabólicas de la glucosa se ve alterada en tejidos como el músculo esquelético, el hígado y el tejido adiposo (Dunaif y col. 1992; Phy y col. 2015), mientras que en el ovario el papel estimulante de la insulina sobre la producción de andrógenos no se modifica (Hernandez y col. 1988). La resistencia a la insulina puede ser atribuida a defectos en la señalización de su receptor (Dunaif, 2001). El receptor a la insulina es un heterotetrámero conformado por dos dímeros α y β unidos entre si por enlaces disulfuros. La subunidad α es extracelular y contiene el dominio de unión a la insulina, la subunidad β atraviesa la membrana celular y posee la actividad de una proteína quinasa (Kahn y col. 1993). La acción de la insulina es iniciada por su unión a la subunidad α del receptor, que induce la activación de la subunidad β de la tirosina quinasa por autofosforilación sobre los residuos de tirosina y ello activa la cascada de señalización que permite el ingreso de la glucosa a la célula. En pacientes con SOPQ la fosforilación de la tirosina disminuye e incrementa

la fosforilación de los residuos de la serina, lo que resulta en la inhibición de la acción de la insulina en las vías metabólicas y ello explica el desarrollo de la resistencia a la insulina y la hiperinsulinemia (Dunaif, 1997; Phy y col. 2015).

En el ovario la fosforilación de la serina estimula la actividad de la 17,20 liasa, la enzima responsable de convertir la 17-hidroxiprogesterona en androstendiona, lo que da lugar a una mayor producción de andrógenos en el ovario (Zhang y col. 1995). La hipófisis también contiene receptores para la insulina y su activación incrementa la secreción de LH y en consecuencia la producción de andrógenos (Pfeifer y Kives, 2009).

En el ovario humano se han identificado receptores para la insulina y para el factor de crecimiento semejante a la insulina tipo 1 (IGF-1R). El IGF-1R es un homólogo de los receptores a insulina pero con menor afinidad. La unión de la insulina al IGF-1R favorece el desarrollo de la resistencia a la insulina y la hiperinsulinemia, tal como ocurre en el SOPQ. La hiperinsulinemia incrementa la formación de receptores IGF-1R (Barbieri y col. 1986) con lo cual se estimula una mayor producción de andrógenos.

3. Programación fetal: Estudios en ovejas (Steckler y col. 2007, 2009; Hogg y col. 2011) y primates (Abbott y col. 1998; 2002; 2007) mostraron que la exposición a un exceso de andrógenos en la vida fetal resulta en la reducción o ausencia de la función ovárica cuando el animal llega a la etapa adulta (Abbott y col. 2007; Franks, 2009). La inyección subcutánea de 5mg de testosterona a ratas desde el día 16 hasta el día 19 de gestación, resulta en obesidad, altas concentraciones en suero de insulina, colesterol y triglicéridos cuando los animales llegan a la vida adulta, sin modificaciones en la concentración de glucosa o de la prueba de tolerancia a la glucosa (Demessie y col. 2008). La inyección de propionato de testosterona (PT) a ratas durante la vida fetal (14.5 al 21.5 de gestación) no tuvo efectos sobre la función reproductiva en la vida adulta, y

sólo se observó disminución en el pool de folículos primordiales. Mientras que el tratamiento con PT durante la vida neonatal (desde el día 1 al día 24 de vida) resultó en anovulación cuando los animales llegaron a la etapa adulta (Tyndall y col. 2012). Estos resultados apoyan la idea de que la programación de andrógenos sobre la función reproductora se produce sólo durante ventanas de tiempo específicos en la vida fetal y neonatal con implicaciones para el desarrollo del SOPQ observado en mujeres.

En pacientes con el SOPQ, el crecimiento folicular llega hasta la etapa de folículo antral mediano, después de lo cual se detiene el crecimiento y la maduración, y los folículos presentan signos de atresia (apoptosis, descamación y disminución de células de la granulosa) (Erickson, 1992). La acumulación del líquido folicular resulta en la expansión del antro. A medida que el folículo aumenta de tamaño, disminuye el número de capas de células de la granulosa que rodean al ovocito dando lugar a la aparición de un quiste con una delgada capa de células de la granulosa. La capa de células de la teca es más grande que la que se encuentra en los folículos normales, lo que explica el incremento en la producción de andrógenos (Chang, 2007). En las células de la teca de mujeres con SOPQ hay una sobreexpresión del complejo P450c17 y de la 3ß-hidroxiesteroide deshidrogenasa, enzimas relacionadas con la producción de andrógenos (Lachelin y col. 1982).

4. Hiperactividad nerviosa: En mujeres con SOPQ la resistencia a la insulina y la obesidad se vinculan con alteraciones en la actividad de los nervios simpáticos que inervan los ovarios (Landsdown y Rees, 2012). Por ello, las hipótesis más recientes sobre la patogénesis del SOPQ plantean que éste se origina por la hiperactividad de las fibras simpáticas.

La inyección de valerato de estradiol (VE) a ratas infantiles (Rosa-E-Silva y col. 2003) o adultas (Barria y col. 1993) activa las neuronas simpáticas periféricas que inervan al ovario, influencia que contribuye al incremento en el contenido de noradrenalina (NA) ovárica, lo que se traduce en el

desarrollo de algunas de las características de diagnóstico del SOPQ (Lara y col. 1993). Cuando se inyectan células productoras del factor de crecimiento neural (NGF) en el ovario de ratas juveniles se altera la dinámica folicular e incrementa la concentración de androstenediona, lo que sugiere que la sobreproducción de NGF puede iniciar la patología ovárica (Lara y col. 2000).

En animales con SOPQ el tratamiento con electro-acupuntura de baja frecuencia disminuye la concentración de NGF y revierte la condición quística (Stener-Victorin y col. 2000). A raíz de estos resultados se han realizado pruebas piloto en algunas mujeres con SOPQ. En un primer estudio, se mostró que después de 16 semanas de someter a la mujer a la estimulación por electro-acupuntura, disminuye la actividad simpática, sin que se modifique la resistencia a la insulina, aunque se desconoce sí en estas pacientes el tratamiento indujo la ovulación (Stener-Victorin y col. 2009). En otro estudio, el tratamiento con electro-acupuntura fue más prolongado lo que resultó en el restablecimiento en los ciclos menstruales y disminución de la concentración de andrógenos (Landsdown y Rees, 2012). Dado que la técnica de electro-acupuntura no es invasiva, parecería ser una buena opción para mujeres en las que los tratamientos farmacológicos no funcionan.

5. Otros factores asociados con el desarrollo del SOPQ: La obesidad, es uno de los factores que se asocia al desarrollo del SOPQ. La incidencia de obesidad en pacientes con SOPQ aumentó del 51%, reportado entre 1987 y 1990, hasta un 75-88% hoy en día (Glueck y col. 2005; Ching y col. 2007; Yildiz y col. 2008; Lim y col. 2013). Algunas mujeres con SOPQ tienen una gran cantidad de grasa visceral y subcutánea, lo que se relaciona con el desarrollo de la resistencia a la insulina (Karabulut y col. 2012). La obesidad empeora el cuadro clínico de hiperandrogenismo, las alteraciones menstruales y la infertilidad (Lim y col., 2013) y puede llegar a desarrollar el síndrome metabólico, que implica la alteración en la tolerancia a la glucosa y el desarrollo de diabetes tipo II (Moran y col. 2010). La obesidad puede desarrollarse por factores genético-

hereditarios y ambientales, que pueden afectar la síntesis de leptina (Velásquez, 2011), hormona secretada por el tejido adiposo que actúa sobre las neuronas ubicadas en el núcleo arcuato del hipotálamo y de esta manera regula la ingesta de alimento y el balance energético. El núcleo arcuato contiene dos grupos distintos de neuronas: Las neuronas que sintetizan el neuropéptido Y (NPY), el cual estimula la ingesta de alimento, inhibe la saciedad, disminuye la pérdida calórica y aumenta la actividad de las enzimas lipogénicas hepáticas y del tejido adiposo, produciendo obesidad y las neuronas que sintetizan propiomelanocortina (POMC), la que estimula la saciedad e inhibe la ingesta de alimentos (Velásquez, 2011).

Aunque se ha planteado que la leptina pudiera participar en el desarrollo del SOPQ, existe controversia al respecto. Algunos autores reportan que no hay diferencias en la concentración de la hormona entre mujeres con o sin el SOPQ (Laughlin y col. 1997) y otros, que la concentración es mayor en las mujeres con SOPQ (Mantzoros y col., 2000). Se propone que la leptina puede ejercer sus efectos directamente sobre las neuronas hipotalámicas productoras de GnRH y, de esta manera, regular la secreción de LH y FSH. También se plantea una acción indirecta al modificar la secreción del NPY, el cual regula la secreción de GnRH, lo que resulta en el incremento de la secreción de LH y de los andrógenos (Jacobs, 1997). No se descarta que la leptina actúe sobre la secreción de esteroides a nivel del ovario (Velásquez, 2011).

En el desarrollo del SOPQ también participa los antecedentes genéticos de la paciente. Las anormalidades reproductivas y metabólicas que caracterizan al SOPQ, tienen una mayor incidencia entre las mujeres de una misma familia (Moran, 2011). Los hermanos y las hermanas de mujeres con SOPQ tienen alta concentración de andrógenos, lo que indicaría el papel genéticamente heredable del síndrome (Lenarcik y col. 2011).

Las alteraciones genéticas asociadas al SOPQ incluyen principalmente al gen que codifica para el receptor a LH (LHCGR), el receptor a andrógenos, el gen CYP11A (que codifica para síntesis del complejo P450scc), el gen de la insulina y su receptor, el inhibidor del activador del plasminógeno-1 y el receptor gamma activador proliferativo del peroxisoma. Recientemente se ha identificado en el tejido adiposo alteración en la expresión del gen que codifica para la interleucina-6, el cual ésta vinculado con alteraciones metabólicas (Huang y Coviello, 2012).

El primer estudio de asociación del genoma del SOPQ fue reportado en mujeres chinas, donde se identificaron 2 loci susceptibles en el desarrollo del SOPQ. El primero se identificó sobre el cromosoma 2, el cual se vincula con el gen que codifica para el LHCGR. El segundo sobre el cromosoma 9, relacionado con el gen asociado con la obesidad (Goodarzi y col. 2012; Welt y col., 2012). La alteración de estos genes identificados en mujeres chinas con SOPQ se asoció con anormalidades en la tolerancia a la glucosa (Lerchbaum y col. 2011).

Mujeres (Xu y col. 2011) y primates hembras no-humanos (Xita y Tsatsoulis, 2006), expuestas *in* útero a altas concentraciones de andrógenos, desarrollan características fenotípicas del SOPQ en la vida adulta. Este evento se relaciona con alteraciones en la metilación del ADN y modificaciones en las histonas de células sanguíneas periféricas y del tejido adiposo. Por lo tanto, la exposición a hormonas sexuales en periodos críticos del desarrollo puede influenciar la expresión de fenotipos metabólicos y reproductivos en la vida adulta, por efectos epigenéticos mediados por factores ambientales o intrauterinos (Xita y Tsatsoulis, 2006; Xu y col. 2011).

Modelos de inducción del Síndrome del Ovario Poliquístico

Hasta el momento no hay consenso sobre cual o cuales mecanismos explican las alteraciones que acompañan al SOPQ. Para estudiar el ciclo reproductivo, la morfología ovárica y los cambios hormonales asociados con la fisiopatología de anovulación crónica que se observa en pacientes con SOPQ se han utilizado diversos modelos experimentales (Singh, 2005).

1. Modelo de luz constante. Ratas hembras adultas expuestas a luz constante desarrollan gradualmente anovulación crónica asociada con el SOPQ (Singh, 1969c; Beys y col. 1995). La intensidad, duración y características espectrales de la luz influencian la tasa de anovulación crónica (Weber y Adler, 1979). La ovulación espontánea es regulada por mecanismos neuroendocrinos controlados por un reloj biológico. El aumento brusco ("pico") en la concentración de la LH es controlado por el ciclo de luz-obscuridad al que está sometido el animal (Everett, 1964; McCormack y Sridaran, 1978). En los animales mantenidos en luz constante la concentración de LH es similar a los controles con ciclo contralado de luz/oscuridad, las concentraciones de la FSH y de progesterona son bajas, mientras que la concentración de estradiol es mayor que en los controles (Lowton y Schwartz, 1967; Takeo, 1984). La exposición a la luz ambiental revierte la condición acíclica y anovulatoria provocada en los animales por la exposición a la luz constante (Shi, 2012).

La cópula estimula la ovulación en ratas mantenidas en luz constante y lo mismo ocurre si los animales son inyectados con progesterona o gonadotropina coriónica humana (hCG) o sometidos a la ovariectomía unilateral. La tasa de preñez es normal y las crías expuestas a condiciones de luz constante no desarrollan ovarios poliquísticos cuando llegan a la pubertad (Singh y col. 1969 a, b, c).

No se conoce el mecanismo por el cual los animales expuestos a luz constante no ovulan. Se postula que esto puede ser debido al estrés continuo provocado por la exposición a la luz constante, que activa al sistema nervioso simpático y resulta en hipertrofia de la glándula adrenal (Singh, 1969c). En animales expuestos a iluminación crónica, los volúmenes absolutos y relativos de la zona fasciculada, así como la concentración sérica de corticosterona incrementan significativamente. Por lo que se concluye que la exposición a ratas hembras a luz constante incrementa el crecimiento y la actividad secretora de las células de la zona fasciculada, que puede ser relacionada a estrés crónico (Miloševic y col. 2005). Otro mecanismo que explica la falta de ovulación en ratas con exposición a luz constante puede ser la disminución en la actividad de la glándula pineal y supresión de la producción de melatonina, ya que su administración induce ovulación en el 70% de ratas expuestas a luz constante (Trentini y col. 1978).

2. Modelo del animal androgenizado. El hiperandrogenismo es la manifestación primaria del SOPQ. Una de las hipótesis sobre la etiología del síndrome es que la exposición a altas concentraciones de andrógenos durante la vida neonatal lleva a desarrollar SOPQ durante la vida adulta (Shi y Vine, 2012). En monos Rhesus la exposición intrauterina a altas concentraciones de andrógenos circulantes altera la maduración ovárica folicular y favorece la formación de quistes cuando estos animales llegan a la etapa adulta (Abbott y col. 2005).

Para inducir el SOPQ en ratas se han utilizado diferentes andrógenos y formas de tratamiento como la inyección diaria o la colocación de implantes subcutáneos de dehidroepiandrosterona (DHEA), androstenediona, PT y 5α-dihidrotestosterona (DHT) (Shi y Vine, 2012).

En la etapa peripuberal de la rata hembra, aumenta la concentración sérica de DHEA (Mahesh y col. 1967; Apter y col. 1994) y el 50% de la testosterona circulante deriva de la conversión de DHEA (Haning y col. 1994). El 25% de las pacientes con SOPQ presentan altas concentraciones de DHEA (Azziz y col. 2009). La inyección prolongada de DHEA a ratas hembras de 22 días de

edad resulta en el aumento del peso de los ovarios, la formación de quistes foliculares que presentan degeneración de la capa de células de la granulosa y aumento en la concentración de DHEA, testosterona, estradiol, FSH, LH y prolactina (Knudsen y col. 1975; Knudsen y Mahesh, 1975; Parker y Mahesh, 1976; Lee y col. 1991; Apter y col. 1994; Trivax y Azziz, 2007; Cussons y col. 2008). Según otros autores, el tratamiento prolongado con DHEA a ratas de 21 días de edad no modifica las concentraciones de FSH y LH (Anderson y col. 1997; Henmi y col. 2001).

Ratas hembras de 21 días de edad inyectadas durante 35 días con PT resulta en el desarrollo de múltiples quistes foliculares con hipertecosis, falta de cuerpos lúteos y aumento del número de folículos preantrales (Beloosesky y col. 2004). En este modelo de androgenización aumenta la concentración sérica de testosterona, LH y prolactina, y disminuyen las de progesterona, estradiol y FSH (Ota y col. 1983). La administración de PT al nacimiento resultó en falta de ciclo estral, ausencia de ovulación, quistes foliculares, incrementó en la concentración sérica de estradiol y LH y disminución en la concentración de FSH (Díaz, 2010).

El implante subcutáneo de una cápsula de liberación continua de DHT, un andrógeno no aromatizable, en ratas de tres semanas de edad, resultó en el incremento en la concentración sérica de leptina y resistencia a la insulina, sin cambios en la de testosterona y estradiol y menor concentración de progesterona, ciclos estrales irregulares, ovarios poliquísticos, aumento en la incidencia de folículos atrésicos con reducida capa de las células de la granulosa e hipertécosis de la capa de las células de la teca interna (Mannerås y col. 2007). En ratas el tratamiento prenatal con DHT o testosterona resulta en desfeminización del hipotálamo e incapacidad para generar el pico preovulatorio de la GnRH/LH inducido por los estrógenos y falta de ovulación (Foecking y col. 2005).

3. Modelo del animal estrogenizado. En el animal adulto la inyección de VE, un estrógeno de larga actividad, provoca cornificación vaginal persistente y ovarios poliquísticos anovulatorios

(Brawer y col. 1986). Los ovarios son pequeños, con folículos secundarios, pocos folículos sanos y ausencia de cuerpos lúteos. La característica más notable es la presencia de quistes ováricos, que presentan pocas capas de células de la granulosa y una gruesa capa de células de la teca (Hemmings y col. 1983). Las estructuras quísticas que se forman en el ovario de la rata, a pesar de que son muy semejantes a las que se observan en la mujer, carecen de ovocito (Lara y col. 2000). Como consecuencia de la falta de cuerpos lúteos el peso de las gónadas de animales con SOPQ es menor que en los animales normales (Brawer y col. 1986; Rosa-E-Silva y col. 2003).

En la rata de 14 días de edad, la inyección de VE resulta en la disminución de la concentración sérica de LH y FSH, lo que se explica por la disminución en la sensibilidad de la hipófisis a la GnRH, por el incremento en la producción de estradiol ovárico o por una mayor sensibilidad de la hipófisis al mecanismo de retroalimentación del estradiol (Rosa-E-Silva y col. 2003). Otros estudios señalan que el VE no modifica la concentración de las gonadotropinas (Schulster y col. 1984; Morales y col. 2010). Estudios *in vitro* muestran que los ovarios de animales adultos con SOPQ inducido con VE, tienen mayor capacidad para secretar estradiol porque la actividad de la aromatasa es mayor, mientras que no se modifica la secreción de progesterona y andrógenos (Barria y col. 1993).

El SOPQ inducido por la inyección de VE a ratas adultas resulta en un incremento en el contenido de NA ovárica, mayor liberación de NA por parte de las terminales nerviosas ováricas y disminución en el número de receptores β-adrenérgicos en células de la teca intersticial y células de la granulosa. Estos cambios preceden a la aparición de quistes foliculares, lo cual sugiere la participación de la inervación simpática en el desarrollo del síndrome (Lara y col. 1993).

Veinticuatro horas después de la inyección de VE a ratas neonatas (36, 60 y 48 horas de nacidas) incrementa la expresión del ARNm del NGF y de su receptor de baja afinidad (p-75). En estos animales hay adelanto de la edad de apertura vaginal (pubertad), aciclicidad estral, y en los ovarios

presencia de quistes foliculares, ausencia de cuerpos lúteos y disminución en el número de folículos primordiales, lo cual sugiere que el VE actúa en los primeros estadios del desarrollo folicular (Sotomayor y col. 2008).

4. Modelo del animal expuesto a estrés. Observaciones clínicas sugieren que el estrés puede ser un factor que prevalece en pacientes con el SOPQ (Lobo y col. 1983). Aunque el estrés no es el único factor que participa en la etiología de la fisiopatología, los estudios sobre el papel de los neurotransmisores involucrados en la regulación de la respuesta reproductiva ante una condición de estrés se han enfocado sobre aquellos que afectan la secreción de GnRH, la razón LH/FSH y la ovulación (Schenker y col. 1992). El estrés por frío activa varias áreas cerebrales involucradas en la regulación de la respuesta neuroendocrina (Bhatnagar y Dallman, 1998).

Cuatro semanas después de haber sometido a ratas adultas a estrés por frío se observa mayor actividad de las fibras simpáticas, evaluada por la alta concentración de NA ovárica, que precede a la aparición de quistes con hipertecosis. Con estos resultados los autores postulan que el desarrollo del SOPQ está relacionado con el incremento de la actividad del sistema nervioso simpático (Dorfman y col. 2003).

El locus coeruleus (LC), junto con el núcleo paraventricular, juegan un papel central en la regulación del eje hipotálamo-hipófisis-adrenal y el sistema nervioso simpático, mediando las respuestas inducidas por los cambios que produce el estrés (Chrousos y Gold, 1992; Valentino y col. 1993). Las neuronas del LC son activadas por diversos factores (Passerin y col. 2000; Osterhout y col. 2005; Kwon y col. 2006), incluido el estrés por frío (Daiguji y col. 1982; Kiyohara y col. 1995; Yuan y col. 2002) que incrementa la secreción de la hormona liberadora de corticotropina y glucocorticoides y resulta en la inhibición del eje hipotálamo-hipófisis-ovario (Anselmo-Franci y col., 1997; Anselmo-Franci y col., 1999; Helena y col., 2002; Kalantaridou y col. 2004).

El estrés por frío provoca alteraciones en el desarrollo folicular similares a las observadas en el SOPQ inducido por la inyección de VE, que se asocian con disfunciones reproductivas que al parecer están mediadas por el LC. En los ovarios de ratas adultas sometidas a estrés crónico por frío durante ocho semanas, se observan quistes foliculares, folículos tipo III o también llamados prequístes y folículos con hipertecosis; así como incremento en las concentraciones de testosterona y estradiol, aciclicidad estral, reducción de la ovulación y concentración de NA ovárica similar a los controles. En ratas con lesión del LC y expuestas a estrés por frío, disminuye la actividad NA en el ovario y previene los efectos producidos por el estrés (Bernuci y col. 2008).

En ratas adultas la exposición a estrés por frío durante tres semanas disminuye el contenido de NA en el ovario, el número de folículos preantrales y la concentración de androstenediona, sin modificar la concentración de progesterona, estradiol y el contenido de NGF. Después de cuatro semanas de exposición a estrés por frío se observa incremento en el contenido de NA ovárica y del NGF, semejante a los efectos que produce la inyección de VE, que se acompaña de incremento en el número de folículos con hipertecosis que anteceden la formación de quistes foliculares (Dorfman y col. 2003).

En la rata adulta expuesta a estrés por frío y restricción del movimiento durante tres semanas, se observa incremento en el contenido de NA en el ganglio celiaco, disminución de la tasa de ovulación e incremento en el número de folículos prequísticos. Después de 11 semanas de estrés aumenta el contenido de NA en el ovario y en el ganglio, con ovulación y morfología ovárica similar a los controles (Paredes y col. 1998).

A partir del postulado de que en los animales con SOPQ, inducido por la inyección de VE, se produce la hiperactividad de las fibras simpáticas evidenciado por las altas concentraciones de NA ovárica, algunos grupos de investigación, incluido el nuestro, hemos mostrado que la eliminación total de la información simpática que transcurre por el nervio ovárico superior (NOS)

disminuye el tono noradrenérgico del ovario, lo que explicaría por qué se restablece el ciclo estral y la ovulación (Barria y col. 1993; Rosa-E-Silva y col. 2003; Morales y col. 2010).

En ratas con SOPQ, inducido con VE en la etapa infantil (10 días de edad), la sección unilateral del NOS realizada a los 24 días de edad, resulta en el restablecimiento de la ovulación por la gónada inervada a pesar de las altas concentraciones de NA ovárica observadas en estos animales. Con base en estos resultados, se sugiere que además del aumento del tono noradrenérgico que llega a los ovarios por el NOS, existen otras vías neurales que llegan a los ovarios y regulan la ovulación espontánea y la patogénesis del SOPQ (Morales y col. 2010).

Muy poco se sabe acerca de los mecanismos en los que participa la información que transcurre por los nervios vago en la regulación de las funciones ováricas. El nervio vago contiene fibras sensoriales que llevarían información desde los ovarios hasta el hipotálamo, la cual participa en la regulación de la secreción de gonadotropinas (Lawrence y col. 1978). Otra información nerviosa que llega a los ovarios por medio del nervio vago regularía los efectos de las gonadotropinas sobre el ovario (Cruz y col. 1986). De acuerdo con Berthoud y Powley (1996), a nivel del ganglio celíaco mesentérico superior (GCMS), donde se localizan los somas de la neuronas que forman al NOS, existe una comunicación entre las fibras simpáticas y parasimpáticas.

A partir de esta evidencia sugerimos que es a nivel del GCMS, donde la información nerviosa que transcurre por el nervio vago modula la función del NOS en la persistencia del SOPQ. Por ello, en el presente estudio se analizó si la actividad monoaminérgica del GCMS, la ovulación espontánea y la esteroidogénesis ovárica son reguladas por la información nerviosa que transcurre por el nervio vago.

PLANTEAMIENTO DEL PROBLEMA

El nervio vago es una de las tres vías nerviosas que participan en la regulación de las funciones del ovario. El 90% de sus fibras llevan información desde la periferia hacia el sistema nervioso central (SNC). Su sección bilateral resulta en un aumento del número de ovocitos liberados por animal ovulante, por lo cual se considera que la participación de la inervación vagal en la regulación de la ovulación, es de tipo inhibitoria.

Se postula que el SOPQ es el resultado de la hiperactividad de las fibras simpáticas que llegan al ovario por el NOS. En la rata, el SOPQ inducido por VE o estrés prolongado por frío, se acompaña del aumento en la actividad de la tirosina hidroxilasa en las neuronas del GCMS y ausencia de ovulación.

En ratas inyectadas con VE la sección bilateral del NOS restablece la respuesta ovulatoria en ambos ovarios, mientras que la sección unilateral la restablece sólo en la gónada inervada. A partir de estos hechos se sugiere que además de la inervación simpática aportada por el NOS, hay otros factores nerviosos que participan en el desarrollo y mantenimiento del SOPQ. Dado que algunas fibras del nervio vago hacen sinapsis con las neuronas simpáticas del GCMS que dan origen al NOS, y que otras inervan directamente al ovario, se sugiere que parte de la regulación que ejerce el nervio vago sobre las funciones del ovario son por sus efectos sobre las neuronas que dan origen al NOS.

Dado que la sección unilateral o bilateral del nervio vago en ratas inyectadas con VE, restablece la ovulación en ambos ovarios, en el presente estudio se analizó si este restablecimiento depende de la regulación que ejerce el nervio vago sobre el sistema monoaminérgico del GCMS.

HIPÓTESIS

Dado que la inducción del SOPQ por la inyección con VE, aumenta la actividad simpática en el GCMS, y que en éste hay sinapsis de fibras nerviosas que transcurren en el NV con neuronas catecolaminérgicas que posiblemente dan origen a fibras que transcurren por el NOS, entonces en la rata con SOPQ la disminución del tono simpático a nivel del GCMS producida por la sección uni o bilateral del nervio vago, sería uno de los mecanismos que explicaría el restablecimiento de la ovulación en esos animales.

OBJETIVO GENERAL

Analizar la participación del nervio vago en el animal sin o con SOPQ inducido por la inyección de VE, en la regulación de la actividad monoaminérgica en el GCMS.

OBJETIVOS PARTICULARES

- Evaluar en ratas prepúberes inyectadas con VE los efectos de la sección uni o bilateral del nervio vago sobre la ovulación, la morfología ovárica y la secreción de hormonas esteroides.
- Evaluar en ratas prepúberes con inyección de VE los efectos de la sección uni o bilateral del nervio vago sobre la concentración de noradrenalina, dopamina, serotonina y sus metabolitos en el GCMS.
- Analizar en ratas prepúberes y adultas los efectos de la vagotomía uni o bilateral sobre la ovulación, la secreción de hormonas esteroides y la concentración de monoaminas en el GCMS.
- Evaluar en ratas adultas inyectadas con VE los efectos de la sección uni o bilateral del nervio vago sobre la ovulación, la secreción de hormonas esteroides y la concentración de monoaminas en el GCMS.

C A P Í T U L O II

Unilateral or bilateral vagotomy induces ovulation in both ovaries of rats with polycystic ovarian syndrome

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REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

RESEARCH Open Access

Unilateral or bilateral vagotomy induces ovulation in both ovaries of rats with polycystic ovarian syndrome

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Abstract

Background: Injecting estradiol valerate (EV) to pre-pubertal or adult female rat results in effects similar to those observed in women with polycystic ovarian syndrome (PCOS). One of the mechanisms involved in PCOS development is the hyperactivity of the sympathetic nervous system. In EV-induced PCOS rats, the unilateral sectioning of the superior ovarian nerve (SON) restores ovulation of the innervated ovary. This suggests that, in addition to the sympathetic innervation, other neural mechanisms are involved in the development/maintenance of PCOS. The aims of present study were analyze if the vagus nerve is one of the neural pathways participating in PCOS development.

Methods: Ten-day old rats were injected with EV dissolved in corn oil. At 24-days of age sham-surgery, unilateral, or bilateral sectioning of the vagus nerve (vagotomy) was performed on these rats. The animals were sacrificed at 90–92 days of age, when they presented vaginal estrous preceded by a pro-estrus smear.

Results: In EV-induced PCOS rats, unilateral or bilateral vagotomy restored ovulation in both ovaries. Follicle-stimulating hormone (FSH) levels in PCOS rats with unilateral or bilateral vagotomy were lower than in control rats.

Conclusions: This result suggests that in EV-induced PCOS rats the vagus nerve is a neural pathway participating in maintaining PCOS. The vagus nerve innervates the ovaries directly and indirectly through its synapsis in the celiac-superior-mesenteric ganglion, where the somas of neurons originating in the SON are located. Then, it is possible that vagotomy effects in EV-induced PCOS rats may be explained as a lack of communication between the central nervous system and the ovaries.

Keywords: Polycystic ovarian syndrome (PCOS), Vagus nerve, Ovarian innervation, Ovulation, Vagotomy

Background

Polycystic ovary syndrome (PCOS) is considered the most common cause of infertility in woman, with approximately 10 percent of women of reproductive age being affected with PCOS [1]. PCOS is characterized by a complex pathophysiology that includes anovulation, oligomenorrhea, follicular cysts, hyper-androgenism, hyper-estrogenism and variable levels of gonadotropins in blood [2-4]. PCOS diagnosis is based on the presence of three main features: the presence of more than 12 cysts in the ovaries, anovulation, and hyper-androgenism [5,6]. In some cases PCOS results in glucose metabolic disorders, cardiovascular diseases, dyslipidemia and cancer [4].

The etiology of PCOS is multifactorial and is attributed to genetic factors as well as primary defects of the hypothalamic-pituitary unit, micro-enviroment of the ovary such defects of intraovarian molecules involved in paracrine/autocrine regulation, such as insulin-like growth factor-I, and an overactive adrenal gland [4,7].

Ovarian functions are regulated by hormonal and neural signals. Hormonal signals regulating ovarian

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functions arise from the pituitary, adrenal, ovaries, thymus and thyroid; while neural signals arrive to the ovaries through the superior ovarian nerve (SON), the plexus ovarian nerve (OPN) and the vagus nerve. Neural signals modulate the effects of hormonal signals on the follicular, luteal and interstitial compartments [8-10].

Experimental models proposed to study the POCS include injecting estradiol valerate (EV), neonatal androgenization, the exposure of animals to constant light, and chronic stress induced by cold [11-13].

EV is a long acting estrogen. Injecting 2 mg of EV to infantile or adult rats results in the interruption of estrous cycle, persistent vaginal cornification, anovulation, formation of follicular cysts, alterations to the basal and pulsatile concentration of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as high concentrations of estradiol (E_2) and testosterone (T) [14-16]. These effects are similar to those observed in women with PCOS.

Injecting luteinizing hormone-releasing hormone (LHRH) to EV-induced PCOS adult rat increased LH levels resulting in spontaneous ovulation [17,18]. There week after surgery unilateral ovariectomy to EV-induced PCOS adult rats, the remaining ovary showed follicles at all stages of development, corpora lutea, and an absence of cystic follicles [19].

In EV-induced PCOS infantile or adult rat, the ovarian content of norepinephrine (NE) is higher than in normal rats. Higher NE concentrations suggest increased activity of the sympathetic nerves and a derangement of sympathetic inputs to the ovary, two factors that contribute to the persistence of PCOS symptoms [20]. Electroacupuncture treatment or bilateral sectioning of the SON to EV-induced PCOS rat reduces sympathetic activity and resets the animals' estrous cycle, LH secretion, steroidogenesis and ovulation [14,15,21,22]. Unilateral sectioning of the SON restores ovulation in the innervated ovary by 80% and 26% by the denervated ovary, suggesting that in addition to an increase in sympathetic activity, other neural pathways reaching the ovaries are involved in the PCOS development [13].

According to Gerendai et al., [10,23], a multi-synaptic neural pathway between the ovary and the central nervous system (CNS) is involved in regulating ovarian functions. In the adult rat, bilateral vagotomy altered the estrous cycle [24], blocked pseudo-pregnancy induction [25], increased the number of ova shed by ovulating adult and pre-pubertal rats [26,27], and in pregnant rats resulted in lower LH basal levels, causing fetal resorption [28]. Taken together, these results suggest that the information arriving to the ovaries through the vagus nerve participates in regulating ovarian functions.

Bilateral abdominal vagotomy to pre-pubertal rats delayed the onset of puberty, and depressed E_2 and progesterone (P_4) response to human chorionic gonadotropin (hCG) *in vitro* [29].

Unilateral vagotomy affects spontaneous ovulation in different ways: in adult rats, sectioning the left vagus nerve resulted in lower ovulation rates, while sectioning the right vagus nerve did not have an apparent effect on ovulation rates or the number of ova shed by ovulating animals [26]. Based on these results, the researchers postulated that the neural information carried by the left vagus nerve plays a more significant role in the ovulatory process than the information carried by the right vagus nerve [26].

In pre-pubertal rats, unilateral vagotomy did not modify ovulation rates or the number of ova shed. Sectioning the right vagus nerve to 28 day old rats resulted in lower E_2 levels and a delay of puberty onset, while sectioning the left vagus nerve had no apparent effects [27].

To our knowledge, the vagus nerve involvement in developing and regulating EV-induced PCOS rat has not been assessed. The aim of the present study was to analyze if the vagus nerve is one of the neural pathways participating in PCOS development. For this purpose we studied the effects of unilateral or bilateral vagotomy on ovarian steroidogenesis and ovulatory response in the EV-induced PCOS rats.

Methods

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines. The Committee of the Facultad the Estudios Superiores Zaragoza approved the experimental protocols. The study was performed using pre-pubertal female rats of the CIIZ-V strain from our own breeding stock. Animals were maintained under controlled lighting conditions (lights on from 05:00 to 19:00 h); with free access to rat chows pellets and tap water.

Animal treatment

Ten-day old rats were injected with a single 0.1 ml corn oil (vehicle Vh) dose or 2 mg EV (Sigma Chem. Co., St. Luis, Mo. USA) dissolved in 0.1 ml corn oil. When Vh or EV injected animals reached 24 days of age they were randomly allotted to one of the following groups: 1) unilateral sectioning of the left (LSVN) or right (RSVN) vagus nerve; 2) bilateral vagotomy or; 3) sham-surgery.

Vagotomy and sham-surgery procedures were performed between 10:00 and 12:00 h. Surgeries were performed following previously described methodologies [26]. In brief, rats were anesthetized with ether and a ventral incision that included skin, muscle and peritoneum was performed. Subsequently, the liver was reflected, the esophagus exposed, and the left, right, or both vagal trunks were cut with fine forceps. Sham-surgery involved

the same procedures except that the vagus trunks were not touched.

After treatment, the age of first vaginal estrous (puberty) was recorded and daily vaginal smears were taken thereafter. Vh-injected animals were sacrificed on the vaginal estrous between 90–92 days of age. EV-injected animals were sacrificed when they presented vaginal estrous at 90–92 days of age, or when they presented vaginal estrous preceeded by a proestrus smear at the similar ages.

Autopsy procedures

Animals were sacrificed by decapitation between 10.00 AM and noon. The blood from the trunk was collected, allowed to clot, and centrifuged during 15 min at 3,000 RPM. The serum was stored at -20°C, until P_4, T, E_2, FSH and LH levels were measured. Following the criterion proposed by Burden y Lawrence [30], at the time of necropsy a distended stomach was considered an index of functional vagotomy.

At autopsy the oviducts were dissected and the number of ova counted with the aid of a dissecting microscope. The results were used to estimate the ovulation rate (number of ovulating animals/number in treatement group) [13].

Ovarian morphology assessment

To assess morphology ovarian, the left ovary from each control or experimental rat was cleaned of adherent fat tissue, immersed in Bouin solution for 24 hours, dehydrated and embedded in paraffin. Ten microns-thick serial histological sections were made and stained with hematoxylin-eosin. All the sections were analyzed for the presence of corpora lutea (CL), healthy antral follicles and follicular cysts with the aid of a Nikon binocular microscope.

Hormone measurement

E_2 (pg/ml), T (pg/ml) and P_4 (ng/ml) serum concentrations were measured using radioimmunoassay (RIA), with kits purchased from Diagnostic Products (Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation were 8.12% and 9.28% for E_2, 9.65% and 10.2% for T and 8.35% and 9.45% for P_4. FSH and LH (ng/ml) levels in serum were measured by the double antibody RIA technique, employing reagents and protocols kindly supplied by the NIADDK National Pituitary Program (Bethesda, MD, USA). Intra- and inter-assay variations were in the order of 5.1% and 6.5% for LH, and 4% and 7.9% for FSH. The results are expressed in terms of NIADDK standards RP-2 FOR-FSH and LH.

Statistical analysis

Data on P_4, T, E_2, FSH and LH concentrations were analyzed using Repeated Measures Analysis of Variance, followed by Dunn's test using the GraphPad Instant 3 program. The number of ova shed by ovulating animals was analyzed using Kruskal-Wallis test, followed by Mann–Whitney *U*-test. The ovulation rate was analyzed using Chi square (Xi^2) test. A p-value of less than 0.05 was considered significant.

Results

Vaginal cycle

Either Vh treatment alone or sham-surgery unilateral or bilateral vagotomy to Vh-injected rats modified the normal 4-day vaginal cycle.

The estrous vaginal smear in EV-injected rats and EV-injected rats with sham-surgery was characterized by prolonged cornified smears, followed by 2–3 days of diestrous smears. Unilateral or bilateral vagotomy did not restore the normal vaginal cycle. All the animals presented a proestrous smear followed by a day of estrous smear on the day of sacrifice.

Ovulatory response

None of the EV injected rats showed spontaneous ovulation while all the Vh-injected animals did (0/11 vs. 12/12, $p < 0.01$ Fisher's exact probability test).

In Vh treated-rats, sham- surgery, unilateral or bilateral vagotomy did not modify ovulation rates. In EV-injected rats 2/28 of the sham-surgery ovulated. EV-injected rats with LSVN or bilateral vagotomy showed a 65 percent ovulation rate (10/15) and the ovulation rate in EV-injected rats with RSVN was 72 percent (11/15) (Figure 1).

The number of ova shed by Vh-injected rats with unilateral or bilateral vagotomy was similar to the Vh-injected sham-surgery group. The number of ova shed by EV treated animals with RSVN or bilateral vagotomy was lower than their respective Vh-injected groups. Compared to Vh-injected animals, LSVN to EV treated animals did not modify ovulation rates (Figure 2).

Steroids and gonadotropin hormones levels

P_4 levels were similar in Vh-injected and EV-injected rats. Sham-surgery, RSVN or BSVN to Vh-injected rats resulted in higher P_4 levels that Vh-injected control group. LSVN to Vh-injected rats resulted in lower P_4 levels than in the Vh-injected sham-surgery group. Sham-surgery, RSVN or BSVN to EV-injected rats resulted in lower P_4 levels than their respective Vh-injected group (Figure 3A).

T levels in Vh-injected and EV-injected rats were similar. Sham surgery to Vh-injected resulted in a non-significant decrease of T levels. T levels in EV-injected rats with sham-surgery, unilateral or bilateral vagotomy (LSVN, RSVN or BSVN) were higher than in EV-injected control group. T levels in EV-injected rats with

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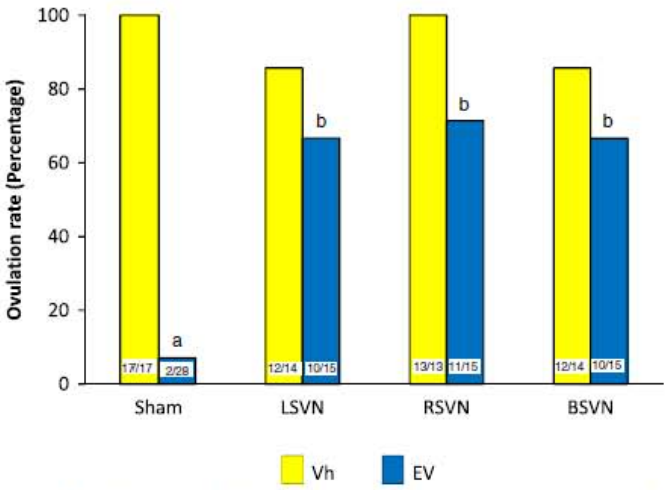

Figure 1 Ovulation rate in rats with LSVN, RSVN or BSVN. Percent of ovulating rats of rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, with sham-surgery (sham) or unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90–92 of life. The numbers at the base of the bars indicate the number of ovulating animals/number of treated animals. a $p < 0.05$ *vs.* Vh sham; b $p < 0.05$ *vs.* EV sham (Xi^2 test).

BSVN were higher than in the EV-sham surgery group. Compared to the EV-sham surgery group, unilateral vagotomy (RSVN or LSVN) did not modify T levels (Figure 3B).

E_2 levels in EV-injected rats were higher than in the Vh treatment group. E_2 levels in the Vh-injected group with RSVN or BSVN were higher than in Vh-injected control rats. BSVN to EV-injected rats resulted in higher E_2 levels than in EV-treated control group (Figure 3C).

EV-treated rats, with or without unilateral or bilateral vagotomy showed lower FSH levels than their respective Vh-injected groups. LH levels in EV-treated rats with unilateral or bilateral vagotomy were higher than in their respective EV-injected groups without surgical procedures (Table 1).

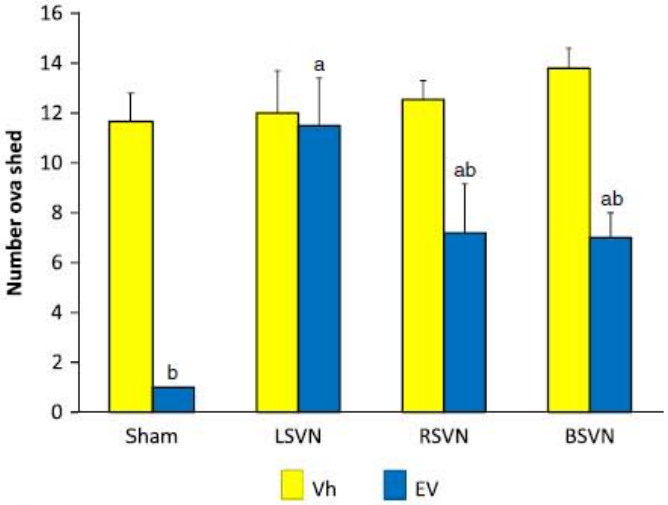

Figure 2 Number ova shed in rats with LSVN, RSVN or BSVN. Mean ± SEM of the number of ova shed in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, with sham-surgery (sham) or unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90–92 of life. a $p < 0.05$ *vs.* EV sham; b $p < 0.05$ *vs.* paired Vh group (Kruskal-Wallis test followed by Mann–Whitney *U*-test).

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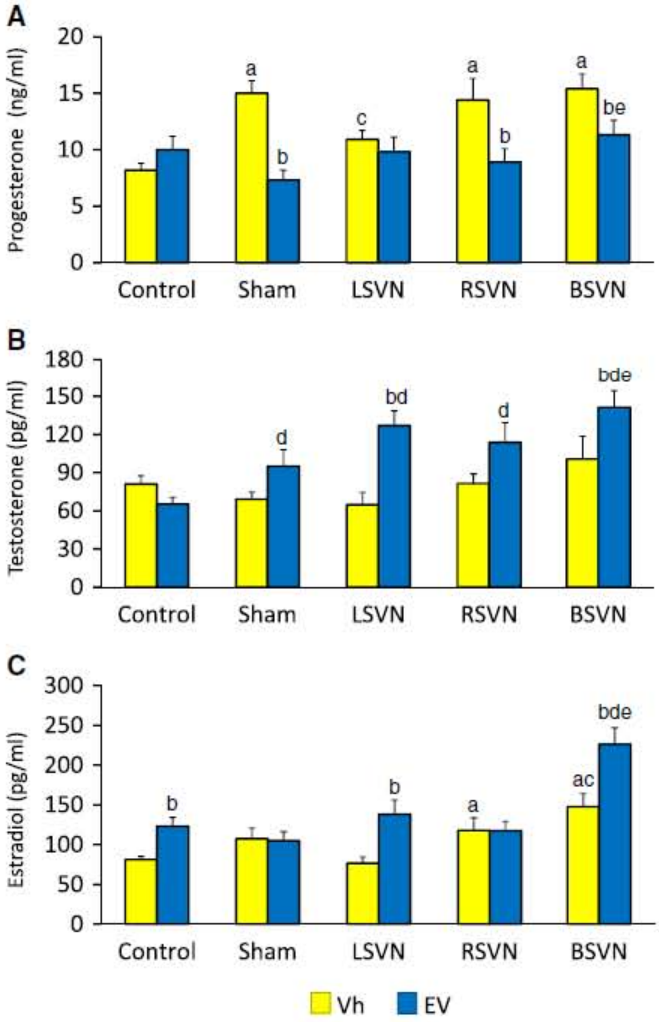

Figure 3 Concentration of steroid hormones. P_4 (**A**), T (**B**) and E_2 (**C**) serum levels in rats with LSVN, RSVN or BSVN. Mean ± SEM of P_4, T and E_2 levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), with sham-surgery (sham) or unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90–92 of life. a $p < 0.05$ *vs.* Vh control group, b $p < 0.05$ *vs.* paired Vh group, c $p < 0.05$ *vs.* sham Vh group; d $p < 0.05$ *vs.* EV control group; e $p < 0.05$ *vs.* sham EV group (Repeated Measures of analysis of variance, followed by Dunn's test).

Ovarian morphology

Figure 4 shows an ovary of a control animal sacrificed on estrous day, where several fresh corpora lutea, as well as some antral follicles can be observed (A). The histological analysis of the ovary of rats with EV- induced PCOS revealed the presence of cystic follicles and no corpora lutes (B). LSVN (C), RSVN (D) and BSVN (E) treatment to EV-induced PCOS rats changed the morphological aspects of the ovaries. Numerous fresh corpora lutea were readily apparent as well as marked attenuation of the cystic condition.

Discussion

The results obtained in the present study suggest that the neural information carried by the vagus nerve plays a role in the mechanisms participating in the regulation of development and maintenance of PCOS.

The development of ovarian patho-physiologic conditions, such as the PCOS, result from alterations in the

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Table 1 FSH and LH serum levels in rats with LSVN, RSVN or BSVN

Group	N	FSH (ng/ml)	LH (ng/ml)
Control Vh	12	10.4 ± 0.9	0.54 ± 0.07
Control EV	11	3.1 ± 0.3[a]	0.35 ± 0.04
Vh + Sham	17	7.7 ± 0.4	0.76 ± 0.04
EV + Sham	28	4.7 ± 0.4[bd]	1.18 ± 0.10[bd]
Vh + LSVN	14	7.28 ± 0.42	0.63 ± 0.05
EV + LSVN	15	4.10 ± 0.42[b]	0.94 ± 0.07[bd]
Vh + RSVN	13	6.14 ± 0.61[ac]	1.01 ± 0.07[ac]
EV + RSVN	15	4.0 ± 0.34[b]	1.14 ± 0.12[d]
Vh + BSVN	14	7.0 ± 0.6	0.82 ± 0.07[a]
EV + BSVN	15	3.2 ± 0.3[b]	1.04 ± 0.08[d]

Mean ± SEM of FSH and LH levels in animals injected with vehicle (Vh) or estradiol valerate (EV) at 10 days of life, untouched (control), with sham-surgery (sham) or unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90–92 of life.
[a] $p < 0.05$ *vs.* Vh control group, [b] $p < 0.05$ *vs.* paired Vh group, [c] $p < 0.05$ *vs.* Vh + Sham group, [d] $p < 0.05$ *vs.* EV control group (Repeated Measures of analysis of variance, followed by Dunn's test).

neuroendocrine axis regulating ovarian function. Several hypothesis have been proposed to explain the etiology of the PCOS, including failings in the pulsatile secretion of gonadotropin release hormone (GnRH) and the resulting deficiencies in ovarian sex steroid synthesis or metabolism [7]; the hyper-activation of the sympathetic fibers arriving to the ovary via the SON [21,31]; and kisspetin related mechanisms [32]. Injecting LHRH to EV-induced PCOS rat induces ovulation, suggesting that alterations to LHRH secretion by the hypothalamus are one of the main conditions that favor PCOS development and maintenance on the female reproductive system [17,18].

The ovarian innervation plays a role modulating the reactivity of the ovaries to gonadotropins. The NE and vasoactive intestinal peptide (VIP) fibers carried by the SON stimulate FSH receptor synthesis [33]. In EV-induced PCOS rat, the bilateral sectioning of the SON [13-15] or the bilateral electro-acupuncture treatment at the T12-L2 segments level [21] result in spontaneous ovulation and lower ovarian NE levels. Despite a drop of NE levels in the denervated ovary, unilateral sectioning of the SON restored ovulation by the innervated ovary; suggesting that lower NE content are not the only factor acting to restore ovulation [13]. Then, it is possible that PCOS onset is triggered by the hyperactivity of the sympathetic ovarian innervation and other non-adrenergic factors, such as VIP [34].

Gerendai et al., [10] suggested that the ovaries and CNS are linked by a neural loop, where the ovaries receive neural information from the CNS via the SON, OPN and vagus nerve. The ovaries send neural information via the SON and celiac-superior mesenteric ganglia (CSMG) and sensitive via by the vagus nerve [35-37].

Bilateral vagotomy to adult [26] and pre-pubertal rats [27], results in a higher ova shed numbers by ovulating animals. On the other hand, unilateral sectioning of the SON results in lower numbers of ova shed by denervated ovary [37]. Present results suggest that in EV-induced PCOS rat the participation of the vagus nerve in regulation ovarian patho-physiology is different than the participation of the SON.

According to Evans and Murray [38] and Agostoni et al., [39], 85–90 percent of vagus nerve fibers carry information from the peripheral organs to the CNS. On the other hand, the SON carries neural information from the CSMG to and from the ovaries [35,36]. In EV-induced PCOS rat, the most striking differences resulting from unilateral sectioning the vagus nerve or the SON are on spontaneous ovulation and hormone secretion changes. Unilateral vagotomy to EV-induced PCOS rat restored ovulation in both ovaries, while unilateral sectioning of the SON restored ovulation only in the innervated ovary. Such difference does not seem related to gonadotropins concentration since EV-induced PCOS rats with unilateral vagotomy had lower FSH levels than Vh injected rats. In turn, unilateral sectioning of the SON in EV-induced PCOS rats resulted in FSH levels similar to the control group [13]. The different types of neural information carried by the vagus nerve and the SON could explain the differences observed in gonadotropin levels in EV-induced PCOS rats submitted to unilateral denervation.

The main Hypothalamic-Pituitary-Adrenal (HPA) axis regulators are the corticotropin-releasing hormone (CRH) and the vassopresine hormone. Both stimulate pituitary adrenocorticotropic hormone (ACTH) secretion and the subsequent secretion of cortisol and P_4 by the adrenal cortex. The stress activation of the HPA axis inhibits female reproductive function [40]. Sham-surgery in Vh-injected rats resulted in higher P_4 levels, suggesting that the increase in P_4 levels resulting from the HPA axis activation also participate in inhibiting ovarian functions.

P_4 levels increases in Vh-injected groups with right or bilateral vagotomy arise from the effects of the sham-surgery, similar to other stressfull situations effects [41]. The adrenals receive vagal innervation directly and by the adrenal nerve originating in the celiac ganglia [42]. Present results suggest that the increase in E_2 levels resulting from injecting EV reduced the ability of the fresh corpora lutea and/or the adrenals ability to secrete P_4.

T levels in EV-induced rats PCOS have been described as higher [13], lower [15,16], and similar to those of control groups [14,17]. Such discrepancies have been explained by the rapid conversion of T to E_2 at the ovary and/or its periphery [15]. In the present study, T levels in EV-induced PCOS rats were similar to control

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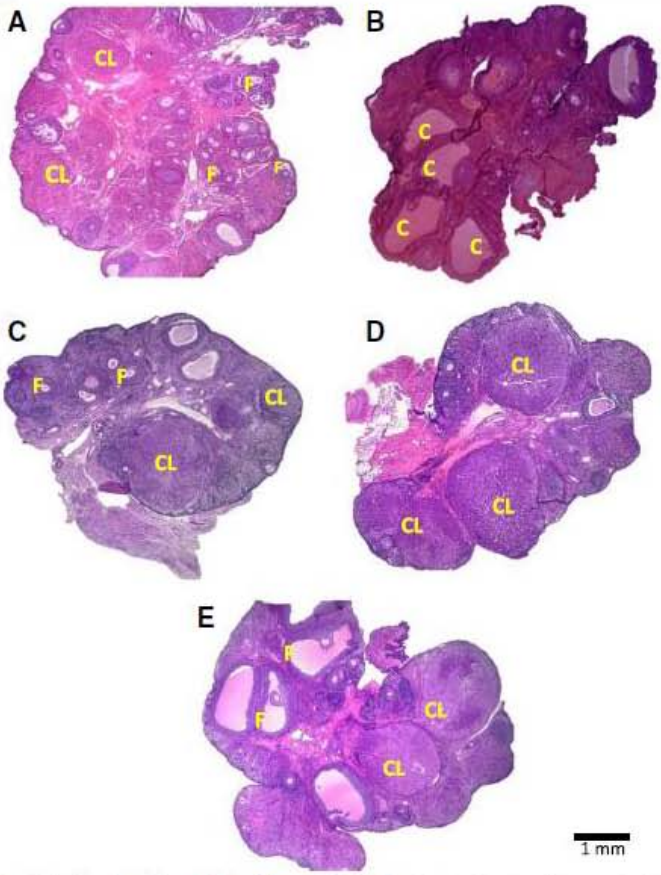

Figure 4 Ovarian histology in EV-induced PCOS rat after of the LSVN, RSVN or BSVN. Micrographs correspond to the largest section of the ovary of 10 μm thick and stained by hematoxylin-eosin, of animals sacrificed at the day with vaginal estrous smear preceeded by a day with proestrous vaginal smear. **A**, Ovary from a Vh-injected rat; **B**, polycystic ovary in EV-injected rats; **C-E**, ovary from an EV-injected rat subjected to unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90–92 of life. F, normal follicle; CL, corpora lutea; C, follicular cystic.

animals. E_2 levels in EV-induced PCOS rats were three times higher than in control rats sacrificed at 13.00 of proestrus [43]. These higher E_2 levels are relatively similar to those observed in previous EV-induced PCOS rat studies [13,15,18].

The higher T and E_2 levels observed in EV-induced PCOS rat with bilateral vagotomy suggest that the vagus nerve plays an inhibitory role on hormone secretion. Then, in EV-induced PCOS rats, the role played by the vagus nerve on T and E_2 secretion is opposite to the role played by the SON, since the bilateral sectioning of the SON decreases T and E_2 levels [13].

Dissen et al., [44] suggested that in humans and rodents the overproduction of ovarian nerve growth factor (NGF) is a component of polycystic ovarian morphology. Persistently higher LH levels in plasma are required for the morphological abnormalities to appear, and under normal conditions, the ovulatory process is facilitated by the ovarian NGF acting via high affinity tyrosine receptor kinase A [44]. However, an excess of ovarian NGF initiates pathological changes in both endocrine and non-endocrine tissues [45]. Therefore, it is possible that bilateral vagotomy treatment disrupted the afferent network involved in the ovarian feedback of GnRH/LH secretion as proposed by Gerendai et al., [10,23].

According to Lara et al., [31], the hyper-activation of the ovarian sympathetic input resulting from EV treatment is related to an overproduction of ovarian NGF and its low affinity receptor in the ovary. Although EV-induced follicular cysts are first detected around 60 days after EV treatment [31,46], activation of the ovaries' sympathetic innervation occurs at least a month before

the formation of follicular cysts [20]. In turn, increases in p75 NGFR synthesis occur as early as 15 days after EV treatment and is shortly followed by increases in NGF synthesis [31]. This suggests that the activation of this ligand/receptor module is an early event in the process by which EV treatment disrupts ovarian function. NGF increased in the sympathetic neurons projecting to the ovary are likely to play a significant role in enhancing the sympathetic outflow to the ovary in EV-treated rats [31]. Then, it is possible that the vagus nerve participates in regulating NGF release by the sympathetic neurons that origin in the CSMG.

Very little is known about the mechanism by which vagotomy alters ovarian function. It has been proposed that the vagus nerve carries sensory fibers that influence gonadotropin secretion by acting on the hypothalamus [28] and modifying the effects of gonadotropin on the ovary [26]. Present results suggest that this regulation depends of physiological environment of the animal. Based on the results presented herein, and according to Berthoud and Powley [47], there is an apparent communication between the sympathetic and parasympathetic fibers at the CSMG level. We suggest that the vagus nerve serves as a communication channel between the ovaries and that, in rats, this channel is closely related to the development and persistence of EV-induced PCOS. The spontaneous ovulation observed in EV-induced PCOS rat with unilateral or bilateral vagotomy is evidence that the neural information carried by the vagus nerve participates, directly or indirectly, in the regulation of the development and persistence of the PCOS.

Since in the PCOS affected animals the mechanisms regulating GnRH secretion are altered [17,18], and these alterations may be modified by vagotomy procedures, present results suggest that neural signal originating from each ovary would indicate the physiological conditions of the ovaries to the CNS, which in turn participates in the regulation of GnRH secretion [37].

Conclusions

The results suggest that in the EV-induced PCOS rats the CSMG is a neural regulation center where the vagus nerve acts on the neurons originating the SON.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RL, LM and RD planned the experiments. LM, RL, DH, CM and RD devised the study and participated in the discussion of the results. RC and MC participated in performing the RIA's to measure the different hormones levels. All authors read approved the final manuscript.

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C A P Í T U L O III

In rats with the polycystic ovary syndrome, the monoaminergic activity in the celiac superior mesenteric ganglion depends on the vagal innervation.

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Running Title: Celiac ganglia monoaminergic activity & vagus nerve

Abstract: One of the mechanisms involved in the polycystic ovarian syndrome (PCOS) development is the hyperactivity of the sympathetic nervous system. In estradiol valerate (EV)-induced PCOS rats, the unilateral section of the superior ovarian nerve (SON) restores the ovulation in the innervated ovary, while the unilateral or bilateral section of the vagus nerve restores ovulation in both ovaries. The somas of the neurons originating the SON are located mainly in the celiac superior mesenteric ganglia (CSMG), which in turn receives innervation by the vagus nerve, suggesting that the neural information arriving to the CSMG through the vagus nerve may modulate the role played by the SON in the persistence of PCOS.

The aim of the present study was to assess the participation of the vagus nerve in the regulation of the monoaminergic activity in the CSMG of rats with EV-induced PCOS. Ten-day old rats injected with EV dissolved in corn oil, at 24-days of age were submitted to unilateral or bilateral vagotomy. The animals were sacrificed at 90-92 days of age, after presenting vaginal estrous smear. In rats with EV-induced PCOS, right or bilateral vagotomy resulted in lower noradrenaline (NA) levels in the CSMG. Present result suggests that in rats with EV-induced PCOS the CSMG serves as communication channel between the vagus nerve and sympathetic innervation in an asymmetric way and this communication is related to the persistence of EV-induced PCOS.

New Findings: What is the central question of this study? The central question of this study is to analyze the participation of the vagus nerve in the regulation of the monoaminergic activity in the celiac superior mesenteric ganglia (CSMG) in rats with polycystic ovarian syndrome (PCOS) induced by estradiol valerate (EV) What is the main finding and its importance? Present results suggest that the vagus nerve, regulate the noradrenergic activity in the CSMG in rats with the EV-induced PCOS

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In rats with the polycystic ovary syndrome, the monoaminergic activity in the celiac superior mesenteric ganglion depends on the vagal innervation.

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New Findings

What is the central question of this study?

The central question of this study is to analyze the participation of the vagus nerve in the regulation of the monoaminergic activity in the celiac superior mesenteric ganglia (CSMG) in rats with polycystic ovarian syndrome (PCOS) induced by estradiol valerate (EV).

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Present results suggest that the vagus nerve, regulate the noradrenergic activity in the CSMG in rats with the EV-induced PCOS

Abstract

One of the mechanisms involved in the polycystic ovarian syndrome (PCOS) development is the hyperactivity of the sympathetic nervous system. In estradiol valerate (EV)-induced PCOS rats, the unilateral section of the superior ovarian nerve (SON) restores the ovulation in the innervated ovary, while the unilateral or bilateral section of the vagus nerve restores ovulation in both ovaries. The somas of the neurons originating the SON are located mainly in the celiac superior mesenteric ganglia (CSMG), which in turn receives innervation by the vagus nerve, suggesting that the neural information arriving to the CSMG through the vagus nerve may modulate the role played by the SON in the persistence of PCOS. The aim of the present study was to assess the participation of the vagus nerve in the regulation of the monoaminergic activity in the CSMG of rats with EV-induced PCOS. Ten-day old rats injected with EV dissolved in corn oil, at 24-days of age were submitted to unilateral or bilateral vagotomy. The animals were sacrificed at 90-92 days of age, after presenting vaginal estrous smear. In rats with EV-induced PCOS, right or bilateral vagotomy resulted in lower noradrenaline (NA) levels in the CSMG. Present result suggests that in rats with EV-induced PCOS the CSMG serves as communication channel between the vagus nerve and sympathetic innervation in an asymmetric way and this communication is related to the persistence of EV-induced PCOS.

Abbreviations. PCOS, polycystic ovary syndrome; EV, estradiol valerate; SON, superior ovarian nerve; LH, luteinizing hormone; GnRH, gonadotropin releasing hormone; OPN, ovarian plexus nerve; CSMG, celiac superior mesenteric ganglia; NA, Noradrenaline; DA, Dopamine; Vh, vehicle; MHPG, 4-hydroxy-3-methoxyphenyl glycol; DOPAC, 3,4-dihydroxyphenilacetic acid; 5-HIAA, 5-hydroxyindole-3-acetic acid; ANOVA, analysis of variance; LSVN, left vagotomy; RSVN, right vagotomy; BSVN, bilateral vagotomy.

Introduction

In humans and other mammal, the exposure to high concentration of androgens or estrogens during prenatal and early postnatal life disrupts normal endocrine functions and decreases fertility [Barraclough and Gorski, 1961; Sotomayor-Zárate et al, 2008].

The polycystic ovary syndrome (PCOS) is the most common cause of infertility in woman, characterized by a complex pathophysiology, including anovulation, oligomenorrhea, follicular cysts, hyper-androgenism, hyper-estrogenism, and variable levels of gonadotropins in blood, glucose metabolic disorders, cardiovascular diseases, dyslipidemia and cancer [Hoyt and Schmidt, 2004; Goodarzi et al, 2011]. The experimental models used in the study of the PCOS includes injecting long lasting estradiol or testosterone esters [Manneras et al, 2007; Morales et al, 2010; Sotomayor-Zárate et al, 2011]; as well as exposure of adults rats to constant light, and chronic cold stress [Paredes et al, 1998; Bernuci et al, 2008]. Injecting EV to infantile or adult rats results in acyclicity, anovulation, polycystic ovaries, hyperandrogenism, characteristics similar to those observed in women with PCOS [Brawer et al, 1978; Lara et al, 1993; Stener-Victorin et al, 2005].

According to Matalliotakis et al., [2006] the etiology of PCOS is multifactorial and is attributed to genetic, nutritional, and environmental factors; as well as primary defects in the reproductive axis, including alterations in the patterns of gonadotropins secretion. The onset of PCOS is associated with

increased activity of the ovarian sympathetic nerves [Barria et al, 1993; Lara et al, 2002; Morales et al, 2010; Rosa-E-Silva et al, 2003; Sotomayor-Zárate et al, 2008]. In adult rats with EV-induced PCOS, the bilateral section of the superior ovarian nerve (SON) resulted in spontaneous ovulation by both ovaries [Barria et al, 1993]. In juvenil rats with EV-induced PCOS, the unilateral section of the SON results in spontaneous ovulation and higher levels of noradrenaline (NA) in the innervated ovary. These results suggest that aside from an increase in ovarian noradrenergic tone in the ovaries, in the pathogenesis of the PCOS participate other neural mechanisms [Morales et al, 2010].

According to Gerendai et al., [2009], a multi-synaptic neural pathway between the ovary and the central nervous system is involved in regulating ovarian functions. In the adult rat, bilateral vagotomy altered the estrous cycle [Chávez et al, 1989], blocked pseudo-pregnancy induction [Burden et al, 1981], increased the number of ova shed by ovulating adult and pre-pubertal rats [Cruz et al, 1986; Morales et al, 2004], and in pregnant rats resulted in lower luteinizing hormone (LH) basal levels, causing fetal resorption [Lawrence et al, 1978]. Taken together, these results suggest that the information arriving to the ovaries through the vagus nerve participates in regulating ovarian functions. The effects of the unilateral or bilateral vagotomy on ovulation suggests that the sensory fibers in the vagus nerve carry information from the periphery to the hypothalamus, which in turn participates in the regulation of gonadotropin releasing hormone (GnRH) and gonadotropin secretion [Gerendai et al, 2009; Lawrence et al, 1978]. Another possibility is that the ovarian vagal innervation modulates the effects of gonadotropins on the ovarian follicles [Cruz et al, 1986].

According to Berthoud and Powley [1996], communication between the sympathetic and parasympathetic fibers is apparent at the celiac-superior-mesenteric ganglion (CSMG) level. The neurons originating the SON and ovarian plexus nerve (OPN) are located in the CSMG [Dissen and Ojeda, 1999]. The vagus nerve may modulate the postganglionic outflow directly or indirectly some or all of the potential modulatory inputs to these postganglionic neurons, allowing the vagal system to

exert a more selective influence on sympathetic outflow. The vagal projections form varicose terminal-like structures suggest the presence of synaptic contacts surrounding each individual ganglion cell [Berthoud and Powley, 1996].

In rats, using the coeliac ganglion-SON-ovary system (CG–SON–O), the noradrenergic or cholinergic stimulation of the CG results in an increase or a decrease in steroid hormones secretion by the ovary [Casais et al, 2001; Delgado et al, 2010; Sosa et al, 2000]. These observations provide evidence that the coeliac ganglion has a direct neural effect on ovarian physiology. In a previous study we showed that unilateral or bilateral vagotomy to 24 day old rats with PCOS induced by EV injection, results in spontaneous ovulation in both ovaries with ovarian morphology similar at to control animals, suggesting that the vagus nerve is a neural pathway participating in maintaining PCOS [Linares et al, 2013]. Since the vagus nerves innervate the ovaries directly and indirectly through its synapsis in the CSMG, where the somas of neurons originating in the SON are located, we presume that at the CSMG the vagus nerve modulates the activity of those neurons originating the SON and the OPN. Then, the aim of the present study was to analyze if the ovulation observed in rats with EV-induced PCOS by the section of the vagus nerves, was accompanied by changes in the monoaminergic activity in the CSMG. For this purpose, we measured the amounts of noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their metabolites in the CSMG of those rats where the unilateral or bilateral section of the vagus nerves restored ovulation.

Methods

Ethical Approval

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines. The Committee of the Facultad the Estudios Superiores Zaragoza approved the experimental protocols. The study was performed using pre-pubertal female rats of the CIIZ-V

strain from our own breeding stock. Animals were maintained under controlled lighting conditions (lights on from 05:00 to 19:00 h); with free access to rat chows pellets and tap water.

Animal treatment

Fifty-six 10-day old rats were injected with either a single dose of 0.1 ml corn oil (vehicle Vh) [n=28] or 2 mg EV (Sigma Chem. Co., St. Luis, Mo. USA) [n=28] dissolved in 0.1 ml corn oil. When the rats injected with Vh or EV reached 24 days of age, groups of seven animals injected with Vh or EV were randomly allotted to one of the following groups: No surgery (Vh and EV), unilateral vagotomy (Vh/left vagotomy, Vh/right vagotomy, EV/left vagotomy, and EV/right vagotomy), or bilateral vagotomy (Vh/ bilateral vagotomy and EV/ bilateral vagotomy).

Vagotomy was performed following methodologies previously described [Cruz et al, 1986]. In brief, between 10:00 and 12:00 h the rats were anesthetized with ether and a ventral incision including skin, muscle and peritoneum was performed. Subsequently, the liver was reflected, the esophagus exposed, and the left, right, or both vagal trunks were cut with fine forceps. After surgery, the abdominal wall was sutured and the animals returned to their cage.

The age of first vaginal estrous (puberty) was recorded in all the treated and untreated animals used in the experiment, and daily vaginal smears were taken thereafter. Vh-injected animals were sacrificed when they presented vaginal estrous proceeded by a proestrus between 90-92 days of age. EV-injected animals were sacrificed when they presented vaginal estrous at 90–92 days of age, following previously described methodologies [Linares et al, 2013].

Autopsy procedures

The animals were sacrificed by decapitation between 10.00 AM and noon. At autopsy, the CSMG was removed and stored at -70 ºC until monoamines and their metabolites were measured using high performance liquid chromatography (HPLC).

Monoamines Levels

The concentration of monoamines (NA, DA, 5-HT) and their metabolites (4-hydroxy-3-methoxyphenyl glycol (MHPG), 3,4-dihydroxyphenilacetic acid (DOPAC) and 5-hydroxyindole-3-acetic acid (5-HIAA)) in the CSMG were measured following methodologies previously described [Ayala et al, 1998; Castro et al, 2001; Quiroz et al, 2013]. In brief, the CSMG was weighed in a precision balance, homogenized in 300 μl of 0.1 N perchloric acid, and centrifuged at 12,000 X g, at 4 ºC for 30 min. The supernatant was filtered using 0.2 μm regenerated cellulose filters. Twenty microliters of this extract were injected into a chromatography column via a Rheodyne injection valve.

The HPLC system consisted of an isocratic pump (L-250 model; Perkin Elmer Co., Norwalk, CT, USA), a Rheodyne injection valve (7125 model; Perkin Elmer Co.), an ultrasphere ODS preanalytical column (5 cm 3 4.6 mm) and a Biophase ODS C-18 analytical (25 cm 3 4.6 mm, 5 mm particle size; Bionalitical Systems Inc., West Lafayette, IN, USA) column. The content of monoamines and their metabolites were detected electrochemically using a LC-4A amperometric detector and a LC-5A glassy carbon traducer cell at a potential of 850 mV (Bionalitical Systems Inc.). The mobile phase consisted of 0.1 M citrate buffer (Merck-México, SA.) at pH 3.0, with 175 mg of 1-octane-sulfonic acid (Sigma Chemical Co., St. Louis, MO, USA), filtered and degassed under vacuum. Immediately after degassing, 20 ml of acetonitrile and 21.5 ml of tetrahydrofurane for chromatography (Merck, Darmastadt, Germany) were added until a total volume of 500 ml was reached. The mobile phase was pumped at a flow rate of 1.2 ml/min. Stock standards (Sigma Chemical Co.) were prepared and diluted with 0.1 M perchloric acid the day of the experiment. The system was calibrated by producing a 0.1 to 2 ng/ml standard range curve. Monoamines and their metabolites were identified by the relative retention times compared to standards. Using a 1020 Perkin-Elmer Nelson integrator, the

concentrations of monamines and their metabolites were determined by comparing standards with the highest peaks obtained from the samples. Results are expressed as pg of neurotransmitter/mg wet tissue. The sensitivity for all neurotransmitters was 0.01 ng.

Neural activity was estimated as previously described, following Shannon et al., [1986], and Kerdelhué et al., [1989] suggestions. Neural activity = [Neurotransmitter Metabolite] / [Neurotransmitter]. Increases in this ratio are considered an indication of greater neurotransmitter turnover and therefore increased neuronal activity [Kerdelhué et al, 1989; Shannon et al, 1986].

Statistical analyses

Results are shown as are expressed as mean ± standard error of the mean (SEM). Comparisons among the concentrations of monoamines, their metabolites, and the monoaminergic activity in the groups different were analyzed using a two-way analysis of variance (ANOVA) followed by Tukey´s multiple comparisons test. P values less than 0.05 were considered to be statistically significant.

Results

Noradrenergic system in the Celiac Superior Mesenteric Ganglia

Compared to the Vh group, the NA levels in the CSMG in EV-treated rats were higher. In EV-treated animals, right or bilateral vagotomy yielded lower NA levels than in EV-treated rats (Figure 1a). MHPG levels in the CSMG of Vh and EV injected animals were similar. In rats injected with Vh, the section of the left vagus resulted in higher MHPG, while the section of the right or bilateral section of vagus nerves resulted in lower levels. In turn, compared to the EV group, MHPG levels were higher in EV-treated rats with left vagotomy and lower in those with right vagotomy (Figure 1b). Noradrenergic activity in the CSMG of Vh-treated and EV-treated animals was similar. In Vh-treated animals with left vagotomy the noradrenergic activity in the CSMG was higher, while it was lower in animals with right or bilateral vagotomy. In comparison with Vh-treated rats with left or bilateral vagotmy, in EV-treatmed animals the noradrenergic turnover in the CSMG was lower in rats

with left vagotomy and higher in those with bilateral vagotomy. Compared to the EV-treated group, the noradrenergic turnover was higher in rats with left or bilateral vagotomy and lower in those with right vagotomy (Figure 1c).

Dopaminergic system in the Celiac Superior Mesenteric Ganglia

DA levels in the CSMG were similar in Vh-treated and EV-treated rats. Unilateral or bilateral vagotomy did not modify DA levels (Figure 2a). The DOPAC levels were higher in the CSMG of EV-treated rats than in Vh-treated ones. The left or right vagotomy in Vh-treated rats resulted in higher DOPAC levels. In EV-treated rats DOPAC levels were higher in animals with left and lower in those with right vagotomy in comparison with Vh-treated submitted to left or right vagotmy, (Figure 2b). The dopaminergic activity was higher in EV-treated than in Vh-treated rats. In Vh-treated rats the left or right vagotomy resulted in higher dopaminergic activity. In comparison with their respective Vh-treated groups, left or bilateral vagotomy to EV-treated rats resulted in higher dopaminergic activity and was lower in those with right vagotomy. Compared to the EV-treated group, the dopaminergic turnover was lower in rats with right or bilateral vagotomy, while it did not modify in those with left vagotomy (Figure 2c).

Serotoninergic system in the Celiac Superior Mesenteric Ganglia

No differences occurred in the 5-HT levels in the CSMG of EV-treated animals. In EV-treated animals, unilateral or bilateral vagotomy resulted in lower 5-HT levels (Figure 3a). In the CSMG of EV-treated rats the bilateral vagotomy resulted in higher 5-HIAA levels (Figure 3b). Left and bilateral vagotomy to Vh-treated rats resulted in higher serotonergic activity. Bilateral vagotomy to EV-treated rats resulted in higher serotonergic activity than in EV-treated ones and Vh-treated rats with bilateral group (Figure 3c).

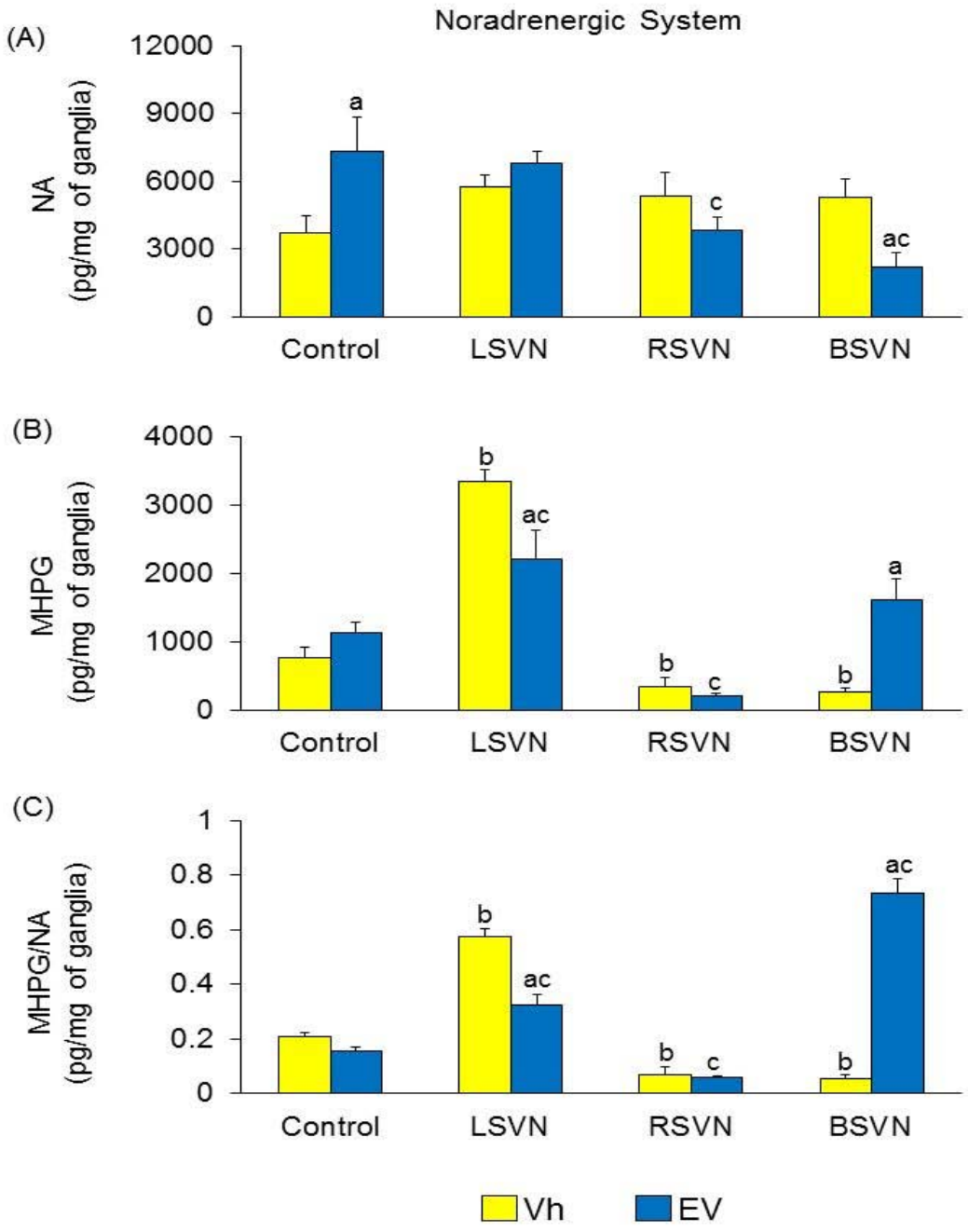

Figure 1 Noradrenergic system. Mean ± SEM (n=7/group) of (A) NA, (B) MHPG and (C) MHPG/NA levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control) or with unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90-92 of life. a $p<0.05$ *vs.* paired Vh group, b $p<0.05$ *vs.* control Vh group, c $p<0.05$ *vs.* control EV group (two-ways ANOVA followed by Tukey´s multiple comparisons test).

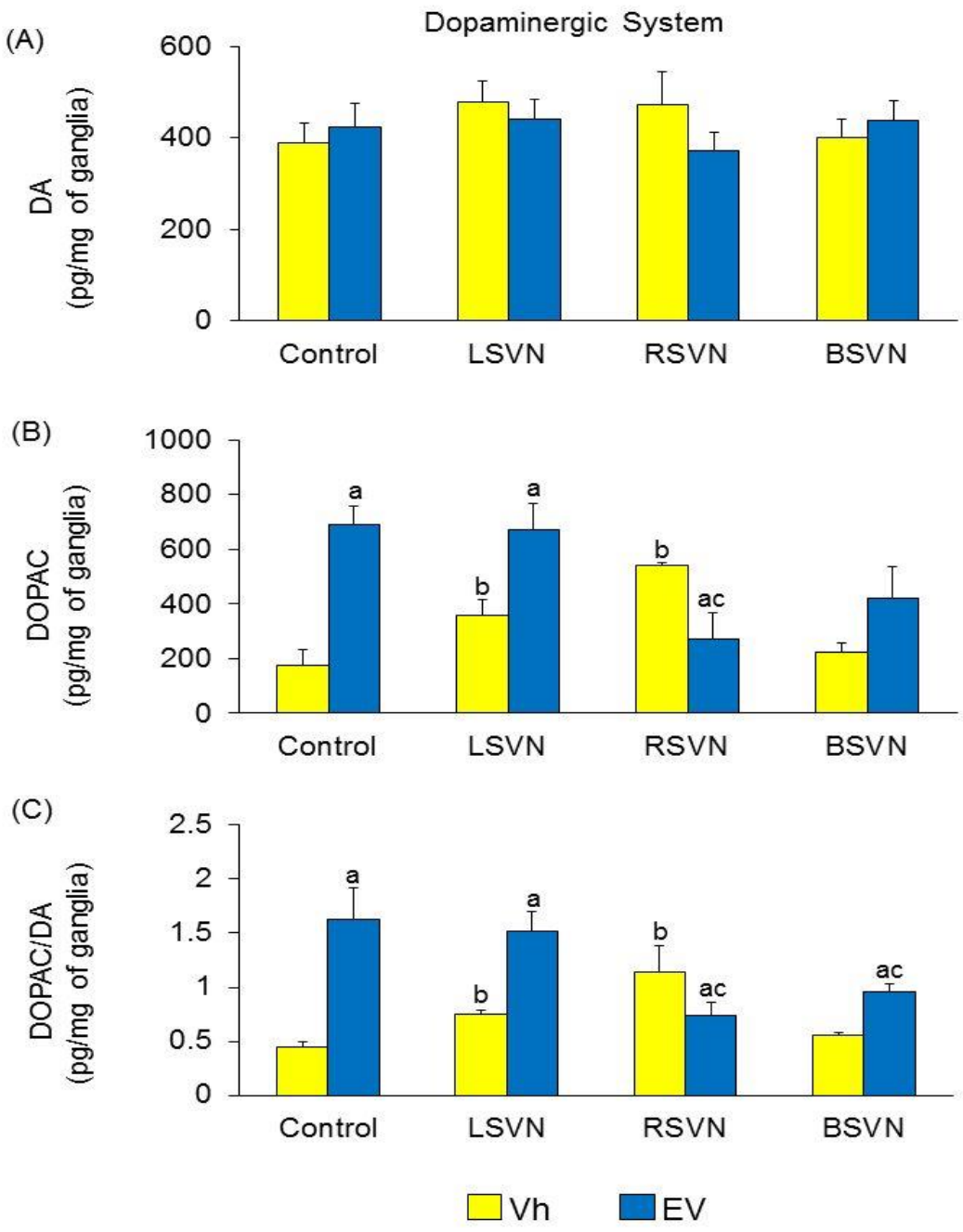

Figure 2 Dopaminergic system. Mean ± SEM (n=7/group) of (A) DA, (B) DOPAC and (C) DOPAC/DA levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control) or with unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90-92 of life. a $p<0.05$ *vs.* paired Vh group, b $p<0.05$ *vs.* control Vh group, c $p<0.05$ *vs.* control EV group (two-ways ANOVA followed by Tukey´s multiple comparisons test).

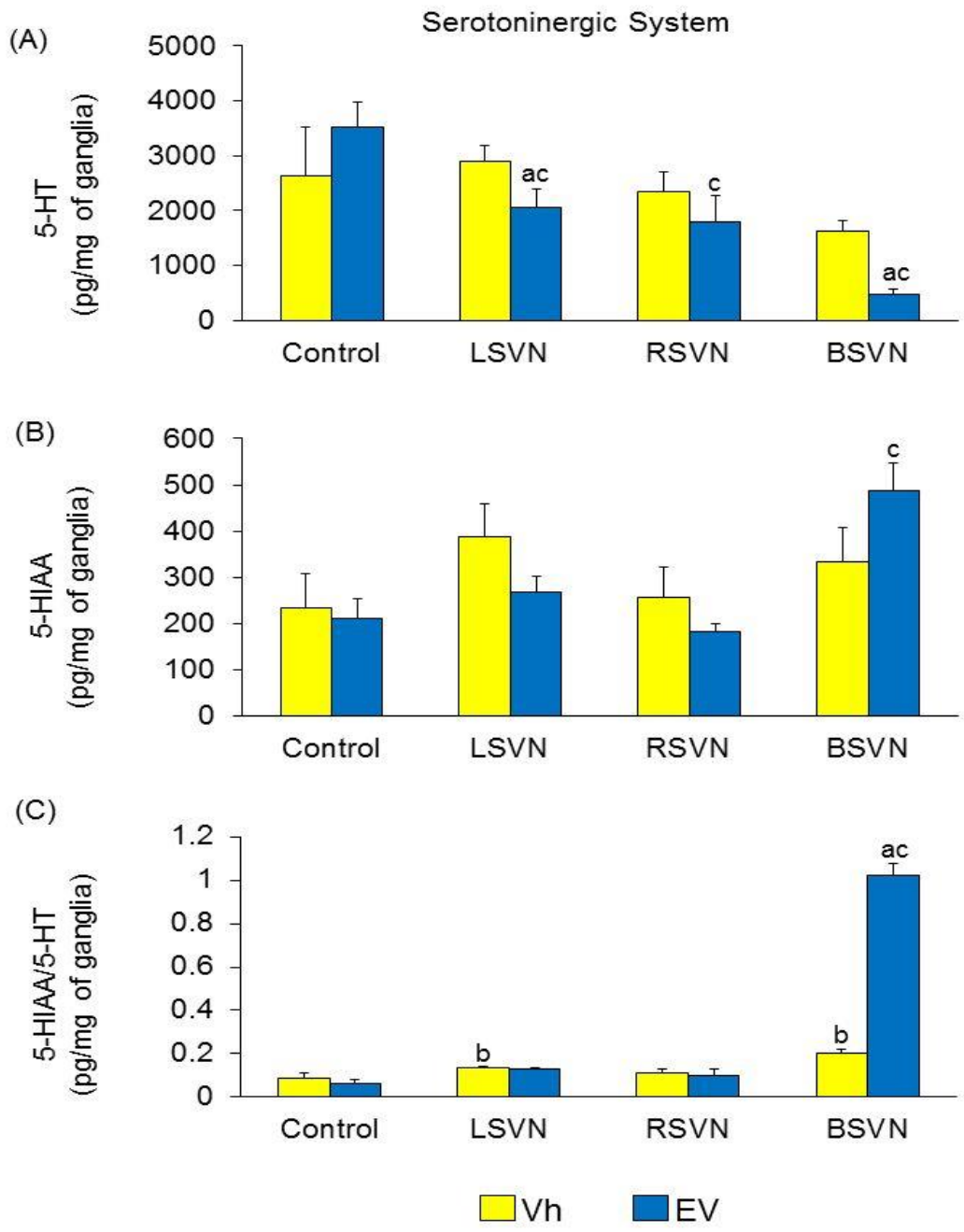

Figure 3 Serotoninergic system. Mean ± SEM (n=7/group) of (A) 5-HT, (B) 5-HIAA and (C) 5-HIAA/5-HT levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control) or with unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 24 of life, sacrificed at day 90-92 of life. a $p<0.05$ *vs.* paired Vh group, b $p<0.05$ *vs.* control Vh group, c $p<0.05$ *vs.* control EV group (two-ways ANOVA followed by Tukey´s multiple comparisons test).

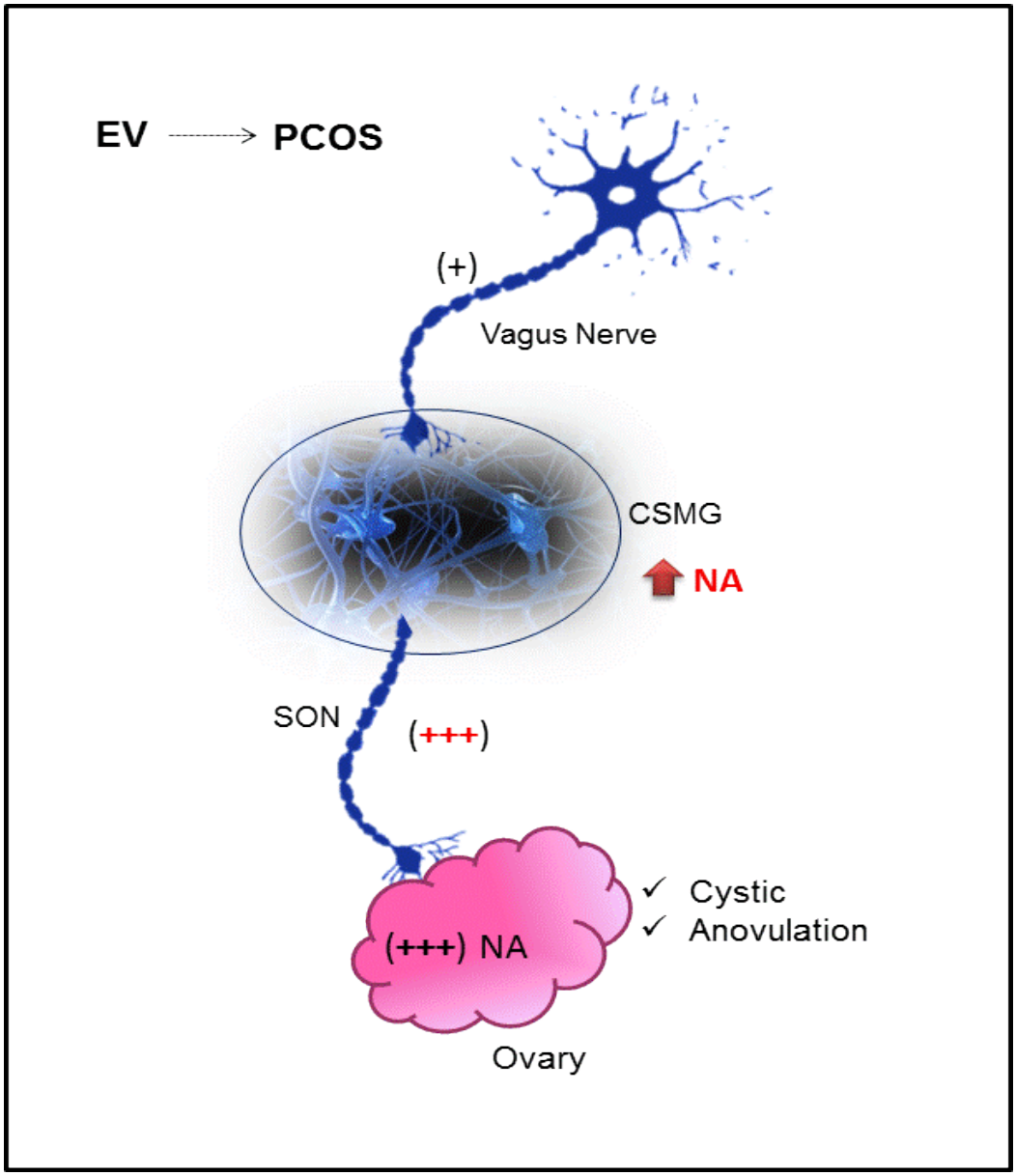

Figure 4 Mechanism the action to vagus nerve in rats with EV- induced PCOS. The schematic shows that in animals with PCOS, induced by administration of EV, the vagus nerve stimulates increased secretion of NA in the CSMG, which is the origin of sympathetic innervation and therefore the site of interaction between the vagal and sympathetic innervation. From the increase in concentration of NA in the CSMG the ovary also is under hiper-noradrenergic tone which will come via the SON and in response the development and persistence of PCOS is presented. PCOS polycystic ovarian syndrome, EV estradiol valerate, NA noradrenaline, CSMG celiac superior mesenteric ganglia, SON superior ovarian nerve.

Discussion

The results obtained in the present study show that injecting EV to pre-pubertal female rats increases NA levels in the CSMG, without changes in DA and 5-HT levels, while right or bilateral vagotomy results in lower NA and 5-HT levels suggesting that the vagus nerve has a stimulant role in the regulation of the CSMG monoaminergic tone.

The EV injection increases ovarian NA content, enhanced NA uptake and release form ovarian nerve terminals [Lara et al, 1993], increase intraovarian synthesis of neural growth factor and its low affinity neurotrophin receptor p-75, on the other hand in the CSMG increase the tyrosine hydroxylase mRNA level [Lara et al, 2000]. In adult female rats, combining cold and restraint stress procedures results in higher CSMG-NA levels than control [Paredes et al, 1998]. In the present study, a similar increase in CSMG NA levels was observed in rats with EV-induced PCOS, supporting the idea that an increase in the noradrenergic system activity is part of the mechanisms elicited by the experimental PCOS inductors.

According to Aneseti et al., [2009], 15-days old rat injected with estradiol cypionate (a long lasting estrogen) significantly increased the ovaries' catecholaminergic innervations, the sympathetic celiac neuronal size, and the expression of the neurotrophin receptor p-75, seven days after treatment. Injecting adult gilts with estradiol 17β during 38 days resulted in lower number of neurons present in the ganglia innervating the ovaries [Jana et al, 2013; Koszykowska et al, 2011a; Koszykowska et al, 2011b]. In the present study, higher estradiol levels resulting from EV treatment lead to NA levels increases in the CSMG 70 days after treatment. Higher NA levels could be explained by noradrenergic neurons size increases described by Anesetti et al., [2009].

Ovarian sympathetic innervation regulated to steroidogenesis, folliculogenesis and corpus luteum development and regression in various species [Chávez-Genaro et al, 2007; Kotwica et al, 1999]. This innervation not only includes the neural components that enter the ovary, as is the case of the SON and OPN, but also intermediate structures such as CSMG, which was capable of receiving and

integrating signals coming from the central nervous system and organizing responses that influence ovarian physiology [Forneris and Aguado, 2002; Sosa et al, 2000]. According to Berthoud and Powley [1996], the vagal pre-ganglionic efferent innervates the ganglion cells of CSMG. These vagal contacts may either play a role by directly modulating the post-ganglionic outflow or by accessing the potential modulatory inputs to these post-ganglionic neurons, thus allowing the vagal system to exert a more selective influence on sympathetic outflow. Aguado [2002] and Gerendai et al., [2002] proposed that the CSMG is part of a multi-synaptic neural network connecting the central nervous system and the ovary. According to Follesa et al., [2007], the acute stimulation of the vagus nerve increased the brain-derived neurotrophic factor and fibroblast growth factor mRNAs in the hippocampus and cerebral cortex, as well in the concentration of NA in the prefrontal cortex of the rat. In the present study changes in the concentration of neurotransmitters and their metabolites in rats with unilateral or bilateral vagotomy may be explained by differences in the neural information carried by the left and right vagus nerve. This interpretation is supported by the effects of unilateral or bilateral vagotomy on ovulation [Cruz et al, 1986; Morales et al, 2004].

Present results suggest that during the PCOS the vagus nerve may regulate the transmission of sensory information from the ovary to the spinal cord. The density of sympathetic nerve fibers is higher in the cystic ovaries of women [Heider et al, 2001], in the ovaries of rats injected with EV [Stener-Victorin et al, 2005] and in pigs with cystic ovaries induced by injecting dexamethasone [Jana et al, 2005; Kozlowska et al, 2013]. Cholinergic [Kozlowska et al, 2009] and sensory [Kozlowska et al, 2011] ovarian innervations are also modified in pigs with cystic ovaries induced by injecting dexametasone.

Previously we showed that in rats with EV- induced PCOS, the vagotomy restore the ovulation; suggesting that the CSMG is a neural regulation center where the vagus nerve acts on neurons originating in the SON and OPN. Present results support this hypothesis and suggest that the vagus nerve carries sensory fibers that participate, directly or indirectly, in regulating the persistence of the PCOS (Figure 4).

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Additional Information

Competing Interests

The authors declare that they have no competing interests.

Author Contribution

RL, LM and RD planned the experiments. RL, YAG, GR, CM, MEA, RD and LM devised the study and participated in the discussion of the results. RL, GR and MEA participated in performing the HPLC to measure the different monoamines levels. All authors approved the final manuscript.

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C A P Í T U L O IV

Uni or bilateral vagotomy to juvenil or adult rats has different effects of on ovulation, hormones levels and monoaminergic activity in the celiac superior mesenteric ganglia

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Abstract:	The vagus nerve is one of the pathways used by the central nervous system (CNS) to send and receive information to and from the ovaries. Vagus nerve fibers have synapses with neurons of the celiac superior mesenteric ganglia (CSMG), the ovary's sympathetic innervation origin. In the present study we analyzed whether the vagus nerve participates in the regulation of CSMG monoaminergic activity, ovulation and ovarian hormone release. Pre-pubertal and adults rats were submitted to unilateral or bilateral vagotomy and sacrificed at 80-82 days of age. Unilateral vagotomy to adult rats resulted in lower number of ova shed than in pre-pubertal rats. Pre-pubertal rats with bilateral vagotomy and adult rats with unilateral vagotomy had lower progesterone levels. Uni or bilateral vagotomy effects on testosterone and estradiol levels were different for pre-pubertal and adult rats. Unilateral vagotomy to pre-pubertal rats resulted in higher noradrenergic activity, while in adult rats the resulting noradrenergic activity was lower. Bilateral vagotomy to pre-pubertal rats resulted in lower dopaminergic activity. In adult rats uni or bilateral vagotomy resulted in higher dopaminergic activity. Vagotomy to pre-pubertal or adult rats decreased serotoninergic activity. These results confirm that the disruption of ovarian innervation modifies ovarian functions depending on the age at which treatment was performed and are the first evidence that the vagus nerve controls the CSMG' monoaminergic system.
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Uni or bilateral vagotomy to juvenil or adult rats has different effects of on ovulation, hormones levels and monoaminergic activity in the celiac superior mesenteric ganglia

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Abstract

The vagus nerve is one of the pathways used by the central nervous system (CNS) to send and receive information to and from the ovaries. Vagus nerve fibers have synapses with neurons of the celiac superior mesenteric ganglia (CSMG), the ovary's sympathetic innervation origin. In the present study we analyzed whether the vagus nerve participates in the regulation of CSMG monoaminergic activity, ovulation and ovarian hormone release. Pre-pubertal and adults rats were submitted to unilateral or bilateral vagotomy and sacrificed at 80-82 days of age. Unilateral vagotomy to adult rats resulted in lower number of ova shed than in pre-pubertal rats. Pre-pubertal rats with bilateral vagotomy and adult rats with unilateral vagotomy had lower progesterone levels. Uni or bilateral vagotomy effects on testosterone and estradiol levels were different for pre-pubertal and adult rats. Unilateral vagotomy to pre-pubertal rats resulted in higher noradrenergic activity, while in adult rats the resulting noradrenergic activity was lower. Bilateral vagotomy to pre-pubertal rats resulted in lower dopaminergic activity. In adult rats uni or bilateral vagotomy resulted in higher dopaminergic activity. Vagotomy to pre-pubertal or adult rats decreased serotoninergic activity. These results confirm that the disruption of ovarian innervation modifies ovarian functions depending on the age at which treatment was performed and are the first evidence that the vagus nerve controls the CSMG' monoaminergic system.

Keywords: vagus nerve, vagotomy, celiac superior mesenteric ganglia (CSMG), ovarian function, monoamines.

Introduction

The ovaries receive sympathetic, parasympathetic and sensorial innervation through the ovarian plexus nerve (OPN), the superior ovarian nerve (SON), and the vagus nerve [1-3]. The nerve release neurotransmitters into the ovaries and play a role in the regulation of steroidogenesis, follicular development and ovulation [4-6].

Morphological evidence of a multi-synaptic neural pathway between the ovary and the central nervous system (CNS) show that the vagus nerve is part of such neural connection [7-9]. Experimental evidence obtained from sectioning the vagus nerve suggest that the information arriving to the ovaries through the vagus nerve participates in regulating ovarian functions in pre-pubertal [10, 11], adult [12-16] and pregnant animals [17, 18].

Neurons originating in the SON and OPN are located in the celiac-superior-mesenteric ganglion (CSMG) [19]. According to Berthoud and Powley [20], the communication between the sympathetic and parasympathetic fibers is apparent at the CSMG level. Then, it is possible that the vagus nerve modulate the postganglionic monoaminergic outflow of the neurons originating in the SON and OPN.

The aims of the present study were to analyze the effects of uni or bilateral vagotomy to pre-pubertal and adult rats on monoaminergic activity of the CSMG, the levels of monoamines in the ovaries, ovulation and hormone levels.

Materials and Methods

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines. The Committee of the Facultad the Estudios Superiores Zaragoza approved the experimental protocols. The study was performed using pre-pubertal female rats of the CIIZ-V

strain from our own breeding stock. Animals were maintained under controlled lighting conditions (lights on from 05:00 to 19:00 h); with free access to rat chows pellets and tap water.

Animal treatment

At 24 (pre-pubertal) or 76 (adult) days of age, animals were randomly assigned to one of the following experimental groups: 1) untouched control; 2) sham-operated; 3) sectioning of the left vagus nerve; 4) sectioning of the right vagus nerve, and 5) bilateral sectioning of the of vagus nerve. Ten animals were used in each experimental group.

Sectioning the vagus nerve and sham surgery procedures were performed between 10:00 and 12:00 h, following previously described methodology [13]. In brief, the rats were anesthetized with ether and laparotomized and a ventral incision, including skin, muscle and peritoneum, was performed. Subsequently, the liver was reflected, and the esophagus exposed. Rats in the vagotomy treatment groups had the left, right, or both vagal trunks cut with fine forceps. Vagus nerves were untouched in the sham-surgery groups of rats. After surgery, the abdominal wall was sutured and the animals returned to their cage. All animals were sacrificed when they reached 80-82 days of age, when they presented vaginal estrous proceeded by a proestro.

A**utopsy procedures**

The animals were sacrificed by decapitation between 10.00 AM and noon. The blood from the trunk was collected, allowed to clot, and centrifuged during 15 min at 3,000 RPM. The serum was stored at -20°C, until progesterone, testosterone and estradiol levels were measured. The oviducts were dissected and the number of ova counted with the aid of a dissecting microscope. The ovaries and the CSMG were removed and stored at -70 ºC until monoamines and their metabolites were measured using high performance liquid chromatography (HPLC). Following the criterion proposed by Burden and Lawrence [12], a distended stomach at the time of necropsy was considered an index of functional vagotomy.

Hormone measurement

Serum concentrations of estradiol (pg/ml), testosterone (pg/ml) and progesterone (ng/ml) were measured using radioimmunoassay, with kits purchased from Diagnostic Products (Los Angeles, CA, USA). The intra- and interassay coefficients of variation were 8.35% and 9.45% for progesterone, 8.12% and 9.28% for estradiol, and 9.65% and 10.2% for testosterone respectively.

Monoamines Levels

The concentration of monoamines (noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their metabolites (4-hydroxy-3-methoxyphenyl glycol (MHPG), 3,4-dihydroxyphenilacetic acid (DOPAC) and 5-hydroxyindole-3-acetic acid (5-HIAA)) in the CSMG and in the ovaries were measured following previously described methodologies [21-23]. In brief, the ovaries and the CSMG were weighed separetly in a precision balance, subsequently homogenized in 300 μl of 0.1 N perchloric acid, and centrifuged at 12,000x g, at 4 ºC for 30 min. The supernatant was filtered using 0.2 μm regenerated cellulose filters. Twenty μl of this extract were injected into a chromatography column via a Rheodyne injection valve.

The HPLC system consisted of an isocratic pump (L-250 model; Perkin Elmer Co., Norwalk, CT, USA), a Rheodyne injection valve (7125 model; Perkin Elmer Co.), an ultrasphere ODS preanalytical column (5 cm 3 4.6 mm) and a Biophase ODS C-18 analytical column (25 cm 3 4.6 mm, 5 mm particle size; Bionalitical Systems Inc., West Lafayette, IN, USA). Monoamines content and their metabolites were detected electrochemically using a LC-4A amperometric detector and a LC-5A glassy carbon traducer cell at a 850 mV potential (Bionalitical Systems Inc.). The mobile phase consisted of 0.1 M citrate buffer (Merck-México, SA.) at pH 3.0, with 175 mg of 1-octane-sulfonic acid (Sigma Chemical Co., St. Louis, MO, USA), filtered and degassed under vacuum. Immediately after degassing, 20 ml of acetonitrile and 21.5 ml of tetrahydrofurane for chromatography (Merck,

Darmastadt, Germany) were added until a total volume of 500 ml was reached. The mobile phase was pumped at a flow rate of 1.2 ml/min. Stock standards (Sigma Chemical Co.) were prepared and diluted with 0.1 M perchloric acid the same day of the experiment.

The system was calibrated by producing a 0.1 to 2 ng/ml standard range curve. Monoamines and their metabolites were identified by the relative retention times compared to standards. Using a 1020 Perkin-Elmer Nelson integrator, the concentrations of monamines and their metabolites were determined by comparing standards with the highest peaks obtained from the samples. Results are expressed as pg of neurotransmitter/mg wet tissue. The sensitivity for all neurotransmitters was 0.01 ng.

Neural activity was estimated following Shannon et al., [24] and Kerdelhué et al. [25] suggestions. Neural activity = [Neurotransmitter Metabolite] / [Neurotransmitter]. Increases in this ratio are considered an indication of greater neurotransmitter turnover and therefore increased neuronal activity [24, 25].

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). The number of ova shed by ovulating animals was analyzed using Kruskal-Wallis test, followed by Mann–Whitney U-test. Treatment effects on ovulation rate (number of ovulating animals/number of treated animals) were analyzed using Fisher's exact probability test. The concentrations of progesterone, testosterone, estradiol, monoamines and their metabolites, as well as the monoaminergic activity in the CSMG and monoamines levels in the ovaries were analyzed by analysis of variance (ANOVA) followed by Tukey test, a p-value ≤ 0.05 was considered significant.

Results

Ovulation (Table 1)

Sham surgery and unilateral or bilateral vagotomy to pre-pubertal or adult rats did not modify the ovulation rate. In adult rats, unilateral vagotomy resulted in lower number of ova shed than in pre-pubertal rats.

Steroids hormones levels

Compared to control animals, sham-surgery to pre-pubertal rats resulted in higher progesterone levels. Compared to sham-rats, bilateral vagotomy resulted in lower progesterone levels. Compared to their corresponding sham-surgery group, uni or bilateral vagotomy to adult rats did not modify progesterone levels. Adult rats with unilateral vagotomy showed lower progesterone than pre-pubertal rats with the same treatment (Figure 1A).

In both age groups, sham-surgery resulted in lower testosterone levels than control. Sectioning the left vagus nerve to pre-pubertal rats resulted in higher testosterone levels than in the corresponding sham-surgery group. In pre-pubertal animals with right or bilateral vagotomy testosterone levels were lower than the analytical sensitivity method. Compared to adult sham-surgery rats, adult rats with right or bilateral vagotomy showed lower testosterone levels. Compared to prepubertal treated group, testosterone levels were lower in adult rats treated with left vagotomy (Figure 1B).

Compared to pre-pubertal sham-surgery rats, rats with left vagotomy showed higher estradiol levels. Adult animals with bilateral vagotomy showed higher estradiol levels than the corresponding sham-surgery group. Compared to pre-pubertal vagotomized animals, estradiol concentrations were lower in adult rats with left vagotomy and higher in rats with right or bilateral vagotomy (Figure 1C).

Noradrenergic activity in the Celiac Superior Mesenteric Ganglia

Compared to control rats, NA levels in the CSMG was higher in pre-pubertal sham-surgery rats. Such increase was abolished by the bilateral section of the vagus nerve. Adult sham-surgery treated rats had lower NA levels in the CSMG than pre-pubertal treated animals. NA levels in uni or bilateral vagotomized adult rats were higher than in sham-surgery treated animals. Adult rats with bilateral vagotomy treatment showed higher NA levels in the CSMG than pre-pubertal treated rats (Figure 2A).

Compared to pre-pubertal sham-surgery treated rats, pre-pubertal rats with unilateral vagotomy showed higher MHPG levels. In pre-pubertal rats with bilateral vagotomy MHPG levels were at the detection limit. Compared to their respective sham-surgery treated group, adult rats with uni and bilateral vagotomy showed higher MHPG levels and noradrenergic activity. Compared to corresponding prepubertal treated group, the MHPG levels and noradrenergic activity were lower in adult rats with unilateral vagotomy and higher in rats with bilateral vagotomy (Figure 2B, 2C).

Dopaminergic system in the Celiac Superior Mesenteric Ganglia

DA levels were not modified by sham-surgery or uni- or bilateral vagotomy treatment in pre-pubertal or adult rats. DA levels were higher in adult rats treated with bilateral vagotomy than in pre-pubertal treated rats (Figure 3A).

Compared to control rats, DOPAC levels were higher in pre-pubertal sham-surgery treated rats. Prepubertal rats with right or bilateral vagotomy showed lower DOPAC levels than pre-pubertal sham-surgery treated rats. Adult rats with uni or bilateral vagotomy showed higher DOPAC levels than sham-surgery rats. Compared to prepubertal vagotomized animals, the DOPAC levels were higher in adult rats treated with bilateral vagotomy (Figure 3B).Compared to control rats, dopaminergic activity in CSMG was higher in pre-pubertal sham-surgery treated rats. Such increase

was absent in pre-pubertal rats treated with bilateral vagotomy. Adult rats with uni or bilateral vagotomy showed higher dopaminergic activity than in sham-surgery animals. Compared to prepubertal vagotomized animals, the dopaminergic activity were higher in adult rats treated with bilateral vagotomy (Figure 3C).

Serotoninergic system in the Celiac Superior Mesenteric Ganglia

Compared to control rats, pre-pubertal rats treated with sham-surgery or uni or bilateral vagotomy did not show changes in 5-HT levels. In adult sham-surgery treated rats 5-HT levels were lower than in the control group. Compared to adult rats with sham-surgery treatment, adult rats with right or bilateral vagotomy showed higher 5-HT levels. Compared to prepubertal treated group, the 5-HT level was higher in adult rats treated with bilateral vagotomy (Figure 4A).

Compared to the control group, pre-pubertal rats treated with sham-surgery showed higher 5-HIAA levels, while the same treatment in adult rats yielded lower 5-HIAA levels. Compared to the pre-pubertal sham-surgery group, right or bilateral vagotomy resulted in lower 5-HIAA levels. In adult rats, animals with left vagotomy showed higher 5-HIAA levels than in rats with sham-surgery treatment. Compared to corresponding prepubertal treated group, the 5-HIAA levels was lower in adult rats treated with sham-surgery or left vagotomy (Figure 4B). Compared to control rats, the serotonergic activity in pre-pubertal sham-surgery treated rats was higher. Serotonergic activity was lower in pre-pubertal rats with right or bilateral vagotomy than in the corresponding sham-surgery group. In adult rats treated with right or bilateral vagotomy the serotonergic activity was lower than in in the corresponding sham-surgery group. Compared to corresponding prepubertal treated group, the serotonergic activity was lower in adult rats treated with sham-surgery, left or bilateral vagotomy (Figure 4C).

Monoamines in the ovary (Table 2)

Compared to control rats, pre-pubertal rats with sham-surgery treatment showed lower NA levels. Compared to pre-pubertal rats with sham-surgery treatment, NA levels were lower in pre-pubertal rats with left vagotomy treatment and higher in pre-pubertal rats with bilateral vagotomy. Compared to adult sham-surgery treated rats, NA levels were lower in adult rats with left vagotomy and higher in rats with right vagotomy.

Compared to control rats, DA levels were higher in adult sham-surgery treated rats. Compared to the corresponding sham-surgery treatment group, pre-pubertal and adult rats with bilateral vagotomy showed higher DA levels.

Compared to control rats, 5-HT levels in the ovaries of pre-pubertal sham-surgery treated rats was lower. Compared to the corresponding sham-surgery treatment group, animals with left or bilateral vagotomy showed lower 5-HT levels. Compared to control rats, 5-HT levels were higher in the ovaries of adult sham-surgery rats. Compared to adult sham-surgery treated rats, 5-HT levels were higher in adult rats with left vagotomy.

Table 1. Ovulation rate (number of animals ovulating/number of treated animals) and ova shed (mean ± SEM (n=10/group)) by untouched (control) rats, with sham-surgery and vagotomy at 24 (pre-pubertal) or 76 (adults) days of age, and sacrificed at day 80–82 of life.

Group	Percent of Ovulation rate	Total number ova shed
Control	100	12 ± 1.1
Sham surgery-24d	91	10 ± 1.3
Sham surgery-76d	85.7	7.5 ± 0.8
Left vagotomy-24d	77.8	9.6 ± 1.8
Left vagotomy-76d	70	5.7 ± 0.7 *****
Right vagotomy-24d	88.9	10.5 ± 1.2
Right vagotomy-76d	80	5.7 ± 1.7 *****
Bilateral vagotomy-24d	90	9.2 ± 0.9
Bilateral vagotomy-76d	100	8.1 ± 1.2

***** $p < 0.05$ vs. 24 day (Fisher test; Kruskal-Wallis followed by Mann–Whitney U-test.

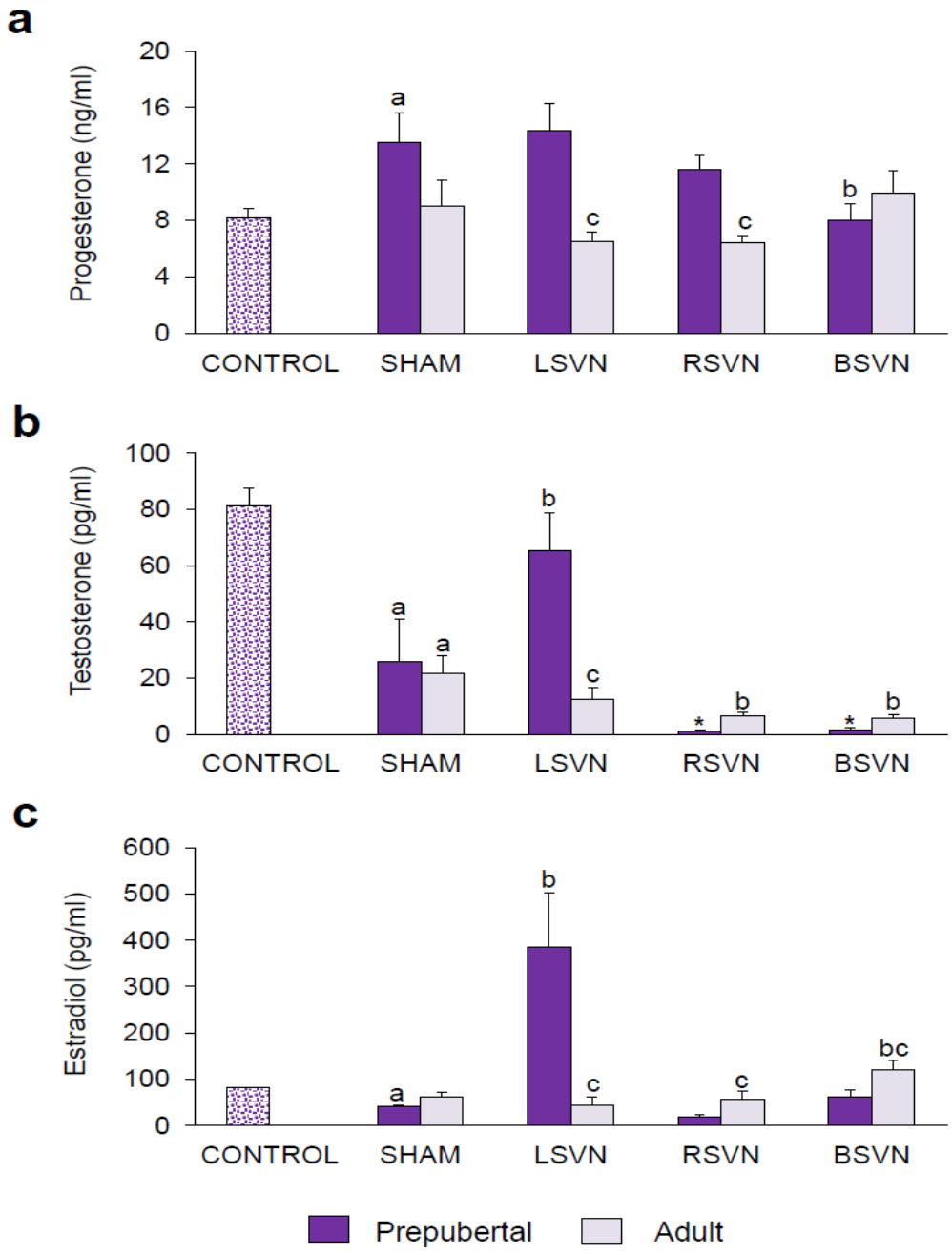

Figure. 1 Mean ± SEM (n=10/group) of Progesterone (a), Testosterone (b) and Estradiol (c) levels in untouched (control) rats, with sham-surgery (sham) unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at 24 (pre-pubertal) or 76 (adults) days of age, and sacrificed at day 80–82 of life. a, $p < 0.05$ vs. control group; b, $p < 0.05$ vs. their sham group; c $p < 0.05$ vs. 24 day. *****, detection limit (ANOVA followed by Tukey´s multiple comparisons test).

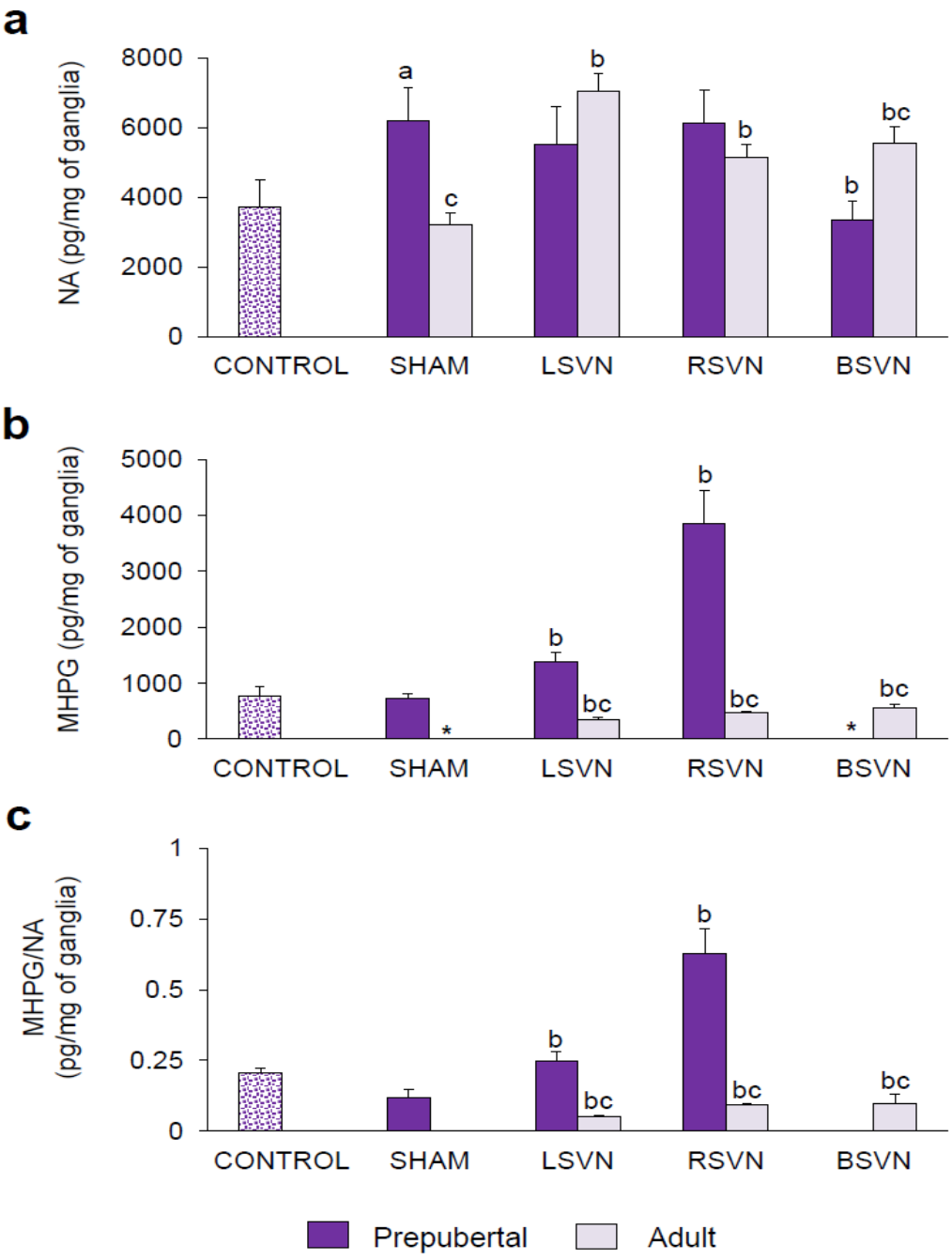

Figure. 2 Mean ± SEM (n=10/group) of (a) NA, (b) MHPG and (c) MHPG/NA levels in untouched (control) rats, with sham-surgery (sham), unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at 24 (pre-pubertal) or 76 (adults) days of age, and sacrificed at day 80–82 of life. a, $p < 0.05$ vs. control group; b, $p < 0.05$ vs. their sham group; c, $p < 0.05$ vs. 24 day. *****, detection limit (ANOVA followed by Tukey´s multiple comparisons test).

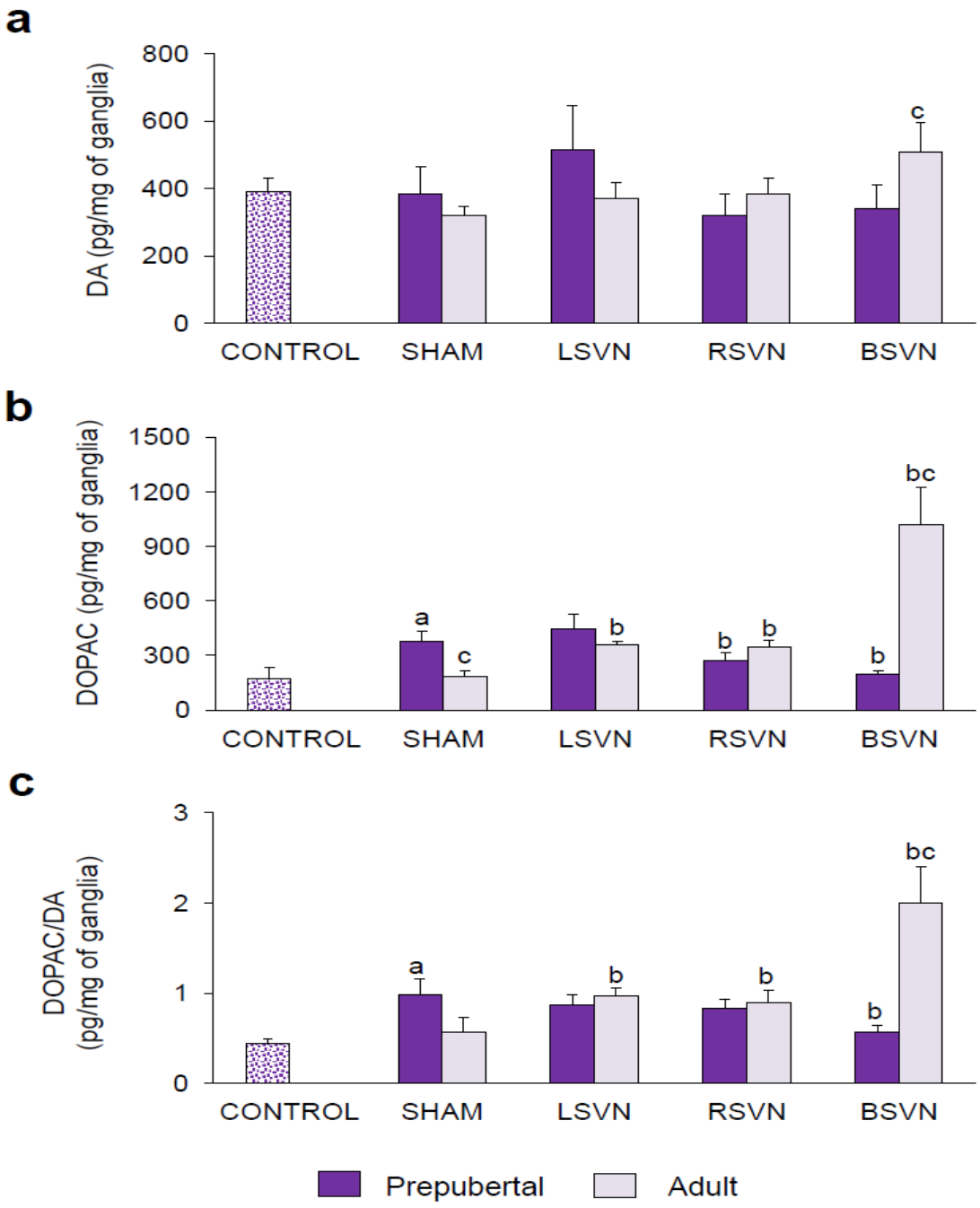

Figure. 3 Mean ± SEM (n=10/group) of (a) DA, (b) DOPAC and (c) DOPAC/DA levels in untouched (control) rats, with sham-surgery (sham), unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at 24 (pre-pubertal) or 76 (adults) days of age, and sacrificed at day 80–82 of life. a, $p < 0.05$ vs. control group; b, $p < 0.05$ vs. their sham group; c, $p < 0.05$ vs. 24 day (ANOVA followed by Tukey´s multiple comparisons test).

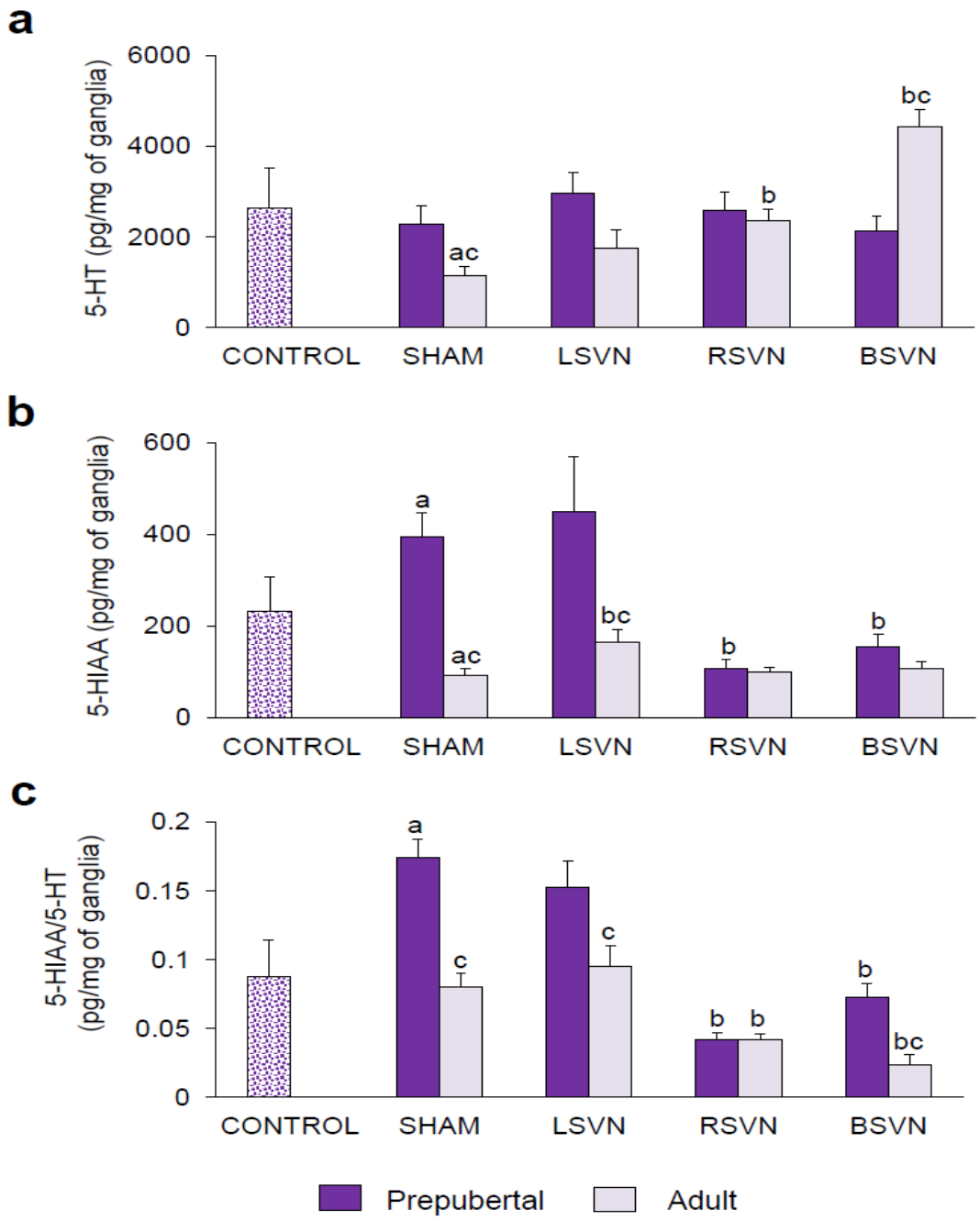

Figure. 4 Mean ± SEM (n=10/group) of (a) 5-HT, (b) 5-HIAA and (c) 5-HIAA/5-HT levels in untouched (control) rats, with sham-surgery (sham) or unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at 24 (pre-pubertal) or 76 (adults) days of age, and sacrificed at day 80–82 of life. a, $p < 0.05$ vs. control group; b, $p < 0.05$ vs. their sham group; c, $p < 0.05$ vs. 24 day (ANOVA followed by Tukey´s multiple comparisons test).

Table 2 mean ± SEM (n=10/group) of Noradrenaline (NA), Dopamine (DA) and Serotonin (5-HT) levels in the ovaries of untouched (control) rats, sham-surgery or vagotomy treatment at 24 (pre-pubertal) or 76 (adults) days of age and sacrificed at day 80–82 of life.

Group	NA	DA	5-HT
Control	1074.6 ± 130.7	61.5 ± 3.1	779.4 ± 163.3
Sham surgery-24d	308.1 ± 108.4 **a**	38.7 ± 6.5	456.6 ± 56.4 **a**
Sham surgery-76d	821.2 ± 88.9	164.1 ± 55.6 **a**	1273.7 ± 128.4 **a**
Left vagotomy-24d	50.9 ± 10.9 **b**	64.8 ± 9.0	239.4 ± 38.7 **b**
Left vagotomy-76d	65.9 ± 2.3 **b**	91.6 ± 15.5	696.1 ± 31.5 **b**
Right vagotomy-24d	414.6 ± 184.7	54.4 ± 5.2	336.8 ± 90.8
Right vagotomy-76d	1170.9 ± 53.8 **b**	71.4 ± 4.9	1012.7 ± 58.4
Bilateral vagotomy-24d	1288.9 ± 42.9 **b**	1061.4 ± 61.9 **b**	126.6 ± 15.7 **b**
Bilateral vagotomy-76d	738.5 ± 11.6	702.9 ± 11.6 **b**	944.3 ± 35

a, $p < 0.05$ vs. control group; b, $p < 0.05$ vs. their sham group, (ANOVA followed by Tukey´s multiple comparisons test).

Discussion

The results of the present study suggest that in pre-pubertal and adult rats the vagal innervation of the CSMG plays a role in regulating noradrenergic, dopaminergic and serotonergic activities, and that this regulatory role depends on the denervation model used (unilateral or bilateral). Similar results were observed in ovulation rates and progesterone, testosterone and estradiol levels. There is evidence of neural connections between the abdominal wall, the celiac, nodose spinal ganglia, and the dorsal motor nucleus of the vagus nerve [26]. Such neural connections could explain the different effects on neurotransmitters levels and monoaminergic turnover, and hormone levels induced by sham surgery in pre-pubertal and adult rats. Flores et al, [27] showed that the neural information arising from different zones of the peritoneum modulates progesterone, testosterone and estradiol secretion in different ways. Present results support such interpretation.

Morales et al. [11], Cruz et al. [13] and Chávez et al. [14] showed that the bilateral vagotomy to pre-pubertal and adult rats results in higher number of ova shed than in rats with sham-surgery treatment. Such effects were not observed in the present study. The differences can be explained by the time between surgery and autopsy in the four studies (In pre-pubertal rats 16 to 19 vs. 56 to 60 days in the present study, and in adult rats 4-6 days vs 25-40 days in the present study).

The ovarian nerves may act via neurotransmitters coupled to the cAMP-generating system to influence the differentiation process by which the ovary performs its functions [6]. Delgado et al [28], using the *ex vivo* coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system, showed that the NAergic stimulation of the CG induced the ovarian release of androstenedione and estradiol, and inhibited progesterone release. The electrical stimulation of the distal part of the severed SON decreased estradiol [29, 30] and testosterone [31] secretion rates from the ovary. In rats, the sub-diaphragmatic vagotomy increases epinephrine levels in plasma, 3, 7 and 14 days after vagotomy treatment, suggesting that vagus nerve has an inhibitory role in of catecholamines regulation [32]. In

rats, the unilateral cervical vagotomy enhances the NA induced activation of left nodose ganglion neurons [33]. The authors suggest that these findings may reflect adaptive responses of the vagal sensory neurons responsible of the monitoring plasma and tissue catecholamine levels.

According to Mravec [34], the sympathoadrenal system is regulated by several mechanisms involving central and peripheral interactions with the parasympathetic nervous system, suggesting that sensory vagal pathways represent a crucial part of the afferent negative feedback loop regulating the activity of the sympathoadrenal system. Since the vagus nerves innervate the ovaries [1] and the CSMG [20], and the soma of the neurons originating in the SON and NPO (both noradrenergic nerves) are in the CSMG, it is possible that the vagus also monitors the ovarian monoaminergic system. In the present study, unilateral and bilateral vagotomy treatment to pre-pubertal and adult rats resulted in different monoaminergic levels in the CSMG and ovaries, and progesterone, testosterone and estradiol levels in serum. Such differences could be explained by the different ages of the animals and the time lapse between treatment and autopsy. Ricu et al (2008) showed that, although the sympathetic nerves can already incorporate NA after birth, the vesicular capacity for catecholamine release does not fully develop until near the time of puberty. Since NA regulates steroidogenic activity [36, 37] and such activity is regulated by vagal information, our results suggest that the changes in catecholaminergic activity in the ovarian nerves resulting from vagotomy, may contribute to the changes in ovarian function.

Based on present results we suggest that the vagus nerve regulates ovarian functions indirectly by regulating monoaminergic activity in the GCMS, the main nervous information relay site between the gonads and the central nervous system.

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Conflict of interest

The authors declare that they have no competing interests.

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C A P Í T U L O V

Participation of the vagus nerve on the monoaminergic system of the celiac-superior mesenteric ganglia of adults rats with polycystic ovarian syndrome

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Participation of the vagus nerve on the monoaminergic system of the celiac-superior mesenteric ganglia of adults rats with polycystic ovarian syndrome

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Abstract

In the present study we analyzed whether the vagus nerve is one of the neural pathways participating in PCOS development through their regulation of CSMG monoaminergic activity. Ten-day old rats were injected with EV dissolved in corn oil. At 76-days of age, experimental rats were submitted to sham-surgery, unilateral, or bilateral vagotomy. The animals were sacrificed at 80-82 days of age, after presenting vaginal estrous smear. In rats with EV-induced PCOS, unilateral or bilateral vagotomy restored ovulation in both ovaries and this was accompanied by a decrease in testosterone levels. The noradrenaline (NA) levels in the CSMG of PCOS rats were higher. Animals with PCOS submitted to unilateral or bilateral vagotomy showed a decrease in concentration NA in the CSMG, without being statistically significant. This result suggests that in rats with EV-induced PCOS the vagus nerve activity regulates monoaminergic CSMG and this paper is related to the development and persistence of EV-induced PCOS.

Keywords: Polycystic ovarian syndrome (PCOS), vagus nerve, vagotomy, celiac superior mesenteric ganglia (CSMG), monoamines.

Introduction

There is evidence on the functional interaction between the peripheral nervous system and the reproductive system (Sosa et al. 2000, 2004, Casais et al. 2006). The ovary receives sympathetic innervation from the celiac superior mesenteric ganglia (CSMG) by two routes: the ovarian plexus nerve (OPN) and the superior ovarian nerve (SON). The CSMG is part of the sympathetic prevertebral chain related with the ovaries (Sosa et al. 2000, 2004; Morán et al. 2009). The ganglia has three major types of cells: the neurons also named principal ganglion cells, the chromaffin cells and the glial cells (Fasano and Niel, 2009) and has a profuse capillary plexus forming circulatory microcircuits among the different ganglionic structures (Tanaka and Chiba, 1996). The CSMG receive innervation by the vagus nerve (Berthoud and Powley, 1996, Sisu et al. 2008). There is evidence that

the activity of the CSMG is modulated by endocrine signals (Casais et al. 2006, Vega et al. 2006, 2010). We have previously shown that the unilateral or bilateral section of the vagus nerve of prepubertal and adult rats modifies the CSMG monoaminergic system in different ways (unpublished data).

The expression of the tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, in the CGMS of rats with polycystic ovarian syndrome (PCOS) induced by the injection of estradiol valerate (EV), is higher than control (Lara et al. 2000). The noradrenaline (NA) levels are increased in the ovaries of rats with EV -induced PCOS (Lara et al. 1993, 2000; Morales et al. 2010), and the ovarian follicular growth is modifying leading to the development of polycystic ovaries (Lara et al. 2002, Rosa-E-Silva et al. 2003).

Unilateral or bilateral vagotomy to 24 day old rats with EV-induced PCOS resulted in spontaneous ovulation in both ovaries (Linares et al, 2013), while the unilateral section of the SON, which neuronal somas are located in the CSMG, restored ovulation only in the innervated ovary (Morales et al, 2010). These results suggest that the vagus nerve modulates the functions of the neurons originating the SON at the CSMG level and therefore this neural information may modulate the SON function in the persistence of PCOS when animals reached adult age.

The aims of present study were to analyze in PCOS rats whether innervation reaching the GCMS through the vagus nerves modulates the monoaminergic activity of the CSMG.

Materials and Methods

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines. The Committee of the Facultad the Estudios Superiores Zaragoza approved the experimental protocols. The study was performed using pre-pubertal female rats of the CIIZ-V strain from our own breeding stock. Animals were maintained under controlled lighting conditions (lights on from 05:00 to 19:00 h); with free access to rat chows pellets and tap water.

Animal treatment

Ten-day old rats were injected with either a single dose of 0.1 ml corn oil (vehicle Vh) or 2 mg EV (Sigma Chem. Co., St. Luis, Mo. USA) dissolved in 0.1 ml corn oil. When the rats injected with Vh or EV reached 76 days of age, groups of ten animals injected with Vh or EV were randomly allotted to one of the following groups: No surgery (Vh and EV), unilateral vagotomy (Vh/left vagotomy, Vh/right vagotomy, EV/left vagotomy, and EV/right vagotomy), or bilateral vagotomy (Vh/ bilateral vagotomy and EV/ bilateral vagotomy). Vagotomy was performed following methodologies previously described (Cruz et al, 1986). In brief, between 10:00 and 12:00 h the rats were anesthetized with ether and a ventral incision including skin, muscle and peritoneum was performed. Subsequently, the liver was reflected, the esophagus exposed, and the left, right, or both vagal trunks were cut with fine forceps. After surgery, the abdominal wall was sutured and the animals returned to their cage. The animals injected with Vh or EV were sacrificed when they presented vaginal estrous at 80–82 days of age, following previously described methodologies (Linares et al, 2013).

To analyze the monoamine activity of CSMG, three animals from each group injected with Vh or VE were injected in the bursa the left or right ovary with True Blue (TB). The injection of TB was performed following previously described procedures [Morán et al, 2005]. In brief, the animals were ether anesthetized between 10:00 am and 12:00 PM, and a ventral incision was performed 2 cm below the last rib, affecting skin, muscle, and peritoneum. The left or right ovary was exposed and 3 μL of TB (Sigma, St. Louis, Missouri, USA) solution at 4%, diluted in distilled water, was injected into the ovarian bursa. To prevent the leakage of the tracer, the needle was kept in the bursa for 1 min after injection treatment. Subsequently, the ovary was carefully cleaned, dried, and returned to the abdominal cavity. The possibility that TB leaked into the abdominal cavity was assessed by exposing the cavity to a fluorescent light, and the animals with TB leakage were excluded from the experiment.

In other groups of three animals injected with Vh or VE the vagus nerve were sectioned uni or billaterally before injecting the TB into the ovarian bursa.

A**utopsy procedures**

Groups of control rats and animals injected with Vh or EV, followed or not by uni or bilateral vagotomy were sacrificed by decapitation between 10.00 AM and 12:00 PM. The blood from the trunk was collected, allowed to clot, and centrifuged during 15 min at 3,000 RPM. The serum was stored at -20°C, until progesterone, testosterone and estradiol levels were measured. Following the criterion proposed by Burden y Lawrence [1977], at the time of necropsy a distended stomach was considered an index of functional vagotomy. At autopsy, the oviducts were dissected and the number of ova counted with the aid of a dissecting microscope. The ovaries and the CSMG was removed and stored at -70 ºC until monoamines and their metabolites were measured using high performance liquid chromatography (HPLC).

The animals injected with TB were sacrificed by perfusion when they presented vaginal estrous at 80–82 days of age. For that, the rats were anesthetized with sodium pentobarbital (40 mg/Kg) IP and intracardiac perfused with 250 mL of cold saline solution (0.9%), followed by the injection of 150 mL solution of 4% paraformaldehyde. After perfusion of the fixative solution, the pre-vertebral CSMG was dissected and kept in the fixative solution overnight (approx. 18 h). The ganglia were cryoprotected successively in 10, 20, and 30% sucrose in phosphate buffer and were serially sectioned at twenty micrometer with the aid of a cryostat kept at -20°C. The sections were analyzed following the same procedure previously described [Morán et al, 2005]. In brief, eight to ten images of the right or left CSMG, from the animals injected with TB, were used to count the number of positively labeled cells. Positive TB cells are defined as cells in which fluorescence is present when the sections were exposed to UV light. Only principal neurons were labeled with TB and SIF neurons are not labeled with the tracer.

The pictures were obtained with a Digital Camera (Optronics 60300, USA) and analyzed with a KS-300 Imaging System 3.0 (Carl Zeiss Vision GmbH, Germany). The imaging system was programmed to generate binary regions, automatically count and integrate them.

Hormone measurement

Serum concentration of estradiol (pg/ml), testosterone (pg/ml) and progesterone (ng/ml) were measured using radioimmunoassay, with kits purchased from Diagnostic Products (Los Angeles, CA, USA). The intra- and interassay coefficients of variation were 8.35% and 9.45% for progesterone, 8.12% and 9.28% for estradiol, and 9.65% and 10.2% for testosterone respectively.

Monoamines Levels

The concentration of monoamines (noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their metabolites (4-hydroxy-3-methoxyphenyl glycol (MHPG), 3,4-dihydroxyphenilacetic acid (DOPAC) and 5-hydroxyindole-3-acetic acid (5-HIAA)) in the CSMG and monoamines in the ovary were measured following methodologies previously described (Quiroz et al, 2013). In brief, the ovary or CSMG were weighed in a precision balance, homogenized in 300 μl of 0.1 N perchloric acid, and centrifuged at 12,000x g, at 4 ºC for 30 min. The supernatant was filtered using 0.2 μm regenerated cellulose filters. Twenty μl of this extract were injected into a chromatography column via a Rheodyne injection valve.

The HPLC system consisted of an isocratic pump (L-250 model; Perkin Elmer Co., Norwalk, CT, USA), a Rheodyne injection valve (7125 model; Perkin Elmer Co.), an ultrasphere ODS preanalytical column (5 cm 3 4.6 mm) and a Biophase ODS C-18 analytical column (25 cm 3 4.6 mm, 5 mm particle size; Bionalitical Systems Inc., West Lafayette, IN, USA). Monoamines content and their metabolites were detected electrochemically using a LC-4A amperometric detector and a LC-5A glassy carbon traducer cell at a 850 mV potential (Bionalitical Systems Inc.). The mobile phase

consisted of 0.1 M citrate buffer (Merck-México, SA.) at pH 3.0, with 175 mg of 1-octane-sulfonic acid (Sigma Chemical Co., St. Louis, MO, USA), filtered and degassed under vacuum. Immediately after degassing, 20 ml of acetonitrile and 21.5 ml of tetrahydrofurane for chromatography (Merck, Darmastadt, Germany) were added until a total volume of 500 ml was reached. The mobile phase was pumped at a flow rate of 1.2 ml/min. Stock standards (Sigma Chemical Co.) were prepared and diluted with 0.1 M perchloric acid the same day of the experiment.

The system was calibrated by producing a 0.1 to 2 ng/ml standard range curve. Monoamines and their metabolites were identified by the relative retention times compared to standards. Using a 1020 Perkin-Elmer Nelson integrator, the concentrations of monamines and their metabolites were determined by comparing standards with the highest peaks obtained from the samples. Results are expressed as pg of neurotransmitter/mg wet tissue. The sensitivity for all neurotransmitters was 0.01 ng.

Neural activity was estimated following Shannon et al., (1986), and Kerdelhué et al., (1989) suggestions. Neural activity = [Neurotransmitter Metabolite] / [Neurotransmitter]. Increases in this ratio are considered an indication of greater neurotransmitter turnover and therefore increased neuronal activity (Shannon et al, 1986; Kerdelhué et al, 1989).

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). The number of ova shed by ovulating animals was analyzed using Kruskal-Wallis test, followed by Mann–Whitney U-test. The ovulation rate was analyzed using Fisher's exact probability test. The progesterone, testosterone, estradiol, monoamines and their metabolites levels, as well as the monoaminergic activity in the CSMG and monoamines levels in the ovary were analyzed using a two-way analysis of variance (ANOVA) followed by Tukey´s multiple comparisons test; a, p-value ≤ 0.05 was considered significant.

Results

Ovulation

None of the EV-injected rats ovulated while all the Vh-injected animals did it (0/7 vs. 7/7, $p < 0.01$ Fisher's exact probability test). Sham-surgery did not modify ovulation rates in Vh injected-rats, while none of the EV-injected rats submitted to sham-surgery ovulated. Most of the EV-injected rats with unilateral or bilateral vagotomy ovulated, and the number of ova shed was similar to Vh-injected rats with similar treatments (Table 1).

Steroids hormone levels

Progesterone levels were similar in Vh-injected control and EV-injected control rats. Compared to EV-injected control group, sham-surgery to EV-injected rats resulted in lower progesterone levels. Compared to EV-injected sham-surgery group, right vagotomy resulted in higher progesterone levels (Figure 1A).

The testosterone levels in EV-injected rats were higher than in the Vh-injected group. The unilateral vagotomy to Vh-injected rats did not modify the testosterone levels. Compared to Vh-injected sham-surgery rats, bilateral vagotomy to Vh-injected resulted in lower testosterone levels. Compared to EV-injected sham-surgery rats, the unilateral or bilateral vagotomy to EV-injected rats resulted in lower testosterone levels (Figure 1B).

Estradiol levels were similar in Vh-injected and EV-injected rats. Sham-surgery to Vh-injected rats resulted in higher estradiol levels that those Vh-injected one. Sectioning the right vagus or both vagus to EV-injected animals resulted in higher estradiol levels than the corresponding sham-surgery group (Figure 1C).

Noradrenergic system in the Celiac Superior Mesenteric Ganglia

NA levels in the CSMG of EV-injected rats were higher than those Vh-injected. Compared to control group, sham-surgery performed to Vh-injected rats resulted in higher NA levels. Such increases were not modify by unilateral vagotomy and they decreased in rats with bilateral vagotomy. In EV-injected rats, sham-surgery and uni- or bilateral vagotomy did not modify the NA levels (Figure 2A).

Compared to the Vh-injected group, MHPG levels were higher in EV-injected rats. Sham-surgery to Vh-injected rats resulted in lower MHPG levels than Vh-injected rats, while unilateral vagotomy resulted in higher MHPG levels and noradrenergic activity. In animals with bilateral vagotomy, MHPG concentration was below the sensitivity of the method. Compared to the EV-injected group, MHPG levels and noradrenergic activity were lower in EV-injected rats with sham-surgery. Sectioning the left vagus or both vagus to EV-injected animals resulted in higher MHPG levels and noradrenergic activity than the corresponding sham-surgery group (Figure 2B, 2C).

Dopaminergic system in the Celiac Superior Mesenteric Ganglia

DA levels in the EV-injected rats were similar to those Vh-injected. Sham-surgery and unilateral vagotomy did not modify DA concentration in Vh-injected rats, while bilateral vagotomy decreased it. Compared to the EV-injected group, sham-surgery decreased DA levels, while sectioning the right vagus to EV-injected animals resulted in higher DA levels than the corresponding sham-surgery group (Figure 3A).

DOPAC levels in the CSMG were higher in EV-injected than in those Vh-injected rats. Compared to Vh-injected sham-surgery rats, the section the left vagus resulted in higher DOPAC levels. Such increase was absent in Vh-injected rats treated with bilateral vagotomy. Compared to the EV-injected group, sham-surgery resulted in lower DOPAC levels. Compared to the EV-injected sham-surgery group, the right or bilateral vagotomy resulted in higher DOPAC levels (Figure 3B).

In EV-injected rats, the dopaminergic activity was higher than in those Vh-injected. Compared to its corresponding sham-surgery group, uni or bilateral vagotomy did not modify the dopaminergic activity in Vh-injected or EV-injected rats (Figure 3C).

Serotoninergic system in the Celiac Superior Mesenteric Ganglia

5-HT levels were similar in Vh-injected and EV-injected animals. Sham-surgery and unilateral vagotomy did not modify 5-HT levels Vh-injected rats, while bilateral vagotomy increased it. In EV-injected rats, sham-surgery, left and bilateral vagotomy did not modify 5-HT levels, while right vagotomy increased it (Figure 4A).

5-HIAA levels were not modified by sham-surgery, left or bilateral vagotomy treatment in Vh-injected rats. Right vagotomy to Vh-injected resulted in lower 5-HT levels than in those with sham-surgery. In EV-injected rats, sham-surgery, unilateral or bilateral vagotomy did not modified 5-HIAA levels (Figure 4B).

The unilateral or bilateral vagotomy to Vh-injected rats did not modify serotoninergic activity. Right and bilateral vagotomy resulted in lower serotonergic activity in EV-injected rats (Figure 4C).

Monoamines system in the ovary

The NA levels in EV-injected rats were higher than in those Vh-injected. Sham-surgery resulted in lower NA levels in Vh-injected group. Unilateral or bilateral vagotomy did not modify the sham-surgery effects.

DA levels were similar in Vh-injected and EV-injected rats. In Vh-injected group, sham-surgery resulted in higher DA levels, while unilateral vagotomy decreased it. Compared to EV-injected sham-surgery group, the right vagotomy resulted in lower DA levels, while was higher in bilateral vagotomy group.

Compared to Vh-injected group, the 5-HT levels were lower in EV-injected control rats. Sham-surgery decreased 5-HT levels in Vh-injected rats, while right vagotomy resulted in higher levels. In EV-injected group, sham surgery resulted in higher 5-HT levels, while sectioning the left vagus resulted in lower 5-HT levels than corresponding sham-surgery group.

Number of positive the neurons in CSMG of rats injected with TB

In the CSMG of the EV-injected rats, the number TB positive neurons were higher than in Vh-injected group, and unilateral or bilateral vagotomy increased the number of positive neurons in both EV and Vh-injected rats (Figure 5).

Table 1 Ovulation rate (number of animals ovulating/number of treated animals) and ova shed (mean ± SEM (n=7/group)) by rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), with sham-surgery (sham) or unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at day 76 of life, sacrificed at day 80–82 of life.

Group	Percent of Ovulation rate	Number ova shed
Vh_Control	100	9.7 ± 1.5
EV_Control	0 **a**	0 **a**
Vh_Sham	75	8.7 ± 0.9
EV_Sham	0 **b**	0 **b**
Vh_LSVN	70	7.0 ± 1.4
EV_LSVN	84.6 **ab**	5.0 ± 1.0 **ab**
Vh_RSVN	81.8	5.6 ± 1.0
EV_RSVN	70 **ab**	7.4 ± 0.7 **ab**
Vh_BSVN	85.7	10.1 ± 1.5
EV_BSVN	100 **ab**	6.3 ± 0.9 **ab**

a $p < 0.05$ vs. Vh or EV control group, b $p < 0.05$ vs. Vh or EV sham group (Fisher test; Kruskal-Wallis test followed by Mann–Whitney U-test).

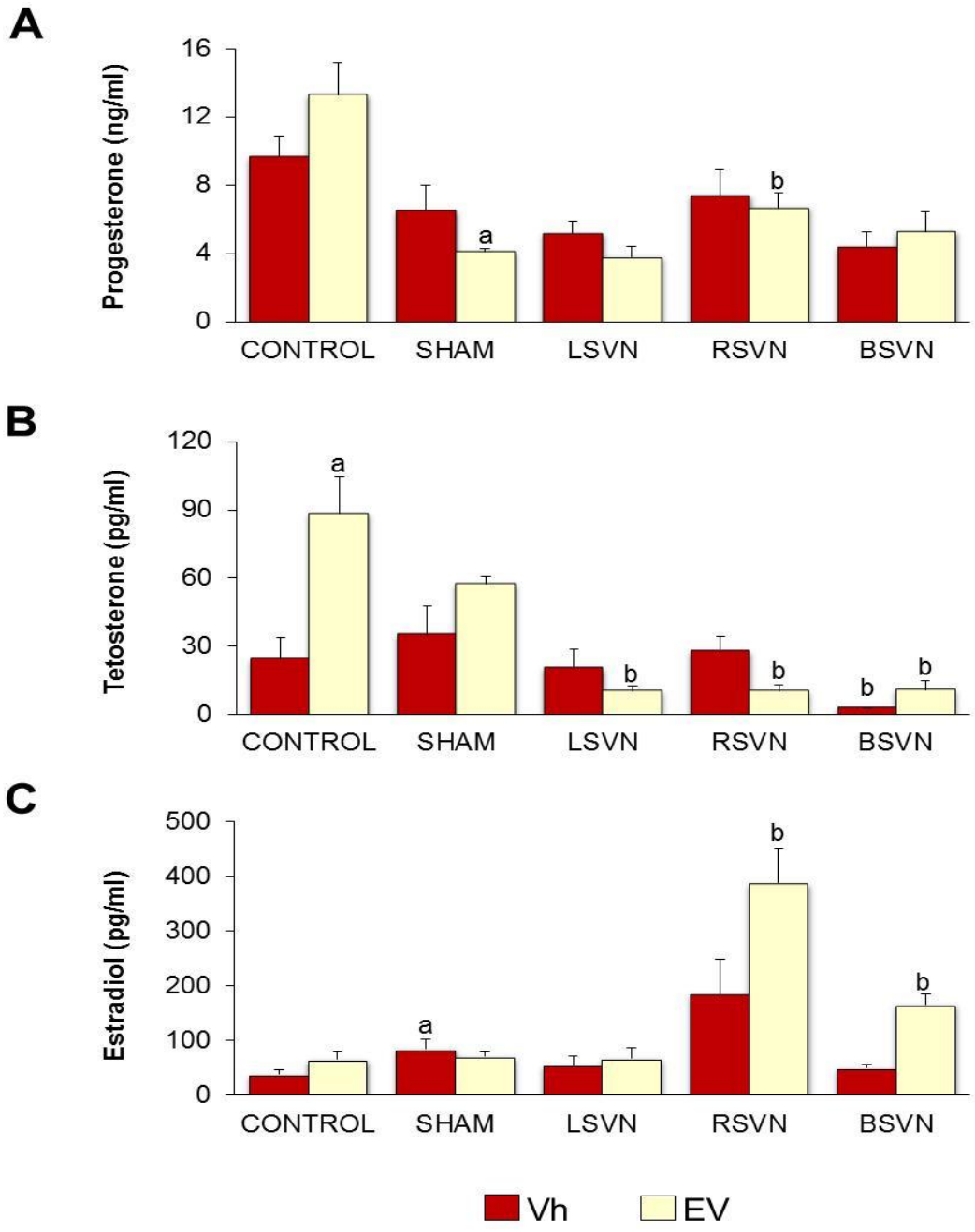

Figure 1 Mean ± SEM (n=7/group) of Progesterone (A), Testosterone (B) and Estradiol (C) levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), with sham-surgery (sham) or unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at day 76 of life, sacrificed at day 80–82 of life. a $p < 0.05$ vs. Vh or EV control group, b $p < 0.05$ vs. Vh or EV sham group. (Two-ways ANOVA followed by Tukey´s multiple comparisons test).

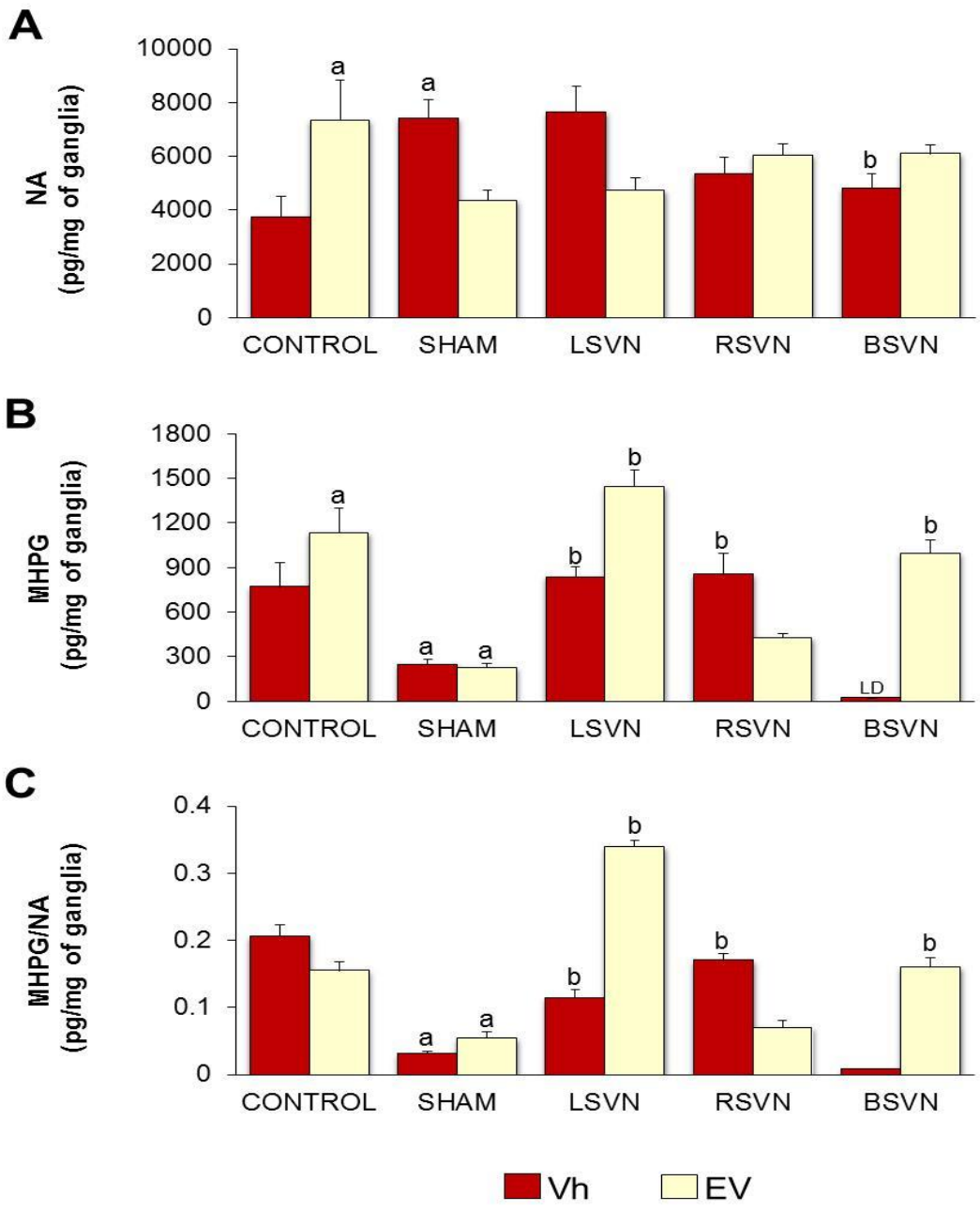

Figure 2 Mean ± SEM (n=7/group) of (A) NA, (B) MHPG and (C) MHPG/NA levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), sham-surgery (sham), unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at day 76 of life, sacrificed at day 80-82 of life. a $p < 0.05$ vs. Vh or EV control group, b $p < 0.05$ vs. Vh or EV sham group. (Two-ways ANOVA followed by Tukey´s multiple comparisons test). LD = detection limit.

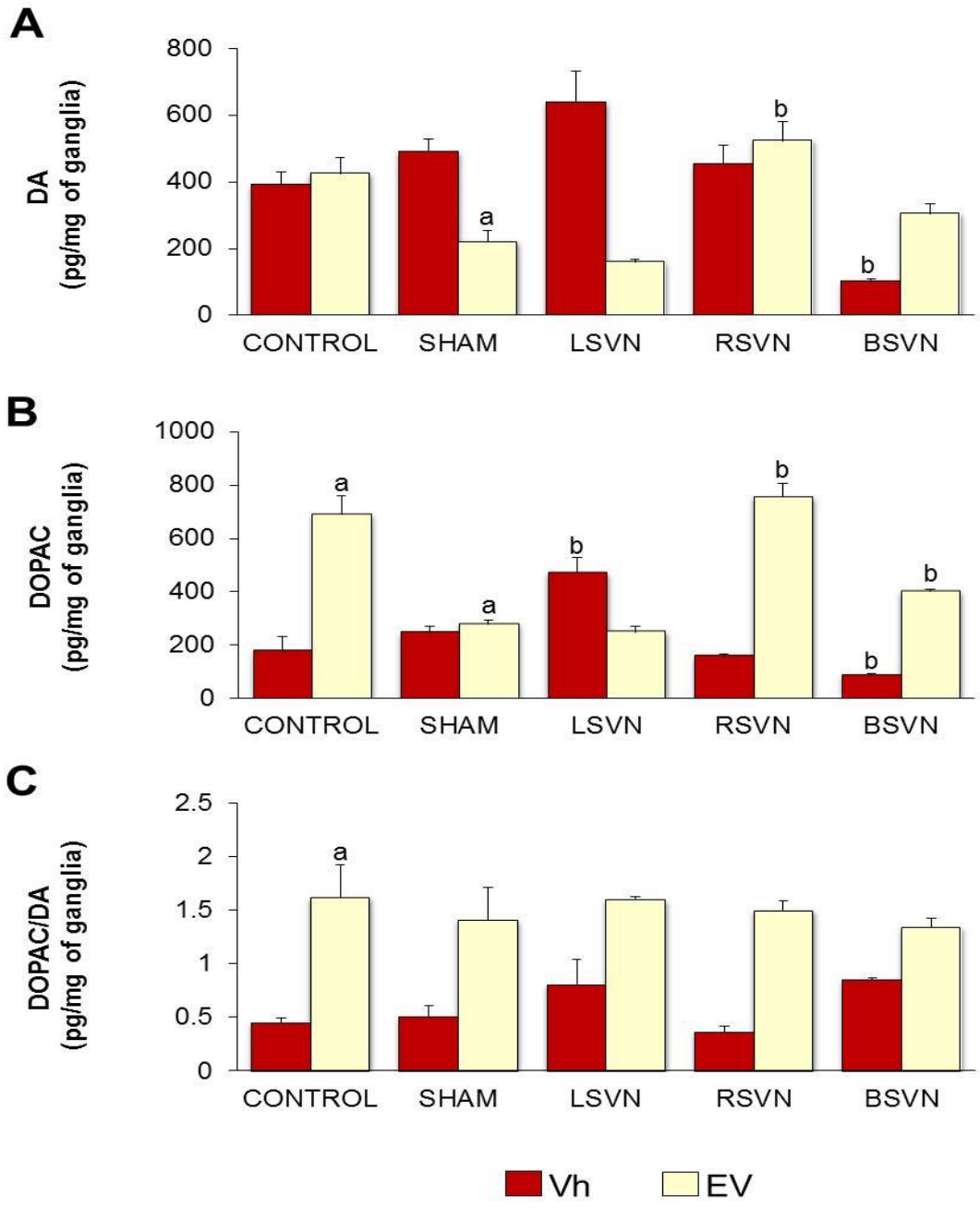

Figure 3 Mean ± SEM (n=7/group) of (A) DA, (B) DOPAC and (C) DOPAC/DA levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), with sham-surgery (sham), unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at day 76 of life, sacrificed at day 80-82 of life. a $p < 0.05$ vs. Vh or EV control group, b $p < 0.05$ vs. Vh or EV sham group. (Two-ways ANOVA followed by Tukey´s multiple comparisons test).

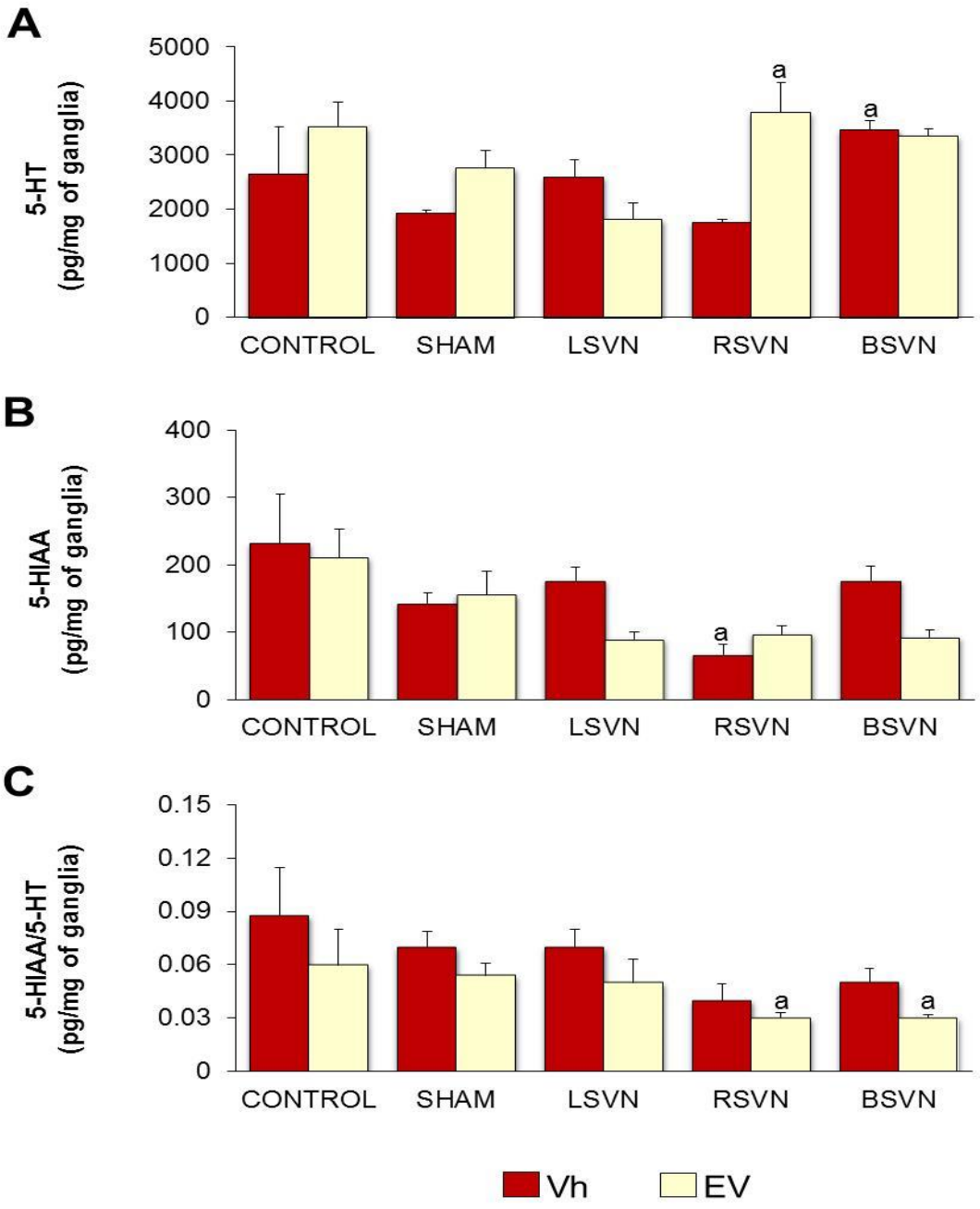

Figure 4 Mean ± SEM (n=7/group) of (A) 5-HT, (B) 5-HIAA and (C) 5-HIAA/5-HT levels in rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), with sham-surgery (sham), unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at day 76 of life, sacrificed at day 80-82 of life. a $p < 0.05$ vs. Vh or EV sham group. (Two-ways ANOVA followed by Tukey´s multiple comparisons test).

Table 2 mean ± SEM (n=7/group) of Noradrenaline (NA), Dopamine (DA) and Serotonin (5-HT) levels in the ovaries of rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control), with sham-surgery (sham) or unilateral (LSVN or RSVN) and bilateral vagotomy (BSVN) at day 76 of life, sacrificed at day 80–82 of life.

Group	NA	DA	5-HT
Vh_Control	1074.6 ± 130.7	61.5 ± 3.1	779.4 ± 163.3
EV_Control	1523.8 ± 185.6 **a**	63.4 ± 8.1	371.8 ± 96.5 **a**
Vh_Sham	558.9 ± 195.7 **a**	161.9 ± 35.0 **a**	453.7 ± 81.3 **a**
EV_Sham	1198.6 ± 134.4	53.9 ± 8.2	736.5 ± 57.8 **a**
Vh_LSVN	498.0 ± 129.4	62.6 ± 13.5 **b**	489.9 ± 72.8
EV_LSVN	1272.2 ± 192.4	80.8 ± 24.8	471.9 ± 69.8 **b**
Vh_RSVN	802.6 ± 95.0	30.5 ± 6.3 **b**	1174.9 ± 141.6 **b**
EV_RSVN	1561.5 ± 163.8	25.0 ± 2.3 **b**	724.8 ± 104.7
Vh_BSVN	500.4 ± 77.3	129.9 ± 20.9	653.2 ± 88.2
EV_BSVN	895.1 ± 381.5	89.2 ± 2.3 **b**	507.3 ± 58.3

a $p < 0.05$ vs. Vh or EV control group, b $p < 0.05$ vs. Vh or EV sham group. Two-ways ANOVA followed by Tukey´s multiple comparisons test.

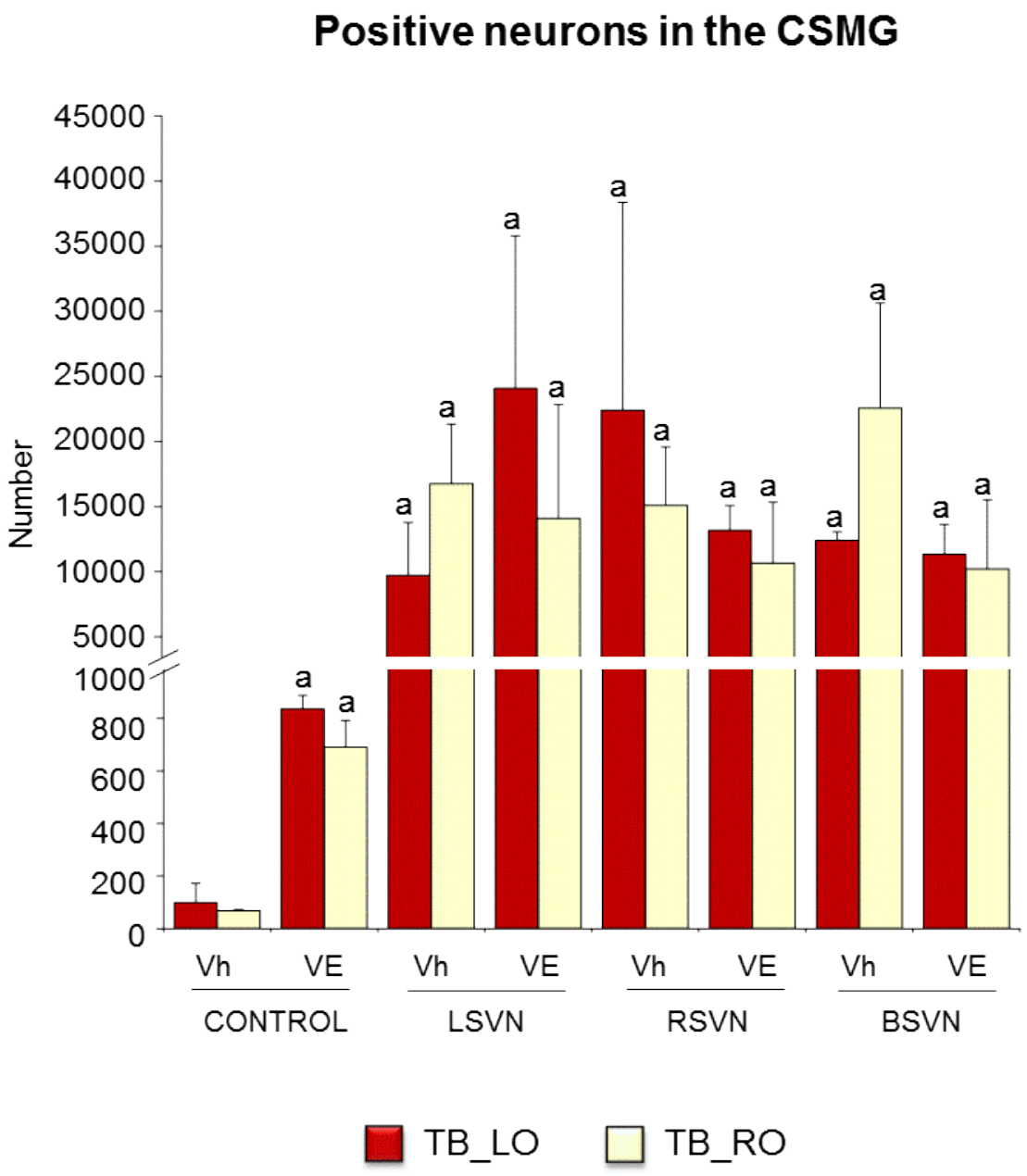

Figure 5 Mean ± SEM (n=3/group) of the number of TB-positive neurons in the CSMG of rats injected with vehicle (Vh) or estradiol valerate (EV) at day 10 of life, untouched (control) and with unilateral (LSVN or RSVN) or bilateral vagotomy (BSVN) at day 76 of life, previous to injected with true blue (TB) in the left (LO) or right (RO) ovary and sacrificed at day 80-82 of life. a $p < 0.05$ vs. Vh or EV control group. Two-ways ANOVA followed by Tukey´s multiple comparisons test.

Discussion

The results obtained in the present study support the hypothesis of that the neural information carried by the vagus nerve plays a role in the mechanisms participating in the regulation of monoaminergic activity of the CGMS and therefore in the development and maintenance of PCOS.

In EV-induced PCOS rat, the bilateral sectioning of the SON (Barria et al. 1993, Rosa-E-Silva et al. 2003, Morales et al. 2010) or the bilateral electro-acupuncture treatment at the T12-L2 segments level (Stener-Victorin et al. 2000) result in spontaneous ovulation and lower ovarian NA levels. Despite a drop of NA levels in the denervated ovary, unilateral sectioning of the SON restored ovulation by the innervated ovary; suggesting that aside from an increase in ovarian noradrenergic tone in the ovaries, in the pathogenesis of the PCOS participate other neural influences arriving to the ovaries (Morales et al. 2010). We have previously shown that uni or bilateral vagotomy to prepubertal EV-induced PCOS rats restored ovulation in both ovaries (Linares et al. 2013), suggesting that in EV-induced PCOS rats the vagus nerve is a neural pathway participating in maintaining PCOS. Similar results were observed in the present study, since the unilateral or bilateral vagotomy to adult rats restored ovulation in more than 70 percent of animals. The section of the left or both vagus nerve to prepubertal rats results in lower concentration of NA in the CSMG (unpublished results). These results suggest that the vagus nerve regulate the persistence of PCOS indirectly through its synapsis in the CSMG with the somas of neurons originating in the SON are located.

In women, one of the diagnostic features of PCOS is hyperandrogenism. In the rats the injection of the EV increases the concentration of testosterone (Barria et al. 1993), as showed in the present study. This increase in testosterone can be explained from the increased concentration of NA in the CSMG, which has a stimulating effect on androgen production, as already reported (Dyer and Erickson, 1985; Delgado et al. 2010). In animals with PCOS and unilateral or bilateral vagotomy

decreases the concentration of NA in CSMG, which may explain the decrease in testosterone levels and restoring ovulation in this animals.

Several hypotheses have been proposed to explain the etiology of the PCOS, including the hyper-activation of the sympathetic fibers arriving to the ovary via the SON (Stener-Victorin et al. 2000; Lara et al. 1993, 2000). The EV injection increases ovarian NA content, enhanced NA uptake and release form ovarian nerve terminals (Lara et al, 1993), increase intraovarian synthesis of neural growth factor and its low affinity neurotrophin receptor p-75, while in the CSMG increases the tyrosine hydroxylase mRNA level (Lara et al, 2000). In the present study, a similar increase in CSMG-NA levels was observed in rats with EV-induced PCOS, supporting the idea that an increase in the noradrenergic system activity is part of the mechanisms elicited by the experimental PCOS inductors.

According to Huang et al (2004) vagotomy may trigger a variety of adaptive biochemical and molecular alterations in vagal afferent neurons of the nodose ganglion leading to the functional plasticity. The changes in the concentration of monoamines and their metabolites in the CSMG of PCOS rats with unilateral or bilateral vagotomy suggest differences in the neural information carried by the left and right vagus nerve, as proposed before (Morales et al. 2004; Linares et al. 2013). Present results suggest that during the PCOS the vagus nerve may regulate the of sensory information transmission from the ovary to the spinal cord. The density of sympathetic nerve fibers is higher in the cystic ovaries of women (Heider et al, 2001), in the ovaries of rats injected with EV (Stener-Victorin et al, 2005) and in pigs with cystic ovaries induced by injecting dexamethasone (Jana et al, 2005; Kozlowska et al, 2013). Cholinergic (Kozlowska et al, 2009) and sensory (Kozlowska et al, 2011) ovarian innervations are also modified in pigs with cystic ovaries induced by injecting dexametasone. According to Lara et al., (2000), the hyper-activation of the ovarian sympathetic input resulting from EV treatment is related to an overproduction of ovarian NGF and its low affinity

receptor in the ovary. Although EV induced follicular cysts are first detected around 60 days after EV treatment (Brawer et al. 1986), activation of the sympathetic innervation of the ovaries occurs at least a month before the formation of follicular cysts (Lara et al. 1993). In turn, increases in p75 NGFR synthesis occur as early as 15 days after EV treatment and is shortly followed by increases in NGF synthesis (Lara et al. 2000). This suggests that the activation of this ligand/receptor modules is an early event in the process by which EV treatment disrupts ovarian function. NGF increased in the sympathetic neurons projecting to the ovary are likely to play a significant role in enhancing the sympathetic outflow to the ovary in EV-treated rats (Lara et al. 2000). Then, it is possible that the vagus nerve participates in regulating NGF release by the sympathetic neurons that origin in the CSMG.

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Authors' Roles

RL, LM and RD planned the experiments. RL, GR, DRV, CM, CM, RD and LM devised the study and participated in the discussion of the results. RL and GR participated in performing the HPLC to measure the different monoamines levels. All authors approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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DISCUSIÓN GENERAL

Los resultados obtenidos en los capítulos II, III y V confirman que en animales inyectados con VE la falta de la información vagal, uni o bilateral, reduce parcialmente la actividad noradrenérgica del GCMS y restablece la ovulación lo que nos sugiere que además de la hiperactividad noradrenérgica otros mecanismos participan en el mantenimiento del SOPQ los que dependen, al menos parcialmente, de la inervación vagal.

A partir de los resultados obtenidos en el capítulo IV sugerimos que en la rata la inervación vagal modula las funciones ováricas en función de la edad en la cual se realizan los estudios y del lapso entre el momento en que se realizan los tratamientos y se evalúan los resultados.

Los ovarios reciben y envían información neural desde y hacia el SNC vía el NOS, el nervio del plexo ovárico y el nervio vago (Gerendai col. 2000). Diversos estudios sugieren la existencia de una comunicación nerviosa entre los ovarios en la que participa el NOS (Gerendai y col. 1998). Según Morán y col. (2005, 2009) el GCMS sería el principal sitio de relevo de la información neural entre las gónadas. En el sistema *ex vivo*, ganglio celíaco-NOS-ovario, la estimulación del ganglio celíaco con NA aumenta la liberación de hormonas ováricas, lo que sugiere que la respuesta ovárica está ligada al estímulo noradrenérgico del ganglio celíaco (Delgado y col. (2010). Los resultados obtenidos en el presente estudio, muestran que en ratas prepúberes o adultas los nervios vago regulan la actividad monoaminérgica del GCMS, lo que puede explicar las modificaciones en la ovulación, y en las concentraciones de hormonas ováricas observadas en este estudio y las descritas por Cruz y col. (1986) y Morales y col. (2004).

La inyección del VE en ratas de 10 días de edad resultó en falta de ovulación, falta de ciclo estral, ovarios poliquísticos, hiperandrogenismo, hiperactividad simpática en el GCMS, resultados

similares a los descritos por Lara y col. (1993); Rosa-E-Silva y col. (2003); Sotomayor-Zarate y col. (2008); Morales y col. (2010).

En los ovarios, el VE estimula la expresión del NGF, lo que resulta en la mayor secreción de NA y el desarrollo del SOPQ (Lara y col. 2000; 2002). Según Gerendai y col. (2005) la inyección de VE no activa a las neuronas simpáticas que se proyectan desde el núcleo paraventricular del hipotálamo hacia los ovarios, pero si activa las neuronas del núcleo del tracto solitario y del núcleo motor dorsal del nervio vago, lo que apoya la idea de un efecto central del VE sobre las neuronas que inervan a los ovarios vía los nervios vago.

Varias hipótesis han sido propuestas para explicar el desarrollo del SOPQ, como son la alteración de la secreción pulsátil de GnRH (Brawer y col. 1986; Matalliotakis y col. 2006), la alteración en la acción de la insulina (Dunaif y col. 1997), modificaciones en la síntesis y metabolismo de esteroides ováricos (Franks y col., 1998) y la hiperactivación de las fibras simpáticas que llegan al ovario por el NOS (Lara y col., 2000).

En ratas con SOPQ inducido por la inyección de VE, la sección bilateral del NOS (Barria y col. 1993; Rosa-E-Silva y col. 2003) o el tratamiento con electro-acupuntura (Stener-Victorin y col. 2000) restablecen la ovulación, lo que es explicado por la disminución en la concentración de NA ovárica como resultado de la sección del NOS o por los efectos de la electro-acupuntura. Morales y col. (2010), mostraron que la sección unilateral del NOS restablece la ovulación en el ovario inervado a pesar de las altas concentraciones de NA, por lo que sugieren que la hiperactividad noradrenérgica no es el único factor que explica el desarrollo del SOPQ y que es posible que otras señales nerviosas que llegan a los ovarios participen en el mantenimiento de la fisio-patología.

En el presente estudio mostramos que en ratas con SOPQ la sección uni o bilateral del nervio vago realizada en la etapa prepúber o adulta restablece la ovulación en más del 70% de los animales, por lo que sugerimos que el nervio vago regula algunos de los mecanismos que participan en la comunicación entre los ovarios y en el desarrollo y persistencia del SOPQ inducido por la inyección de VE.

El GCMS, origen de la inervación simpática, es inervado por fibras vagales (Berthoud and Powley, 1996; Sisu y col. 2008). El principal linaje celular del GCMS son neuronas noradrenérgicas (Dail y Barton, 1983), las cuales están implicadas en la etiología del SOPQ (Lara y col. 1993; Gerendai y col. 2005). En ratas prepúberes con SOPQ, la disminución en la concentración de NA del GCMS, resultado de la vagotomía uni o bilateral explicaría el restablecimiento de la función ovulatoria.

En mujeres la principal característica de diagnóstico del SOPQ es el hiperandrogenismo, lo cual también se observa en ratas inyectadas con VE (Barria y col. 1993; Morales y col. 2010). En nuestro estudio la sección uni o bilateral del nervio vago en ratas adultas con SOPQ, disminuyó la concentración de testosterona, mientras que en ratas prepúberes la vagotomía bilateral la incrementó, sin embargo en ambos modelos se restableció la ovulación. Morales y col. (2004) mostraron que la vagotomía uni o bilateral en ratas prepúberes, sin ninguna patología, resulta en la disminución de la concentración de estradiol, mientras que la vagotomía unilateral no modificó la concentración de progesterona pero la bilateral resultó en una disminución. Efectos contrarios se observaron en el presente estudio. Tales diferencias pueden ser explicadas porque la adaptación del sistema de regulación ovárica ante la falta de una o varias señales neuroendocrinas dependen del tiempo transcurrido entre la cirugía y la autopsia.

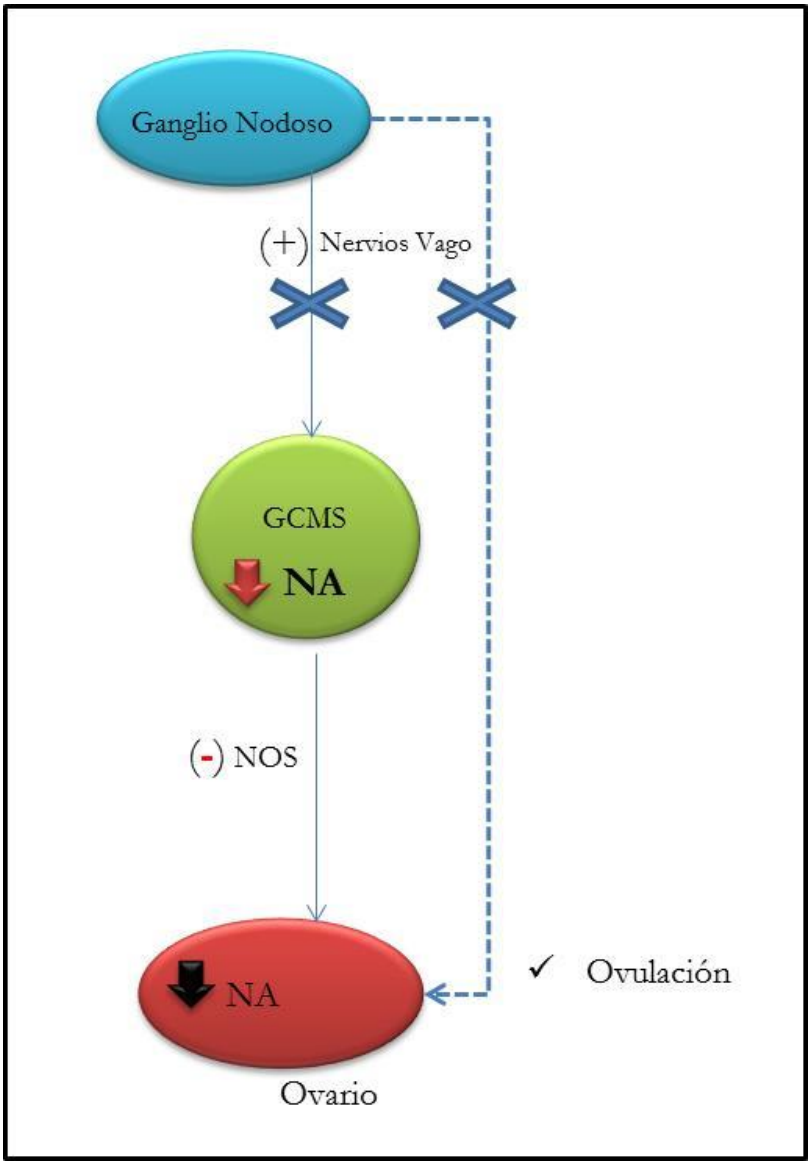
La acetilcolina es el principal neurotransmisor que viaja por el nervio vago (Klein y Burden, 1988). El estímulo colinérgico del ganglio celíaco resulta en el incremento en la concentración de NA ovárica (Daneri y col. 2013). La estimulación del nervio vago aumenta la tasa de disparo de las neuronas serotonérgicas del núcleo dorsal del rafe y la actividad de las neuronas de NA en el LC (Manta y col. 2009). En el presente estudio los cambios observados en el sistema monoaminérgico del GCMS y el ovario de ratas con SOPQ pueden ser explicados por las modificaciones en los circuitos de neurotransmisión que podría regular la inervación vagal.

Tomando en conjunto los resultados obtenidos en el presente estudio, sugiero que en la rata la inervación vagal del GCMS modula de manera inhibitoria la actividad monoaminérgica del ganglio, lo que se traduce en cambios en los mecanismos neuroendocrinos que regulan la ovulación y la secreción de esteroides ováricos y que varían en función del grado de desarrollo y del estado endocrino del animal. En cambio, en las ratas inyectadas con VE la inervación vagal del GCMS modularía de manera estimulante al sistema monoaminérgico. Dado que la eliminación parcial o total de la inervación vagal disminuye el tono noradrénergico del ganglio, el restablecimiento de la ovulación en estos animales apoya la idea de la participación de la inervación en el desarrollo y la persistencia de las alteraciones ováricas inducidas por la inyección de VE.

CONCLUSIONES GENERALES

- En ratas prepúberes y adultas inyectadas con VE, la sección uni o bilateral del nervio vago induce ovulación.
- En animales prepúberes inyectados con VE, la actividad monoaminérgica del GCMS disminuye por la sección del nervio vago.
- Los resultados nos permiten proponer que el nervio vago podría regular las funciones ováricas a través de cambios en la actividad monoaminérgica del GCMS.

Esquema que muestra el posible mecanismo por el cual el nervio vago regula la actividad noradrenérgica del ganglio en la rata inyectada con VE.



El esquema muestra que el nervio vago se origina del ganglio nodoso y que puede llegar al ovario por una via directa (línea azul punteada) o de manera indirecta (línea azul continua). En ratas inyectadas con VE el nervio vago podría regular de manera estimulante la concentración de NA en el GCMS, por lo que la vagotomía resulta en la disminución de la concentración de NA en el GCMS y asu vez disminuye el tono noradrenérgico del NOS y ello explicaría al menos en parte el restablecimiento de la ovulación. GCMS, ganglio celíaco mesentérico superior; NA, noradrenalina; NOS, nervio ovárico superior.

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