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**VNiVERSiDAD
D SALAMANCA**



TESIS DOCTORAL CON MENCIÓN INTERNACIONAL

**Influencia sexual en la modulación
cardiovascular ejercida por el sistema
serotonérgico periférico en rata**

**Influence of sex on cardiovascular
modulation by the peripheral serotonergic
system in the rat**

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Bajo la dirección de:

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Dr. José Ángel García Pedraza y Dra. Mónica García Domingo, Profesores Titulares del Departamento de Fisiología y Farmacología de la Universidad de Salamanca y miembros del Instituto de Investigación Biomédica de Salamanca (IBSAL),

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Que el trabajo “**Influencia sexual en la modulación cardiovascular ejercida por el sistema serotoninérgico periférico en rata**”, que presenta la graduada en Farmacia Dña. Anaïs Clara Terol Úbeda para optar al Grado de Doctor por la Universidad de Salamanca, ha sido realizado bajo su dirección en el Laboratorio de Farmacología (Departamento de Fisiología y Farmacología) de la Facultad de Farmacia de la Universidad de Salamanca y, considerándola finalizada, autorizan su presentación para que sea juzgada por el tribunal correspondiente.

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Esta Tesis Doctoral cumple, de acuerdo con la normativa vigente de la Universidad de Salamanca, los requisitos establecidos para su presentación en el formato de compendio de publicaciones. Se incluyen dos artículos de investigación publicados y un artículo aceptado para su publicación en revistas científicas indexadas en el *Journal Citation Reports* (JCR) en los que la doctoranda figura como primera autora. Asimismo, se incorporan dos anexos correspondientes a otras publicaciones en las que ha participado la doctoranda.

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A mis padres y a mi hermano,



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En cierto modo, una tesis se concluye cuando quien la realiza ya no es la misma persona que cuando la comenzó. La persona que formuló la pregunta inicial ha aprendido tanto durante el proceso que ahora podría plantearse otras preguntas distintas. Cerrar una tesis implica también aceptar algo esencial: el conocimiento siempre permanece abierto. La tesis no es la última palabra sobre un tema, si no la primera palabra propia dentro de una conversación mucho más larga.

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ABREVIATURAS

5

5-CT: 5-carboxamidotriptamina

5-HIAA: 5-hidroxiindolacético

5-HT: 5-hidroxitriptamina, serotonina

A

AC: adenilato ciclasa

ACh: acetilcolina

ADP: adenosina difosfato

AM: receptor de adrenomedulina

AMPc: adenosina monofosfato cíclico

Ang II: angiotensina II

AT₁: receptor de angiotensina II tipo 1

ATP: adenosina trifosfato

B

BHE: barrera hematoencefálica

C

CGRP: péptido relacionado con el gen de la calcitonina

CLR: receptor *calcitonina-like*

COX: ciclooxigenasa

D

DAG: diacilglicerol

DRG: ganglios de la raíz dorsal

E

ECV: enfermedades cardiovasculares

EDH: hiperpolarización dependiente de endotelio

eNOS: óxido nítrico sintasa endotelial

ER α : receptor de estrógeno α

F

FDA: *Food and Drug Administration*

G

GDP: guanosina difosfato

GIRK: canales de potasio rectificadores de entrada acoplados a proteína G

GMPC: guanosina monofosfato cíclico

GPCR: receptor acoplado a proteína G

GTP: guanosina trifosfato

I

IP₃: inositol trifosfato

ISRS: inhibidores selectivos de la recaptación de 5-HT

IUPHAR: Unión Internacional de Farmacología

K

K_{ATP}: canales de K⁺ dependientes de ATP

M

MAO: monoaminooxidasa

MAPK: proteínas cinasas activadas por mitógenos

MLCK: cinasa de la cadena ligera de miosina

N

NA: noradrenalina

NAD⁺: nicotinamida adenina dinucleótido

NANC: no adrenérgico no colinérgico

NIH: *National Institutes of Health*

NO: óxido nítrico

NPY: neuropéptido Y

O

OMS: Organización Mundial de la Salud

P

PIP₂: fosfatidilinositol bifosfato

PKA: proteína cinasa A

PKG: proteína cinasa G

PLC: fosfolipasa C

R

RAMP: proteína modificadora de la actividad de receptores

RCP: proteína componente del receptor

S

SERT: transportador de serotonina

SHR: ratas espontáneamente hipertensas

SNA: sistema nervioso autónomo

SNC: sistema nervioso central

SNE: sistema nervioso entérico

SNP: sistema nervioso periférico

SNPS: sistema nervioso parasimpático

SNS: sistema nervioso simpático

SP: sustancia P

SRAA: sistema renina-angiotensina-
aldosterona

T

TG: ganglios del trigémino

TPH: triptófano-5-hidroxilasa

TRPV1: receptores de potencial transitorio
vaniloide

V

VIP: polipéptido vasoactivo intestinal

VMAT: transportador vesicular de
monoaminas

RESUMEN

La serotonina (5-HT) es una neurohormona que regula el tono vascular por mecanismos directos e indirectos, a través del sistema nervioso autónomo y sensorial, mediante una amplia familia de receptores serotoninérgicos. Los mecanismos serotoninérgicos implicados en la modulación de la neurotransmisión simpática y sensorial CGRPérgica en la vasculatura sistémica han sido caracterizados por nuestro grupo y otros autores en ratas macho. Sin embargo, el sexo biológico influye tanto en el metabolismo de la 5-HT y la expresión de sus receptores, como en la neurotransmisión simpática y sensorial CGRPérgica, lo que limita la comprensión y el desarrollo de dianas farmacológicas eficaces para el sexo femenino en patologías cardiovasculares y neurovasculares.

La presente Tesis Doctoral tiene como objetivo determinar si los mecanismos serotoninérgicos involucrados en la regulación cardiovascular presentan diferencias dependientes del sexo biológico en ratas. Para ello, en ratas hembra *pithed*, se ha estudiado el papel diferencial de los distintos receptores de 5-HT sobre las respuestas vasculares obtenidas por liberación endógena de noradrenalina y del péptido relacionado con el gen de la calcitonina (CGRP), así como la posible implicación de mediadores dependientes de endotelio en la respuesta noradrenérgica.

Los resultados obtenidos muestran que el sexo biológico influye en el tono vascular basal y en los mecanismos serotoninérgicos que regulan tanto la neurotransmisión simpática vascular como la inervación sensorial CGRPérgica perivascular, modificando el tipo y subtipo de receptores de 5-HT implicados, su localización en la unión neuroefectora, y las vías indirectas involucradas. Estos hallazgos podrían abrir nuevas estrategias terapéuticas dependientes del sexo en el tratamiento de enfermedades cardiovasculares y neurovasculares.

ABSTRACT

Serotonin (5-HT) is a neurohormone that regulates vascular tone by direct and indirect mechanisms *via* the autonomic and sensory nervous systems, acting through a large family of serotonergic receptors. The serotonergic mechanisms involved in the modulation of sympathetic and sensory CGRPergic neurotransmission in the systemic vasculature have been characterised by our group and others in male rats. However, biological sex influences 5-HT metabolism and receptor expression, as well as sympathetic and sensory CGRPergic neurotransmission, which limits the understanding and development of effective pharmacological targets for the female sex in cardiovascular and neurovascular disorders.

The aim of the present Doctoral Thesis was to determine whether the serotonergic mechanisms involved in cardiovascular regulation display sex-dependent differences in rats. To do so, female pithed rats were used to study the role of different 5-HT receptors on vascular responses elicited by the endogenous release of noradrenaline and calcitonin gene-related peptide (CGRP), along with the possible involvement of endothelium-dependent mediators in the noradrenergic response.

The results show that biological sex influences basal vascular tone and the serotonergic mechanisms regulating both vascular sympathetic neurotransmission and perivascular sensory CGRPergic outflow, modifying the 5-HT receptor types and subtypes involved, their localisation at the neuroeffector junction, and the indirect pathways implicated. Altogether, these findings may contribute to the development of novel sex-dependent therapeutic strategies for cardiovascular and neurovascular diseases.

INTRODUCCIÓN

1. 5-Hidroxitriptamina

1.1. Descubrimiento y caracterización de la serotonina

La serotonina (5-hidroxitriptamina, 5-HT) es una indolamina biógena multifuncional altamente conservada a lo largo de la evolución, presente en una amplia variedad de reinos taxonómicos, incluyendo bacterias, hongos, protozoos, plantas y animales. En organismos primitivos, su función se ha relacionado principalmente con la regulación del balance energético (Gonçalves *et al.*, 2022; Yabut *et al.*, 2019; Zhao & Piatkevich, 2023). En los mamíferos, la 5-HT actúa como neurotransmisor y neurohormona, desempeñando un papel crucial en las funciones neurológicas, la homeostasis cardiovascular, la motilidad gastrointestinal, la actividad endocrina y la respuesta inmune, estando implicada tanto en el mantenimiento normal de estas funciones como en los procesos patológicos que puedan afectarlas (Nichols & Nichols, 2008; Soslau, 2022).

Los primeros indicios de la existencia de una sustancia vasoactiva presente en la sangre se remontan a finales del siglo XIX. Ludwig y Schmidt (1868) fueron los primeros en observar experimentalmente un marcado efecto vasoconstrictor tras perfundir sangre desfibrinada por vía intraarterial en perros. Posteriormente, Brodie (1900) demostró que la inyección de suero sanguíneo en gatos inducía vasoconstricción acompañada de un reflejo vagal y parada respiratoria, efectos que no se reproducían tras la administración de plasma. Inicialmente se sugirió que esta actividad vasoactiva podría atribuirse a la adrenalina, sin embargo, en 1912 se determinó que dicha sustancia era distinta de las catecolaminas y que su liberación al plasma se producía a partir de las plaquetas durante la coagulación (Zhao & Piatkevich, 2023).

A comienzos del siglo XX se avanzó en la identificación química de dicha sustancia vasoactiva. En 1933 se aisló, a partir de las células enterocromafines de la mucosa intestinal, una sustancia con potente actividad contráctil, a la que se denominó enteramina (Vialli &

Erspamer, 1933). Años más tarde, se sugirió que la estructura química de la enteramina contenía un anillo indólico (Erspamer, 1946). 5-hidroxitriptamina, sin embargo, no fue aislada en sangre hasta 1948, siendo caracterizada poco después por Rapport, Page y Green, quienes la denominaron serotonina, en referencia a su procedencia y acción vascular (sero = *serum*; tonin = vasoconstricción) (Rapport *et al.*, 1948). Esta monoamina fue sintetizada por primera vez en 1951, lo que permitió su estudio farmacológico y la comprobación de que todas las propiedades descritas del compuesto original se cumplían (Hamlin & Fischer, 1951). De manera paralela, Erspamer y Asero aislaron la forma pura de enteramina, identificándola como 5-HT (Erspamer & Asero, 1952).

En los años siguientes, la 5-HT fue identificada como neurotransmisor tras su detección en extractos de cerebro de mamíferos (Twarog & Page, 1953), siendo el tercer neurotransmisor descubierto, después de acetilcolina (ACh; 1933) y noradrenalina (NA; 1946) (Zhao & Piatkevich, 2023). El avance en el estudio fisiológico y farmacológico de la serotonina permitió que, a finales del siglo XX, se conociera que 5-HT provocaba respuestas complejas, e incluso contrapuestas, dentro del sistema cardiovascular (Saxena & Villalón, 1990), lo que despertó un interés creciente en la investigación biomédica acerca de la implicación de serotonina en la homeostasis y la fisiopatología de diversos sistemas del organismo.

1.2. Características químicas, metabolismo y localización de la serotonina

La 5-HT, con denominación química 3-(2-aminoetil)-1H-indol-5-ol y fórmula molecular $C_{10}H_{12}N_2O$ (Figura 1), presenta un peso molecular de 176,09 g/mol. La presencia del grupo hidroxilo en posición 5 del núcleo indólico y de la amina primaria protonada a pH fisiológico confiere a la 5-HT un carácter marcadamente hidrofílico. Como consecuencia, la serotonina no atraviesa la barrera hematoencefálica (BHE), de naturaleza lipofílica, lo que condiciona su síntesis independiente a nivel central y periférico (Hensler, 2012).

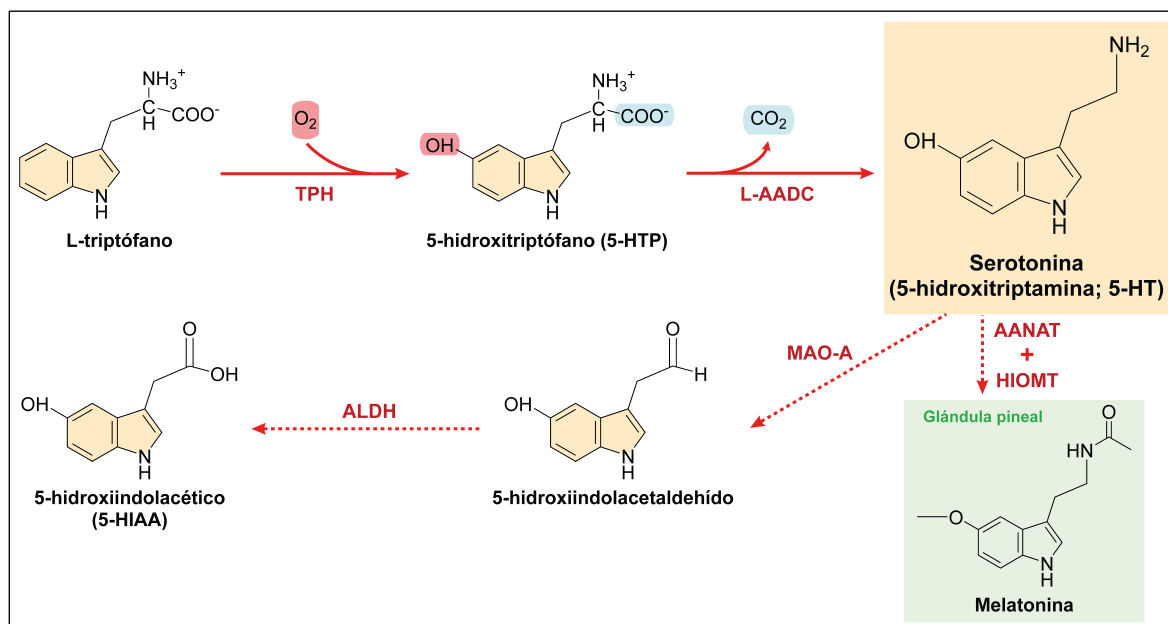


Figura 1. Biosíntesis y metabolismo de serotonina. AANAT, arilalquilamina N-acetiltransferasa; ALDH, aldehído deshidrogenasa; HIOMT, 5-hidroxiindol-O-metiltransferasa; L-AADC, L-aminoácido aromático descarboxilasa; MAO-A, monoaminooxidasa A; TPH, triptófano-5-hidroxilasa.

La biosíntesis de la 5-HT se inicia a partir del aminoácido esencial L-triptófano, que es hidroxilado en posición 5 del anillo indólico mediante la enzima triptófano-5-hidroxilasa (TPH) para dar lugar a 5-hidroxitriptófano. Seguidamente, la cadena lateral de este intermediario es descarboxilada por la L-aminoácido aromático descarboxilasa, originando 5-HT (Figura 1; Hensler, 2012). Una vez sintetizada, la 5-HT es transportada y almacenada en vesículas intracelulares mediante el transportador vesicular de monoaminas (VMAT), lo que la protege de la degradación intracelular por las monoaminooxidases (MAO) (Flórez *et al.*, 2025).

La disponibilidad de triptófano y la actividad de la TPH constituyen los principales factores limitantes de la síntesis de serotonina. Aproximadamente el 5 % del triptófano presente en la dieta se destina para la producción de 5-HT, mientras que la mayor parte se destina a la síntesis de quinurenina, una sustancia bioactiva que cumple funciones clave en la producción de nicotinamida adenina dinucleótido (NAD^+), la regulación del sistema inmune y la actividad neuronal, donde tiene un papel neuroprotector en condiciones fisiológicas (Höglund *et al.*, 2019; Pires *et al.*, 2022; Yabut *et al.*, 2019).

La TPH presenta dos isoformas (Figura 2): TPH1, predominante a nivel periférico, y TPH2, cuya expresión se restringe al sistema nervioso central (SNC) y al sistema nervioso entérico (SNE). Debido a la impermeabilidad de la BHE a la serotonina, se estima que aproximadamente el 95 % de la 5-HT del organismo se sintetiza a nivel periférico, mientras que el 5 % restante se produce de forma independiente en el SNC (Yabut *et al.*, 2019; Zhang *et al.*, 2025).

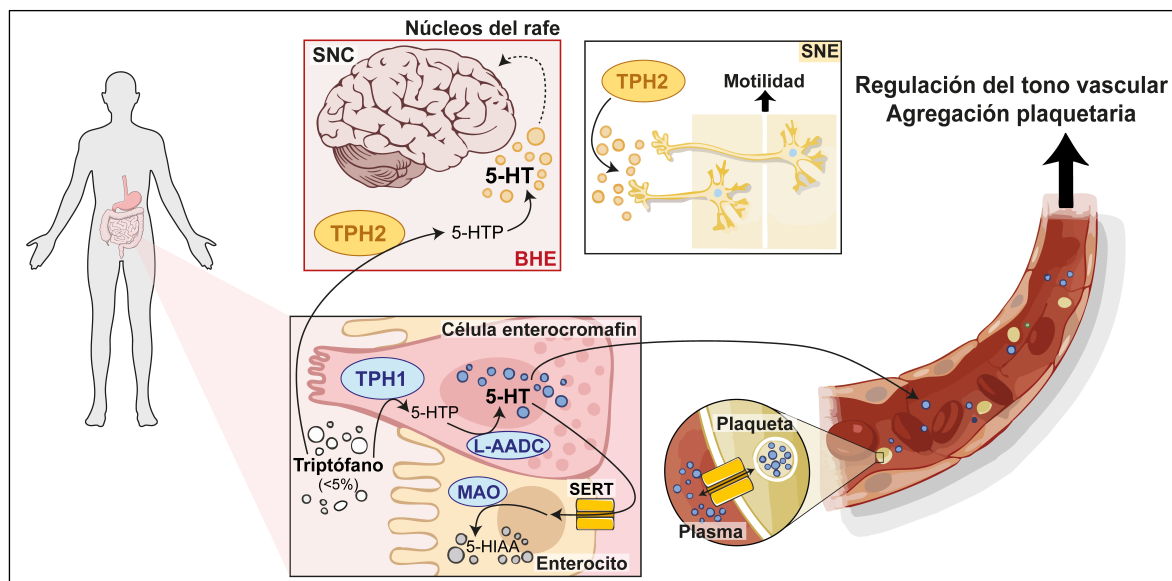


Figura 2. Organización anatómica de la síntesis y la distribución de la serotonina (adaptado de Yabut *et al.*, 2019). 5-HIAA, 5-hidroxiindolacético; 5-HTP, 5-hidroxitriptófano; L-AADC, L-aminoácido aromático descarboxilasa; BHE, barrera hematoencefálica; MAO, monoaminooxidasa; SERT, transportador de serotonina; SNC, sistema nervioso central; SNE, sistema nervioso entérico; TPH1, triptófano-5-hidroxilasa 1; TPH2, triptófano-5-hidroxilasa 2.

En los mamíferos, alrededor del 90 % de la serotonina corporal se sintetiza y se almacena en las células enterocromafines del tracto gastrointestinal. 5-HT es liberada a nivel intestinal en respuesta a estímulos nerviosos, químicos (gastrina) o mecánicos asociados a la ingesta, y puede interactuar con los receptores localizados en el tracto gastrointestinal o acceder a la circulación portal (Gonçalves *et al.*, 2022). En sangre, la mayor parte de serotonina se almacena en las plaquetas a través del transportador de serotonina (SERT), dado que estas carecen de capacidad biosintética propia. De este modo, las plaquetas desempeñan un papel clave en la regulación de la concentración sérica de 5-HT (Conde

et al., 2023). Durante la agregación plaquetaria, se liberan grandes cantidades de 5-HT, que actúan localmente sobre las células endoteliales y el músculo liso vascular.

Además del intestino y las plaquetas, la serotonina se localiza en mastocitos, células β pancreáticas, osteoclastos y adipocitos, así como en otros tejidos periféricos, incluyendo la pared de los vasos sanguíneos, pulmones y corazón (Flórez *et al.*, 2025; Gonçalves *et al.*, 2022).

En el SNC, la serotonina es sintetizada por las neuronas serotoninérgicas a partir de L-triptófano, que accede al cerebro mediante transporte facilitado, y se almacena en vesículas sinápticas. Los cuerpos celulares de estas neuronas se localizan en los núcleos del rafe, desde donde sus proyecciones axonales se distribuyen de forma extensa, innervando prácticamente todas las regiones del SNC (Hensler, 2012). Asimismo, en la glándula pineal, la serotonina es el principal precursor de la síntesis de melatonina, hormona clave en la regulación de los ciclos circadianos. Aunque la síntesis central de serotonina es independiente, diversos estudios han demostrado la presencia de SERT en células endoteliales de la BHE, lo que sugiere la posibilidad de un intercambio desde la periferia hacia el SNC, de modo que los niveles centrales de 5-HT no dependerían exclusivamente de su síntesis local (Nakatani *et al.*, 2008; Roux & Couraud, 2005).

Una vez liberada, la serotonina puede ser recaptada por SERT, un transportador de alta afinidad dependiente de sodio, cloro y energía (Rudnick & Sandtner, 2019), o ser inactivada rápidamente mediante desaminación oxidativa por las MAO, principalmente MAO-A. Este último proceso genera 5-hidroxiindolacetaldehído, que a su vez se oxida por la aldehído deshidrogenasa, dando lugar al ácido 5-hidroxiindolacético (5-HIAA) (Figura 1; Hensler, 2012).

1.3. Receptores serotoninérgicos

El papel multifuncional de serotonina está mediado por su unión a diversos receptores específicos de membrana. Tanto la serotonina como sus receptores están presentes en el

SNC y en el sistema nervioso periférico (SNP), así como en multitud de tejidos no neuronales del intestino, sistema cardiovascular y células sanguíneas (Hoyer *et al.*, 2002).

Los receptores serotoninérgicos fueron caracterizados por primera vez en 1957, cuando los efectos de 5-HT en íleon de cobaya fueron bloqueados por morfina (M) y Dibenzylamine® (fenoxibenzamina; D), diferenciando dos receptores con mecanismos diferentes: receptores 5-HT-M, localizados en las terminales nerviosas parasimpáticas, que mediaban la liberación de ACh, y receptores 5-HT-D, localizados en el músculo liso (Gaddum & Picarelli, 1957). La aplicación de técnicas bioquímicas y funcionales, así como los estudios de clonación molecular, identificaron y caracterizaron nuevos receptores de 5-HT, que en ocasiones han sido nombrados de diferente manera por distintos grupos de investigación. Con el fin de unificar y evitar ambigüedades, los receptores de 5-HT fueron clasificados por el Comité de Nomenclatura de la Unión Internacional de Farmacología (IUPHAR) basándose en criterios (i) operacionales, (ii) transduccionales y (iii) estructurales (Barnes *et al.*, 2021; Hoyer *et al.*, 1994, 2002):

- i. Las características operacionales recogen el perfil farmacológico (empleo de agonistas y antagonistas selectivos para determinar eficacia y afinidad al receptor), la funcionalidad (observación de las respuestas inducidas al activar el receptor) y los datos de unión a radioligandos (para cuantificar la densidad y ubicación de los receptores en tejidos).
- ii. El mecanismo de acción y características transduccionales (segundos mensajeros sintetizados tras producirse la interacción ligando-receptor).
- iii. La estructura molecular (secuencia de nucleótidos que codifica el gen del receptor y su secuencia de aminoácidos).

De este modo, se han identificado hasta siete familias principales de receptores de serotonina (Tabla 1), denominados 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆ y 5-HT₇

(Andrade *et al.*, 2025; Barnes *et al.*, 2021; Hoyer *et al.*, 1994, 2002). Algunos de estos receptores tienen múltiples subtipos, los cuales comparten características estructurales y transduccionales, pero con un perfil operacional (farmacológico) muy diferente; algunos de estos subtipos (5-HT_{1e} y 5-HT_{5b}) no tienen reconocida hasta el momento ninguna acción fisiológica. De acuerdo con las normas de la IUPHAR, un receptor se nombrará con letras mayúsculas cuando sus características operacionales y estructurales se hayan definido y se haya establecido como receptor endógeno, en caso contrario, el receptor se nombrará con letras minúsculas (Hoyer *et al.*, 1994).

Tabla 1. Tipo de receptor serotoninérgico y mecanismo efector.

Receptor serotoninérgico		Tipo de receptor y mecanismo transduccional
5-HT ₁	5-HT _{1A}	Proteína G₁₀
	5-HT _{1B}	
	5-HT _{1D}	
	5-HT _{1e}	
	5-HT _{1F}	
5-HT ₅	5-HT _{5A}	No determinado
	5-HT _{5b}	
5-HT ₂	5-HT _{2A}	Proteína G_{q/11}
	5-HT _{2B}	
	5-HT _{2C}	
5-HT ₃		Canal iónico
5-HT ₄		Proteína G_s
5-HT ₆		
5-HT ₇		

AC, adenilato ciclasa; AMPc, adenosina monofosfato cíclico; [Ca²⁺], concentración de calcio intracelular; DAG, diacilglicerol; IP₃, inositol trifosfato; PLC, fosfolipasa C; →, activación; →, inhibición.

Con la excepción de los receptores 5-HT₃, que forman parte de la superfamilia de canales iónicos activados por ligando (Tabla 1), todos los demás receptores de serotonina pertenecen a la familia A de receptores acoplados a proteínas G (*G-protein coupled receptor*, GPCR). Estructuralmente, los GPCR están constituidos por siete hélices α hidrofóbicas transmembrana, un dominio amino terminal extracelular y una región carboxilo terminal responsable de interactuar con multitud de proteínas reguladoras intracelulares. La familia A de GPCR (receptores similares a la rodopsina) posee los sitios de unión para los distintos ligandos agonistas y antagonistas en el dominio transmembrana de la proteína (Figura 3; Flórez *et al.*, 2025; Liu *et al.*, 2024; Nichols & Nichols, 2008).

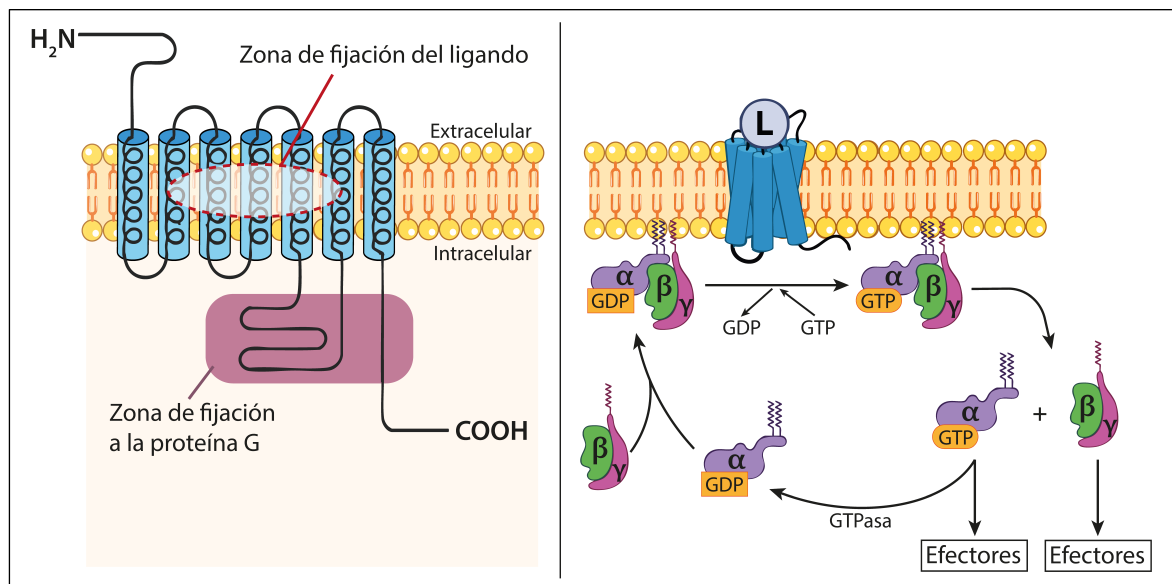


Figura 3. Estructura de los receptores acoplados a proteínas G (GPCR) y ciclo de las proteínas G. GDP, guanosina difosfato; GTP, guanosina trifosfato; L, ligando.

La señalización de los GPCR se produce por su habilidad de interaccionar con una proteína G heterotrimérica, formada por las subunidades $G\alpha$, $G\beta$ y $G\gamma$. Inicialmente, en un estado inactivo, la subunidad $G\alpha$ está unida a guanosina difosfato (GDP) y asociada al complejo $G\beta\gamma$. Al activarse el GPCR por la unión de un ligando, GDP es reemplazado por guanosina trifosfato (GTP) en la subunidad $G\alpha$, lo que provoca su disociación de $G\beta\gamma$. Estas subunidades activadas interactúan con distintos efectores (Figura 3).

Los receptores serotoninérgicos acoplados a proteínas G muestran un acoplamiento preferente a distintas familias de subunidades $G\alpha$, lo que determina la activación de vías de señalización específicas (Tabla 1). Así, la activación de las subunidades $G\alpha_s$ o $G\alpha_i$ conlleva la estimulación o inhibición, respectivamente, de la actividad enzimática de la adenilato ciclasa (AC); las subunidades $G\alpha_o$ regulan canales iónicos; mientras que las subunidades $G\alpha_{q/11}$ se relacionan con la estimulación de la fosfolipasa C (PLC). Por su parte, las subunidades $G\alpha_{12/13}$ activan la proteína Rho, implicada en la regulación del citoesqueleto celular (Glukhova *et al.*, 2018; Imamdin *et al.*, 2023). Además, el complejo $G\beta\gamma$ puede modular múltiples mecanismos efectores, incluyendo la PLC, los canales de potasio y de calcio dependientes de voltaje y la vía de las proteínas cinasas activadas por mitógenos (MAPK) (Hilger *et al.*, 2018).

A pesar de la complejidad derivada de la diversidad de los receptores de 5-HT, la existencia de agonistas, antagonistas y radioligandos específicos de cada tipo/subtipo de receptor ha permitido caracterizar muchas de las funciones fisiológicas de 5-HT (Sharp & Barnes, 2020).

1.3.1. Receptores 5-HT₁

La familia de receptores 5-HT₁ está compuesta por cinco subtipos diferentes, que comparten entre el 40 y el 63 % de la secuencia genética y están acoplados a proteínas $G\alpha_{i/o}$, que inhiben la formación de adenosina monofosfato cíclico (AMPC). Además, a través de $G\alpha_o$ inhiben la apertura de los canales de calcio voltaje dependientes, y la activación del complejo $G\beta\gamma$ provoca la apertura de los canales de potasio rectificadores de entrada acoplados a proteína G (GIRK), lo que conduce a la hiperpolarización de la membrana neuronal (Barnes *et al.*, 2021; Hoyer *et al.*, 2002). Los subtipos 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} y 5-HT_{1F}, a diferencia de los 5-HT_{1e}, han sido confirmados en una gran variedad de tejidos dentro del SNC, tracto gastrointestinal y sistema cardiovascular (Tabla 2; Barnes *et al.*, 2021; Guzel & Mirowska-Guzel, 2022).

Tabla 2. Tipos y subtipos de receptores de serotonina: clasificación, localización, efectos fisiológicos, agonistas y antagonistas principales de los receptores serotoninérgicos.

Receptor de 5-HT	Localización	Funciones principales	Agonistas	Antagonistas
5-HT₁				
5-HT _{1A}	SNC: rafe, corteza, hipocampo VSM	Inhibición neuronal Ansiólisis Hipotensión central	8-OH-DPAT Buspirona Indorrenato	WAY-100635
5-HT _{1B}	SNC, TG Terminales simpáticas VSMC	(-) trigeminal y liberación de NA VC	CGS-12066B CP-93,129 Sumatriptán	GR 55562
5-HT _{1D}	SNC Terminales simpáticas VSMC	Heterorreceptor en aurícula (-) liberación de NT	L-694,247 PNU 109291 Sumatriptán	Ergotamina LY310762 GR 127935
5-HT _{1E}	SNC	-	BRL 54443	Metiotepina
5-HT _{1F}	SNC, TG Corteza renal	(-) trigeminal	LY344864 BRL 54443 Lasmiditán	Metisergida GR 127935
5-HT₂				
5-HT _{2A}	SNC, SNP: VSM, uterino y bronquial VSMC, plaquetas Tracto GI	Excitación neuronal VC, agregación plaquetaria, taquicardia	TCB-2	Ketanserina Ritanserina Sarpogrelato
5-HT _{2B}	VSM de íleon, fundus y endotelio	Contracción del fundus VD NO-dependiente	BW723C86	
5-HT _{2C}	SNC Plexos coroideos	Regulación de LCR	MK212	
5-HT ₃	SNC SNP: nervio vago, neuronas entéricas	Neuroexcitación parasimpática Emesis, náuseas	1-PBG 2-metil-5-HT	MDL 72222 Ondansetrón
5-HT ₄	SNC Tracto GI, corazón y vejiga	Relajación VSM y GI	Cisaprida BIMU8	GR-125487
5-HT₅				
5-HT _{5A}	SNC Ganglio simpático cardíaco	Control motor Ansiedad (-) cardíaca	5-CT Ergotamina	SB 699551
5-HT _{5B}	-	-		
5-HT ₆	SNC Ganglios cervicales superiores	Aumento de ACh central Memoria	WAY-181,187 WAY-208466	Ro 04-6790 SB 399885
5-HT ₇	SNC Tracto GI VSM y ganglios simpáticos	Regulación de los ritmos circadianos Relajación VSM y GI	5-CT AS-19 LP-12	SB 258719

(-), inhibición; 5-CT, 5-carboxamidotriptamina; ACh, acetilcolina; GI, gastrointestinal; LCR, líquido cefalorraquídeo; NA, noradrenalina; NO, óxido nítrico; NT, neurotransmisores; SNC, sistema nervioso central; SNP, sistema nervioso periférico; TG, ganglios del trigémino; VC, vasoconstricción; VD, vasodilatación; VSM, músculo liso vascular; VSMC, células del músculo liso vascular.

1.3.2. Receptores 5-HT₂

Los receptores 5-HT₂ comprenden tres subtipos distintos, 5-HT_{2A}, 5-HT_{2B} y 5-HT_{2C}, que comparten el 46-50 % de su secuencia genética, y se acoplan preferentemente a proteínas G $\alpha_{q/11}$, que estimulan la actividad de la PLC, promoviendo la hidrólisis del fosfatidilinositol bifosfato (PIP₂) en inositol trifosfato (IP₃) y diacilglicerol (DAG), que indirectamente incrementan la concentración de Ca²⁺ intracelular. A nivel funcional, la activación de receptores 5-HT₂ se relaciona con la excitación neuronal por el bloqueo de canales de K⁺, y otras vías de señalización no canónicas (fosfolipasa A₂, MAPK) (Barnes *et al.*, 2021; Hoyer *et al.*, 2002). Los receptores 5-HT_{2A} (receptores 5-HT-D previamente) están ampliamente distribuidos en el SNC y en tejidos periféricos, donde median respuestas contráctiles en la musculatura lisa vascular y no vascular, y participan en la agregación plaquetaria (Barnes *et al.*, 2021; Sharp & Barnes, 2020). La expresión de los receptores 5-HT_{2B} es principalmente periférica, encontrándose a lo largo del tracto gastrointestinal y sistema cardiovascular (Barnes *et al.*, 2021). El receptor 5-HT_{2C} se localiza mayoritariamente en los plexos coroideos, donde regula el intercambio de iones entre el cerebro y el líquido cefalorraquídeo, y se ha sugerido su presencia en tejidos periféricos (Tabla 2; Barnes *et al.*, 2021; Morán *et al.*, 2008; Nichols & Nichols, 2008).

1.3.3. Receptores 5-HT₃

El receptor 5-HT₃ (inicialmente descubierto como receptor 5-HT-M) es el único miembro de la familia de receptores serotoninérgicos que forma parte de la superfamilia de receptores ionotrópicos (Tabla 1). Se trata de un canal iónico formado por cinco subunidades, idénticas o no (desde A hasta E), dispuestas alrededor de una cavidad central y unidas por lazos de cisteína. Únicamente la subunidad 5-HT_{3A} forma receptores 5-HT₃ homoméricos funcionales, y es necesaria la presencia de al menos una subunidad 5-HT_{3A} para las formas heteroméricas (Barnes *et al.*, 2021; Guzel & Mirowska-Guzel, 2022; Hoyer *et al.*, 2002). Estos receptores se expresan en tejidos neuronales centrales y periféricos, donde median una despolarización rápida a través de la entrada de Na⁺ y Ca²⁺ y la salida de

K⁺, además de ser relevantes en otros sistemas tales como el gastrointestinal, donde antagonistas selectivos de receptores 5-HT₃ han revolucionado la terapéutica antiemética, especialmente en pacientes sometidos a quimioterapia (Tabla 2; Barnes *et al.*, 2021; Guzel & Mirowska-Guzel, 2022).

1.3.4. Receptores 5-HT₄, 5-HT₅, 5-HT₆ y 5-HT₇

Los receptores 5-HT₄, 5-HT₆ y 5-HT₇ se unen preferentemente a proteínas G_s y promueven la formación de AMPc, sin embargo, se organizan como diferentes clases de receptores al compartir menos del 35 % de su secuencia genética. En la actualidad existen tanto agonistas como antagonistas selectivos para estos receptores (Tabla 2; Barnes *et al.*, 2021; Hoyer *et al.*, 2002). Los receptores 5-HT₄ están distribuidos en el SNC y en la periferia, incluyendo el corazón, el tracto gastrointestinal, las glándulas adrenales y salivares, la vejiga urinaria y los pulmones. Los receptores 5-HT₆ se expresan exclusivamente en el SNC, donde desempeñan una función en la cognición, aprendizaje, control del apetito y trastornos convulsivos. Los receptores 5-HT₇ muestran una distribución vascular extensa, además de expresarse en células musculares no vasculares y en el SNC (Barnes *et al.*, 2021; Hoyer *et al.*, 2002; Sharp & Barnes, 2020).

La familia de receptores 5-HT₅ están acoplados a proteínas G $\alpha_{i/o}$. Se han descrito dos subtipos de receptores 5-HT₅, sin embargo, solo el receptor 5-HT_{5A} se ha identificado en humanos. El receptor 5-HT_{5b}, que es funcional en roedores, consta de codones de parada prematuros que impiden la formación de un receptor funcional en humanos. Basándose en ratones *knockout* para el receptor 5-HT_{5A}, estudios de expresión y el uso de antagonistas selectivos, su actividad se ha relacionado con la modulación del dolor, un incremento de la actividad locomotora y comportamiento exploratorio, así como con acciones inhibitoras de la neurotransmisión simpática cardíaca en ratas (Barnes *et al.*, 2021; García-Pedraza *et al.*, 2020; González-Hernández *et al.*, 2023; Nichols & Nichols, 2008).

1.3.5. Variantes e isoformas de los receptores serotoninérgicos

Desde la propuesta de clasificación de los receptores de 5-HT realizada por Hoyer y colaboradores en 1994, que constituye la base del sistema actualmente adoptado por la IUPHAR (Barnes *et al.*, 2021), se han descrito posibles variantes adicionales y mecanismos que incrementan la complejidad del sistema serotoninérgico. En este sentido, algunas publicaciones han propuesto la existencia de un receptor 5-HT_{1P} localizado a nivel intestinal, pero aún no ha sido completamente establecido (Guzel & Mirowska-Guzel, 2022).

Asimismo, la diversidad de los receptores de 5-HT se amplía con la existencia de isoformas producidas por modificaciones postranscripcionales (*splicing* alternativo). Se han descrito hasta 7 isoformas funcionales del receptor 5-HT_{2C}, cuatro variantes funcionales del receptor 5-HT₄ y cuatro del receptor 5-HT₇. Aunque estas isoformas no presentan diferencias sustanciales en sus propiedades operacionales, su distribución difiere tanto a nivel central como periférico. La relevancia funcional de estas isoformas no se conoce con precisión, aunque se ha sugerido su implicación en procesos como la desensibilización de receptores a nivel celular o la activación de vías efectoras alternativas (Barnes *et al.*, 2021).

1.4. Acciones fisiológicas de la serotonina y relevancia farmacológica

La diversidad de receptores de 5-HT, su amplia distribución tisular y su acoplamiento a distintos sistemas efectoras explican la implicación de la serotonina en numerosos procesos fisiológicos y patológicos.

1.4.1. Acciones centrales de 5-HT

En el SNC, la serotonina actúa como neurotransmisor modulando, de forma directa o indirecta, un amplio espectro de funciones cerebrales (Barnes *et al.*, 2021; Sharp & Barnes, 2020).

A nivel del eje hipotálamo-hipófisis-adrenal, la 5-HT regula la secreción de diversas hormonas, incluyendo la hormona adrenocorticotropa, la hormona del crecimiento, la prolactina y la melanocortina, principalmente a través de los receptores 5-HT₁ y 5-HT₂. También participa en la termogénesis y el control de la temperatura corporal (5-HT₁ y 5-HT₇), así como en la regulación del ciclo sueño/vigilia, donde 5-HT promueve la vigilia y reduce las fases REM (*Rapid Eye Movement*). En este contexto, los antagonistas no selectivos de receptores 5-HT_{2A/2C} ritanserina y ketanserina incrementan el sueño profundo en individuos con patrones de sueño normales. El núcleo hipotalámico ventromedial, que regula la ingesta y la saciedad, está innervado por neuronas serotoninérgicas, donde la activación de receptores 5-HT_{2C}, y en menor medida de los 5-HT₆, disminuyen el apetito y el peso corporal (Burke & Heisler, 2015).

La 5-HT participa en los procesos de aprendizaje y memoria a través de diversos subtipos de receptores serotoninérgicos. En modelos experimentales, la administración de antagonistas de receptores 5-HT_{1A}, 5-HT_{1B} y 5-HT₃ o de los agonistas de receptores 5-HT_{2A}, 5-HT_{2C} y 5-HT₄, se ha asociado con una mejora del aprendizaje y una atenuación del deterioro cognitivo. Además, la interacción entre 5-HT y dopamina se relaciona con el circuito de recompensa, la motivación y el refuerzo conductual. La relevancia de la neurotransmisión serotoninérgica en los procesos cognitivos y emocionales hace que sus alteraciones se asocien al desarrollo de patologías neuropsiquiátricas, tales como ansiedad, depresión, trastornos obsesivo-compulsivos, déficit de atención/hiperactividad, migraña, adicción y agresividad (Pourhamzeh *et al.*, 2021). En humanos, estas alteraciones se han relacionado con concentraciones reducidas de 5-HIAA, y, en definitiva, de 5-HT (Jayamohananan *et al.*, 2019).

La modulación de la nocicepción por la 5-HT ocurre tanto a nivel espinal como supraespinal, a través de diversos subtipos de receptores, incluyendo 5-HT₁, 5-HT₂, 5-HT₃ y 5-HT₇. La relevancia de esta modulación se evidencia especialmente en la fisiopatología de la migraña, donde los fármacos agonistas de receptores 5-HT_{1B/1D} (triptanes) y 5-HT_{1F}

(ditanes) reducen la vasodilatación y la inflamación neurogénica intra y extracraneal asociadas al ataque migrañoso. Por otra parte, la inducción de la emesis mediada por receptores 5-HT₃ involucra mecanismos tanto centrales, principalmente en el área postrema y el núcleo del tracto solitario, como periféricos, a través de las terminaciones del nervio vago en el tracto gastrointestinal (Barnes *et al.*, 2021; Navari & Aapro, 2016; Pourhamzeh *et al.*, 2021).

Dado que SERT reduce la biodisponibilidad de 5-HT en el medio extracelular, este transportador se ha consolidado como una de las principales dianas farmacológicas en el tratamiento de trastornos neuropsiquiátricos, e incluso en la migraña y el dolor (Pourhamzeh *et al.*, 2021). En la actualidad, los inhibidores selectivos de la recaptación de 5-HT (ISRS) constituyen la primera línea terapéutica en el tratamiento de la depresión.

1.4.2. Acciones periféricas de 5-HT

A nivel periférico, la 5-HT está implicada en la actividad de múltiples órganos y sistemas, incluyendo el aparato respiratorio, el tracto gastrointestinal, el sistema cardiovascular y procesos relacionados con la hemostasia. Estas acciones se ejercen mediante la activación de receptores serotoninérgicos específicos, que modulan el tono del músculo liso, la agregación plaquetaria, la transmisión sensorial y la liberación de neurotransmisores.

Tracto gastrointestinal. Cinco de las siete familias de receptores de 5-HT (5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄ y 5-HT₇) se expresan ampliamente en este sistema, tanto en neuronas del SNE como en el músculo liso y células epiteliales del tracto gastrointestinal. Las funciones de la 5-HT en el tracto gastrointestinal son extensas e incluyen la regulación del peristaltismo, la secreción intestinal, la sensibilidad visceral y los mecanismos de la saciedad. En este contexto, los receptores 5-HT₄ median la estimulación de terminaciones nerviosas en el músculo intestinal y esofágico, mientras que los receptores 5-HT₃ participan en la estimulación ganglionar. Adicionalmente, la activación de receptores 5-HT_{2A} y 5-HT_{2B}, presentes en las fibras musculares del intestino y el *fundus* gástrico, respectivamente,

produce contracción directa de la pared gastrointestinal (Del Colle *et al.*, 2020; Flórez *et al.*, 2025). En los últimos años, se ha puesto de manifiesto la relevancia de la interacción entre la 5-HT y la microbiota intestinal, consolidando el concepto del eje 5-HT-intestino-cerebro. También se ha demostrado que la 5-HT liberada en el tracto gastrointestinal puede influir en la composición y función de la microbiota, la cual presenta mecanismos de recaptación de 5-HT. De forma recíproca, la microbiota intestinal puede regular la disponibilidad de triptófano. Esta relación bidireccional entre el sistema serotoninérgico y la microbiota intestinal se ha propuesto como un mecanismo potencial implicado en el origen de enfermedades tanto neuropsiquiátricas como gastrointestinales. En este sentido, se han descrito alteraciones en la microbiota intestinal en pacientes con trastornos del espectro autista o depresión en comparación con individuos control (Del Colle *et al.*, 2020; Margolis *et al.*, 2021).

Agregación plaquetaria. Las plaquetas desempeñan un papel crucial en la hemostasia y la formación de trombos. En respuesta a un daño endotelial, situaciones de isquemia o a la activación de receptores 5-HT_{2A} principalmente, las plaquetas liberan la 5-HT contenida en sus gránulos densos junto a otros mediadores proagregantes, como el factor Von Willebrand, adenosina difosfato (ADP), Ca²⁺, NA y P-selectina. La 5-HT liberada ejerce un efecto vasoconstrictor directo mediante la activación de receptores 5-HT_{2A} vasculares (Figura 4), lo que contribuye a reducir el flujo sanguíneo en la zona lesionada y a estabilizar el trombo (Marcinkowska *et al.*, 2022; Rieder *et al.*, 2021).

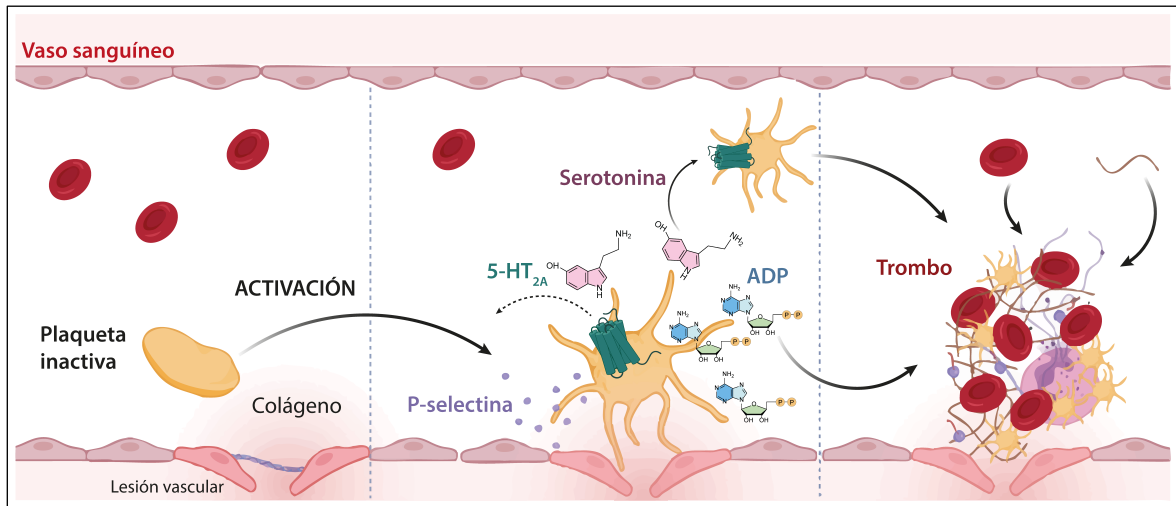


Figura 4. Mecanismo de formación del agregado plaquetario, incluyendo el papel de la serotonina y del receptor 5-HT_{2A} (adaptado de Marcinkowska *et al.*, 2022). ADP, adenosina difosfato.

Metabolismo óseo. Los osteoblastos, osteocitos y fibroblastos del periostio expresan receptores de 5-HT, específicamente los subtipos 5-HT_{2A/2B/2C} (Al Saedi *et al.*, 2020; Jonnakuty & Gragnoli, 2008). Estudios moleculares han demostrado que la 5-HT puede modular la actividad osteoblástica, potenciando la acción de la hormona paratiroidea sobre la fosfatasa alcalina, lo que favorece la diferenciación y maduración de los osteoblastos. Asimismo, se ha evidenciado la presencia de SERT en osteoblastos y osteocitos, lo que sugiere un control local de la disponibilidad de 5-HT en el tejido óseo (Jonnakuty & Gragnoli, 2008).

Metabolismo glucídico y lipídico. La serotonina producida en las células enterocromafines, los islotes pancreáticos y el tejido adiposo actúa a nivel local y sistémico modulando la homeostasis glucídica y lipídica a través de distintos receptores de 5-HT. En las células β pancreáticas, la 5-HT se secreta juntamente con la insulina, y se ha demostrado que una reducción de la síntesis local de 5-HT disminuye la secreción de esta hormona, afectando al control de la producción endógena de glucosa, a su captación y a su conversión a glucógeno y lípidos (Wyler *et al.*, 2017). Adicionalmente, se ha propuesto que la 5-HT mejora el metabolismo glucídico en el músculo esquelético mediante la “serotonilación” de la proteína Rab4, implicada en el tráfico vesicular intracelular de glucosa (Al-Zoairy *et al.*,

2017). En el tejido adiposo, la 5-HT periférica ejerce efectos dependientes del estado metabólico, estimulando la lipólisis durante el ayuno, promoviendo la lipogénesis en dietas ricas en grasa, e inhibiendo la termogénesis (Wyler *et al.*, 2017).

Acciones cardiovasculares. La 5-HT actúa como neurohormona en el sistema cardiovascular, donde desencadena respuestas complejas y, en ocasiones, opuestas, incluyendo bradicardia o taquicardia, hipotensión o hipertensión, y vasodilatación o vasoconstricción. Estas respuestas dependen de múltiples factores, entre los que se incluyen la especie animal, el tono vascular basal, el lecho vascular estudiado, la dosis del agonista serotoninérgico administrado, las condiciones experimentales y, de manera más determinante, la naturaleza de los receptores implicados, así como el nivel en el que se localizan. Estos niveles incluyen (i) el SNC, donde la 5-HT actúa como neurotransmisor en los núcleos del rafe; (ii) las vías simpáticas y parasimpáticas; (iii) los nervios sensoriales; y (iv) la acción directa sobre los vasos sanguíneos y el corazón (González-Hernández *et al.*, 2023; Villalón, 2019; Villalón & Centurión, 2007). Estos efectos están mediados fundamentalmente por las familias de receptores 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT_{5A} y 5-HT₇ (Figura 5), mientras que los receptores 5-HT₆ no participan, ni a nivel central ni periférico, en la regulación cardiovascular (González-Hernández *et al.*, 2023; Villalón, 2019).

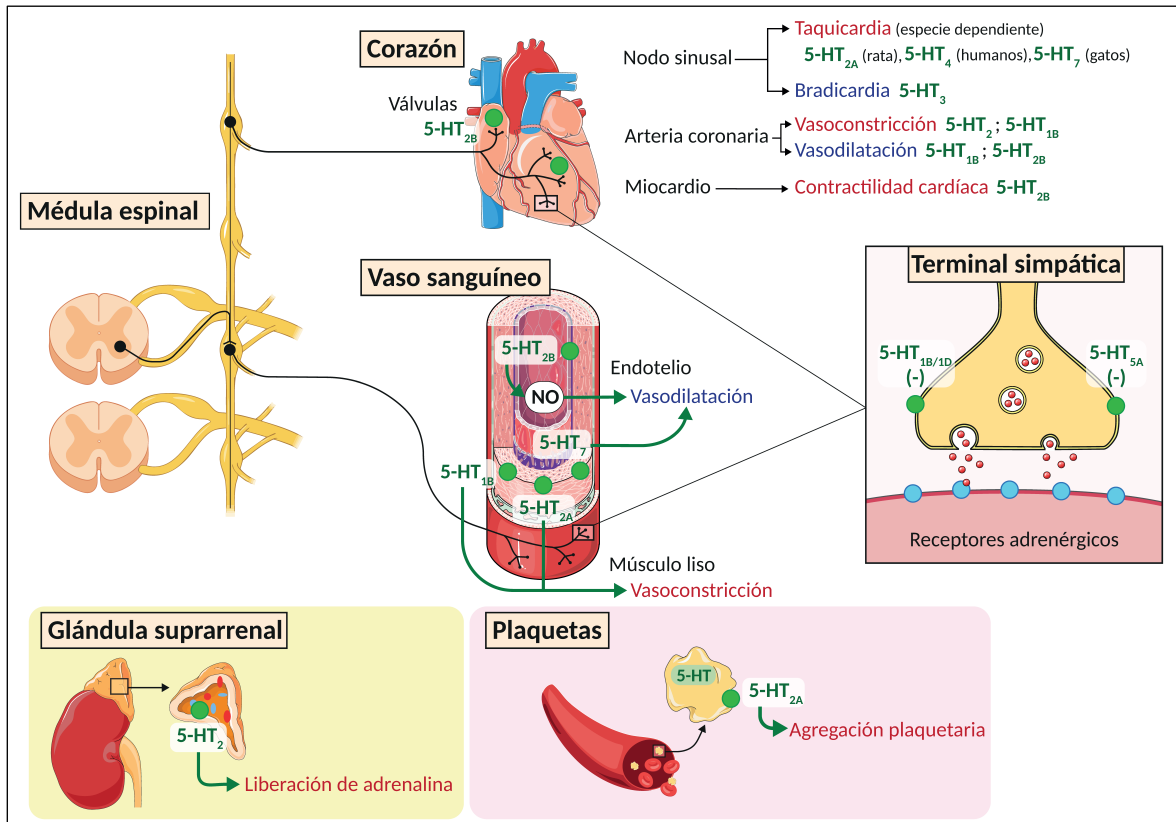


Figura 5. Efectos cardiovasculares de la 5-HT (adaptado de Ramage & Villalon, 2008). NO, óxido nítrico.

La función cardiovascular puede ser regulada por la 5-HT mediante mecanismos centrales que influyen sobre el equilibrio autonómico. La administración directa de 5-HT en el SNC activa de manera predominante dos subtipos de receptores serotoninérgicos: los receptores 5-HT_{1A}, asociados con la inhibición del tono simpático (bradicardia e hipotensión) y la estimulación de la actividad cardíaca vagal; y los receptores 5-HT_{2A}, que inducen excitación simpática (taquicardia e hipertensión) (González-Hernández *et al.*, 2023; Villalón, 2019). En consonancia con estos mecanismos, las patologías psiquiátricas asociadas a alteraciones en la neurotransmisión serotoninérgica suelen ir acompañadas de un desequilibrio autonómico, que se manifiesta clínicamente mediante alteraciones cardiovasculares, como arritmias, taquicardias o cambios en la presión arterial (González-Hernández *et al.*, 2023).

La administración intravenosa en bolo de 5-HT en animales anestesiados induce el reflejo de von Bezold-Jarisch, caracterizado por bradicardia e hipotensión. Esta respuesta

cardiovascular se desarrolla en tres fases. En una primera fase se observa una combinación de bradicardia y una respuesta vasodepresora transitoria, atribuida a la activación de receptores 5-HT₃ localizados en las fibras aferentes parasimpáticas del nervio vago, lo que conlleva la inhibición de las neuronas simpáticas a nivel del tronco encefálico. La segunda fase cursa con vasoconstricción, resultado de la activación directa de los receptores 5-HT_{2A} en los vasos sanguíneos, acompañada de una bradicardia refleja. Finalmente, la tercera fase se caracteriza por una vasodilatación sostenida en el tiempo, mediada principalmente por la activación de receptores 5-HT₇ localizados en las células musculares lisas vasculares (González-Hernández *et al.*, 2023; Ramage & Villalon, 2008).

La 5-HT produce efectos cronotrópicos e inotrópicos positivos en humanos mediante la activación de los receptores 5-HT₄ expresados en los miocitos cardíacos (Jonakuty & Gragnoli, 2008). No obstante, dependiendo de la especie animal y de las condiciones experimentales, la 5-HT puede inducir taquicardia mediante la activación directa de otros subtipos de receptores, incluyendo los receptores 5-HT_{2A} en ratas, 5-HT₄ en cerdos y 5-HT₇ en gatos (Figura 5; González-Hernández *et al.*, 2023). Adicionalmente, estudios en modelos animales modificados genéticamente han puesto de manifiesto la relevancia de la 5-HT en el desarrollo y la función cardíaca. Así, los ratones *knockout* para la enzima TPH1, que presentan concentraciones circulantes de serotonina reducidas al 4-8 % de los valores normales, desarrollan miocardiopatía sin alteraciones morfológicas evidentes. En contraste, los ratones *knockout* para el receptor 5-HT_{2B} presentan defectos morfogénéticos cardíacos graves que conducen a la muerte embrionaria o neonatal (Jonakuty & Gragnoli, 2008).

A nivel vascular, la 5-HT se ha caracterizado clásicamente por su capacidad para inducir respuestas vasoconstrictoras, mediadas por la activación directa de receptores 5-HT₁ o 5-HT₂ en el músculo liso vascular, o por potenciar la acción de agentes vasoconstrictores como la angiotensina II (Ang II) o la prostaglandina F_{2α} (Figura 6; Flórez *et al.*, 2025). En arterias coronarias y carótidas humanas, la acción vasoconstrictora de 5-HT se debe a la

activación de receptores 5-HT_{1B} y 5-HT_{2A}, mientras que en las arterias craneales el receptor 5-HT_{1B} constituye el principal mediador de esta respuesta (González-Hernández *et al.*, 2023; Kaumann & Levy, 2006).

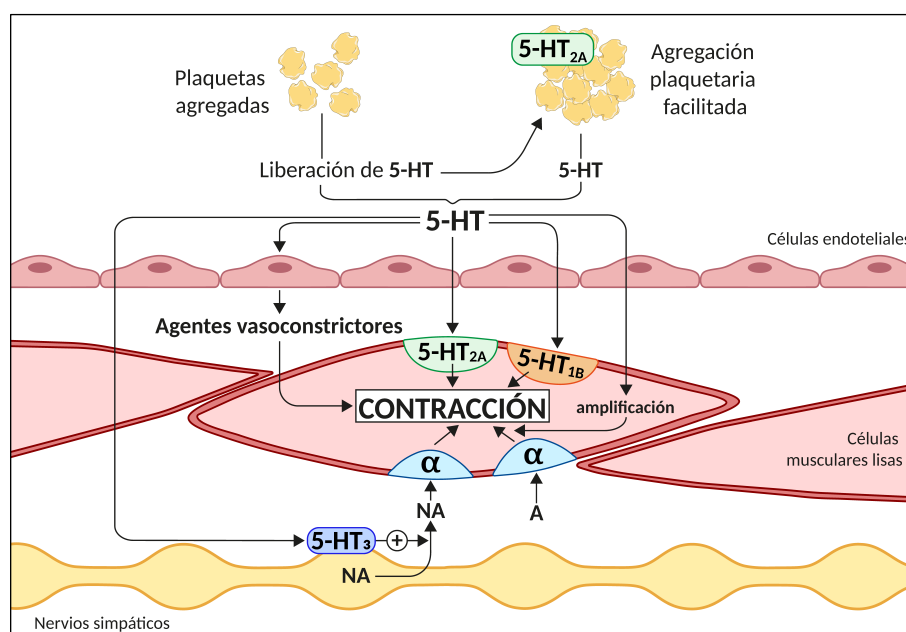


Figura 6. Efectos vasoconstrictores directos e indirectos de 5-HT (adaptado de Vanhoutte, 1987). α, receptor α₁ adrenérgico; A, adrenalina; NA, noradrenalina.

La acción vasopresora asociada a los receptores 5-HT_{2A} no solo deriva de su activación directa en el músculo liso vascular, sino también de la liberación de adrenalina desde la médula adrenal y la consiguiente activación de receptores α₁ adrenérgicos localizados en el músculo liso vascular (Figura 6; Villalón, 2019). Numerosos estudios han puesto de manifiesto que la 5-HT actúa como un potente agente vasoactivo liberado durante la agregación plaquetaria en respuesta a diferentes estímulos, como inflamación e infecciones, y que juega un papel importante en la fisiopatología de diversas enfermedades cardiovasculares (ECV), entre ellas la hipertensión pulmonar y la enfermedad vascular periférica (Matsumoto *et al.*, 2025; Nagatomo *et al.*, 2004).

En lo referente a las acciones vasodilatadoras mediadas por 5-HT (Figura 7), estas son especialmente evidentes cuando el tono simpático está incrementado, y participan en los cambios vasculares que se producen en las arterias craneales durante la migraña (Kaumann

& Levy, 2006). Los receptores 5-HT₇ presentes en la musculatura lisa vascular son los principales involucrados en la vasodilatación directa inducida por serotonina. Asimismo, los receptores 5-HT_{1A}, 5-HT_{1B} y 5-HT_{2B} pueden facilitar la liberación de óxido nítrico (NO) como mediador vasodilatador (Feng *et al.*, 2025; Flórez *et al.*, 2025; Ramage & Villalon, 2008). Muchas de las acciones vasodilatadoras de 5-HT se han relacionado con la activación de la enzima óxido nítrico sintasa endotelial (eNOS), aunque también pueden depender de la producción de prostaciclina, a través de la ciclooxigenasa (COX) tipo 2, o de la hiperpolarización dependiente de endotelio (EDH), ocasionada tras la apertura de canales de K⁺ dependientes de ATP (K_{ATP}) (García-Pedraza *et al.*, 2016; Villalón, 2019; Wei *et al.*, 2018).

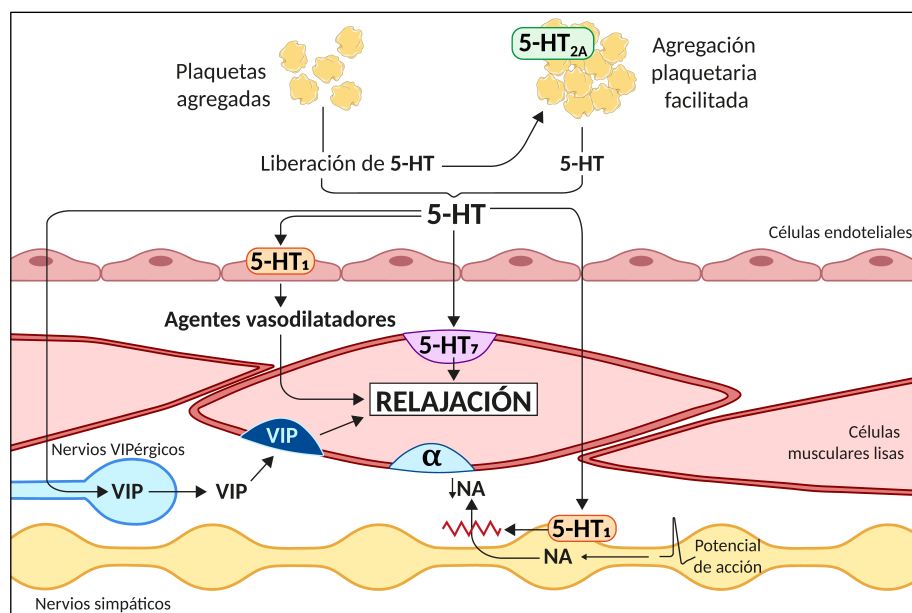


Figura 7. Efectos vasodilatadores directos e indirectos de 5-HT (adaptado de Vanhoutte, 1987). α, receptor α₁ adrenérgico; NA, noradrenalina; VIP, péptido intestinal vasoactivo.

Nuestro grupo y otros equipos de investigación han puesto de manifiesto en distintos territorios vasculares que la activación de receptores serotoninérgicos específicos modifica de forma diferencial el flujo sanguíneo regional:

a) Vasculatura del tren posterior. En ratas normotensas, la respuesta vasoconstrictora inducida por 5-HT está mediada por la activación de receptores 5-HT_{2A} (Calama *et al.*,

2002), mientras que en ratas espontáneamente hipertensas (SHR) esta vasoconstricción no sólo implica la participación adicional de receptores 5-HT_{2C}, sino que además presenta una magnitud significativamente mayor (Calama *et al.*, 2004). Por el contrario, las respuestas vasodilatadoras en este territorio se deben a la activación de receptores 5-HT_{1B/1D}, que promueven la liberación de adrenalina desde las cápsulas suprarrenales y la posterior activación de receptores β_2 adrenérgicos (Calama *et al.*, 2002, 2003).

b) Territorio mesentérico. En el mesenterio autoperfundido de rata, la acción vasoconstrictora de 5-HT se produce principalmente por la activación de receptores 5-HT_{2B} y/o 5-HT_{2C} (Fernández *et al.*, 2000). En estudios *in vitro* con arterias mesentéricas de ratas desprovistas de endotelio, la 5-HT induce vasoconstricción a través de los receptores 5-HT_{2A}, implicando la inhibición de canales de K⁺ dependientes de voltaje (Sung *et al.*, 2013).

c) Territorio renal. Los efectos vasculares de la 5-HT en el riñón varían en función de la especie animal y/o la patología subyacente. En estudios de riñón autoperfundido *in situ* de rata, la 5-HT induce vasoconstricción por activación de receptores 5-HT₂, principalmente 5-HT_{2C}, con participación de la Ang II (Morán *et al.*, 1997, 2008). En ratas hipertensas, esta respuesta vasoconstrictora es significativamente mayor y está mediada por la activación de los receptores 5-HT_{2A}, al igual que ocurre en modelos de diabetes de larga evolución (Morán *et al.*, 2009; Restrepo *et al.*, 2011). En arterias renales aisladas de rata, se ha demostrado la participación de los receptores 5-HT_{2A} y 5-HT_{2B} en la vasoconstricción inducida por 5-HT (Watts & Thompson, 2004), mientras que en las arterias renales de conejo los receptores 5-HT_{1B/1D} median esta respuesta (Hinton *et al.*, 1999). Por el contrario, la vasodilatación inducida por 5-HT en el riñón autoperfundido *in situ* de rata está mediada por receptores 5-HT_{1D}, que incrementan la producción de NO (García-Pedraza *et al.*, 2015b). En ratas en tratamiento crónico con sarpogrelato, un antagonista de receptores 5-HT₂ (García-Pedraza *et al.*, 2016), la vasodilatación renal depende de la activación de receptores 5-HT_{1B}, 5-HT_{1D} y 5-HT₇, que involucran,

respectivamente, la síntesis/liberación de NO, la producción de prostaciclina derivada de la COX, y la activación de canales K_{ATP} .

2. Modulación de la neurotransmisión periférica por el sistema serotoninérgico

El SNP comprende las estructuras nerviosas situadas fuera del SNC y está constituido por nervios y ganglios que conectan el cerebro y la médula espinal con el resto del organismo. De acuerdo con su estructura y función, el SNP se subdivide en el sistema nervioso autónomo (SNA), el sistema nervioso sensorial y el sistema nervioso somático (fibras eferentes destinadas al control de la musculatura esquelética). Tanto el SNA como el sensorial participan en funciones involuntarias y en el mantenimiento de la homeostasis de múltiples sistemas, incluyendo la regulación del tono vascular, la presión arterial y la frecuencia cardíaca, la motilidad y secreción gastrointestinal, el control respiratorio, la función endocrina, el metabolismo y la respuesta inmune (Murtazina & Adameyko, 2023; Wehrwein *et al.*, 2016). Estos efectos se producen mediante la liberación de diversos neurotransmisores en las uniones neuroefectoras autónomas y sensoriales, principalmente NA, ACh y diversos neuropéptidos, cuya liberación y actividad postsináptica están moduladas por el sistema serotoninérgico (Cuesta *et al.*, 2014; García *et al.*, 2005; García-Pedraza *et al.*, 2013, 2017; González-Hernández *et al.*, 2023; Marichal-Cancino *et al.*, 2020; Morán *et al.*, 1998).

Entre los modelos experimentales de animal entero más representativos para estudiar la influencia del sistema serotoninérgico sobre los sistemas de neurotransmisión periférica a nivel cardiovascular, se encuentra el modelo de rata *pithed* (desmedulada). Esta técnica elimina la influencia del SNC, preservando la funcionalidad del SNP, lo que permite la estimulación controlada de las fibras simpáticas que inervan la vasculatura sistémica y el corazón, de las fibras nerviosas parasimpáticas cardíacas, así como de las vías sensoriales perivasculares (Cuesta *et al.*, 2014; Fernández-González *et al.*, 2022; García *et al.*, 2005;

García-Pedraza *et al.*, 2013, 2017, 2018, 2022; Gillespie & Muir, 1967; González-Hernández *et al.*, 2023; Morán *et al.*, 1994, 1998, 2010).

2.1. Sistema nervioso simpático

El SNA se organiza funcionalmente en tres componentes principales: el sistema nervioso simpático (SNS), el sistema nervioso parasimpático (SNPS) y el SNE. El SNA es un sistema predominantemente eferente que transmite impulsos desde el SNC hacia los órganos periféricos, aunque también integra vías aferentes que transmiten información sensitiva al SNC. La mayoría de los órganos reciben inervación dual simpática y parasimpática, con respuestas generalmente opuestas (Flórez *et al.*, 2025; Murtazina & Adameyko, 2023).

El SNS se caracteriza por fibras preganglionares cortas que contienen ACh y proyectan a ganglios simpáticos, desde los cuales emergen fibras posganglionares más largas, de naturaleza adrenérgica. En contraste, el SNPS presenta fibras preganglionares largas y colinérgicas que establecen sinapsis en ganglios situados próximos a los órganos diana, desde donde parten fibras posganglionares cortas de tipo colinérgico (Figura 8). Por su parte, el SNE constituye una red neuronal intrínseca capaz de funcionar de manera relativamente autónoma respecto al SNC (Murtazina & Adameyko, 2023; Waxenbaum *et al.*, 2025).

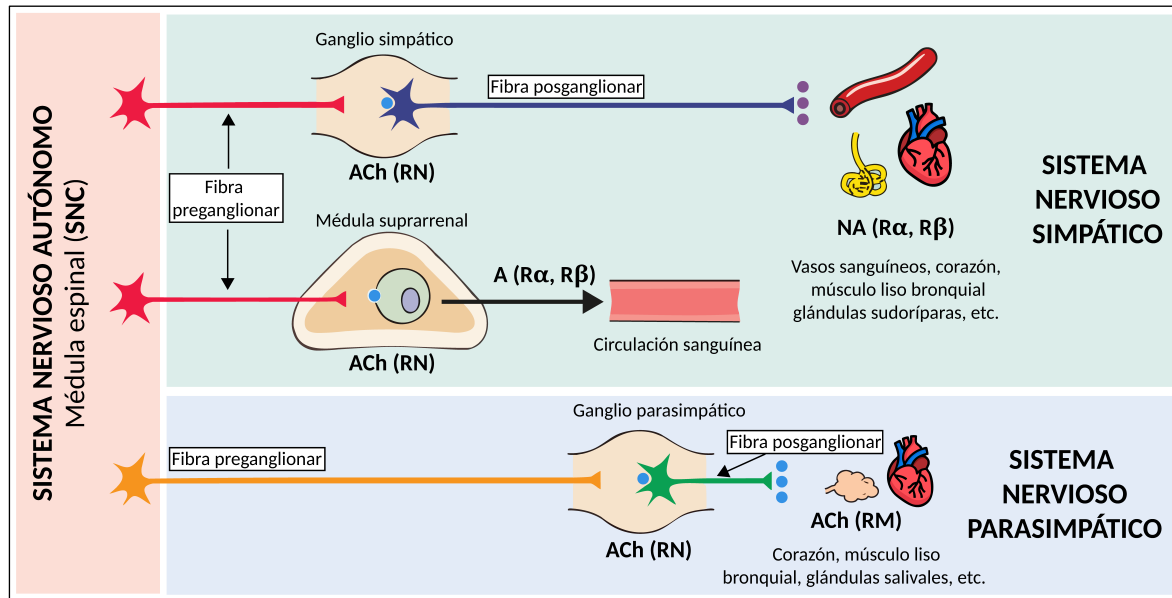


Figura 8. Sistema nervioso simpático y parasimpático y sus sinapsis periféricas. A, adrenalina; ACh, acetilcolina; NA, noradrenalina; R α , receptores α adrenérgicos; R β , receptores β adrenérgicos; RM, receptores muscarínicos; RN, receptores nicotínicos.

En el sistema cardiovascular, la NA ejerce un efecto vasopresor principalmente por la activación de receptores α_1 adrenérgicos en el músculo liso vascular (Figura 9), y efectos inotrópicos y cronotrópicos positivos a través de receptores β_1 cardíacos, ambos pertenecientes a la familia de los GPCR. Los receptores α_1 adrenérgicos se acoplan a proteínas G $\alpha_{q/11}$, produciendo aumento de Ca $^{2+}$ intracelular por estimulación de la PLC, mientras que los receptores β_1 adrenérgicos se acoplan a proteínas G α_s , lo que incrementa la producción de AMPc y de la proteína cinasa A (PKA). Esta última fosforila múltiples proteínas implicadas en el acoplamiento excitación-contracción del miocardio, incluidos los canales de Ca $^{2+}$ tipo L y proteínas reguladoras del retículo sarcoplásmico, facilitando un aumento de la entrada y el manejo de Ca $^{2+}$ (Flórez *et al.*, 2025).

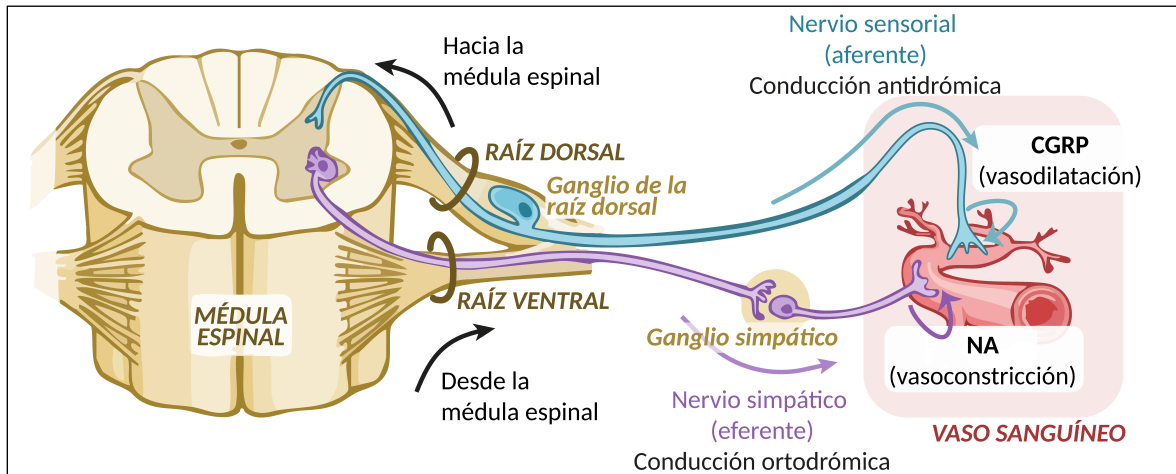


Figura 9. Neurotransmisión simpática y CGRPérgica a nivel vascular. La activación del sistema nervioso simpático induce vasoconstricción por liberación de noradrenalina (NA), mientras que la activación de las fibras sensoriales primarias libera el péptido relacionado con el gen de la calcitonina (CGRP) por conducción antidrómica, favoreciendo la vasodilatación.

La interrelación entre 5-HT y la neurotransmisión simpática cardíaca y vascular está ampliamente documentada. Desde que se describió la capacidad de bajas concentraciones de 5-HT de inhibir la liberación de NA inducida por estimulación eléctrica en vena safena de perro (McGrath, 1977), numerosos estudios han demostrado la interacción entre ambos sistemas tanto a nivel central (De Deurwaerdere & Di Giovanni, 2021; Fink & Göthert, 2007; Rutz *et al.*, 2007) como periférico (Fernández-González *et al.*, 2022; García *et al.*, 2005; García-Pedraza *et al.*, 2013, 2017, 2018, 2022; González-Hernández *et al.*, 2023; Hernández-Abreu *et al.*, 2020; Morán *et al.*, 1994, 1998, 2010). Esta interacción se sustenta, en parte, por la interconexión entre las neuronas serotoninérgicas y adrenérgicas en el SNC, así como por la expresión de múltiples tipos de receptores de 5-HT en las terminaciones nerviosas adrenérgicas periféricas (Fink & Göthert, 2007; Guiard *et al.*, 2008).

La presencia del sistema serotoninérgico en estructuras del SNS ha sido confirmada mediante estudios inmunohistoquímicos, que han identificado a la 5-HT en ganglios simpáticos, donde puede ser recaptada por SERT y liberada, además de sintetizarse en menor medida (Pierce *et al.*, 1996). Así, la 5-HT puede ser recaptada y liberada desde las terminaciones nerviosas simpáticas en la vasculatura sistémica, liberándose junto a NA y modulando la transmisión noradrenérgica (Ramage & Villalon, 2008).

En ratas macho *pithed*, 5-carboxamidotriptamina (5-CT; agonista 5-HT_{1/5/7}) reproduce el efecto inhibidor de serotonina sobre la neurotransmisión simpática vascular, por activación específica de receptores presinápticos 5-HT_{1B} y 5-HT_{1A/1D} (Figura 7). Por el contrario, la activación de los receptores presinápticos 5-HT₃ aumentan la liberación de NA en la vasculatura sistémica (Figura 6; Morán *et al.*, 1994, 1998; Villalón *et al.*, 1998). Asimismo, se ha demostrado que 5-HT inhibe la neurotransmisión noradrenérgica cardíaca en ratas *pithed* mediante la activación de receptores presinápticos 5-HT_{1B/1D} y 5-HT_{5A}, reduciendo las respuestas taquicárdicas obtenidas por estimulación eléctrica selectiva de los nervios simpáticos cardíacos (Sánchez-López *et al.*, 2003, 2004; Villalón *et al.*, 1999).

Adicionalmente, nuestro grupo de investigación ha demostrado que la 5-HT modula la neurotransmisión simpática en diferentes territorios vasculares autoperfundidos *in situ* de ratas macho. En estos modelos, la activación de receptores presinápticos 5-HT_{1D} inhibe la liberación de NA provocada por estimulación eléctrica a nivel renal (García-Pedraza *et al.*, 2015a) y en el tren posterior de rata (Calama *et al.*, 2005), mientras que en el mesenterio esta inhibición se produce a través de los receptores pre y/o postsinápticos 5-HT_{1D} (García-Pedraza *et al.*, 2017).

Resulta de particular interés que el perfil farmacológico de los receptores de 5-HT que modulan la neurotransmisión noradrenérgica no sólo varía entre los distintos territorios vasculares, sino que también se ve modificado en presencia de patologías cardiovasculares (como hipertensión y diabetes), y de tratamientos con fármacos que afectan al sistema serotoninérgico, como la fluoxetina (ISRS) o el sarpogrelato. A pesar de esta variabilidad, los receptores de la familia 5-HT₁ son los principales implicados en la inhibición de la neurotransmisión noradrenérgica, aunque los subtipos específicos (5-HT_{1A/1B/1D/1F}) involucrados y la participación de los receptores 5-HT₇ varían en función del modelo experimental (Fernández-González *et al.*, 2023, 2024; García *et al.*, 2005; García-Pedraza *et al.*, 2013). Por su parte, el perfil potenciador de la neurotransmisión simpática está

ausente o se media a través de los receptores 5-HT_{2A} (García *et al.*, 2005; García-Pedraza *et al.*, 2021; Morán *et al.*, 2010).

2.2. Sistema nervioso sensorial CGRPérgico

El sistema nervioso sensorial está constituido principalmente por neuronas cuyos somas se localizan en los ganglios de la raíz dorsal (DRG), adyacentes a la médula espinal, y en los ganglios del trigémino (TG), que inervan el territorio craneofacial. Estas neuronas presentan una morfología pseudounipolar característica, con un axón bifurcado que se divide en una rama periférica y una rama proyectada al SNC (Mei *et al.*, 2025; Murtazina & Adameyko, 2023). Su función principal es transmitir información sensorial desde la periferia al SNC; sin embargo, estas neuronas también pueden actuar como efectoras, liberando mediadores almacenados en sus terminaciones periféricas mediante conducción antidrómica (Figura 9). En estas terminaciones se han identificado múltiples neuropéptidos con acciones vasculares diferenciadas, entre los que se incluyen mediadores vasoconstrictores como el neuropéptido Y (NPY); vasodilatadores como el polipéptido vasoactivo intestinal (VIP), las encefalinas, la sustancia P (SP) y el péptido relacionado con el gen de la calcitonina (CGRP); así como otros péptidos, como la somatostatina, para los que no se han descrito acciones directas sobre la presión arterial. Estos neuropéptidos pueden expresarse tanto en fibras sensoriales como en fibras autonómicas no adrenérgicas no colinérgicas (NANC) (González-Hernández *et al.*, 2016; Marichal-Cancino *et al.*, 2020).

El CGRP es un neuropéptido de 37 aminoácidos descubierto en 1982 mediante técnicas de biología molecular, como producto del procesamiento alternativo del RNA mensajero del gen de la calcitonina (Figura 10A). Estructuralmente existen dos isoformas, CGRP- α y CGRP- β , que, aunque comparten acciones biológicas, se codifican a partir de dos genes distintos. En humanos estas isoformas difieren en tres aminoácidos, mientras que en ratas sólo difieren en uno. A pesar de su alto grado de homología, la síntesis y expresión de CGRP- β se concentra en el SNE, células del sistema inmune y la glándula pituitaria, mientras que

CGRP- α se expresa principalmente en las fibras sensoriales primarias tipo C desmielinizadas y A δ mielinizadas del SNC y periférico. Esta última isoforma presenta una abundante distribución perivascular y, por tanto, es la principal estudiada a nivel neurovascular (Argunhan & Brain, 2022; Bonura *et al.*, 2023; González-Hernández *et al.*, 2016).

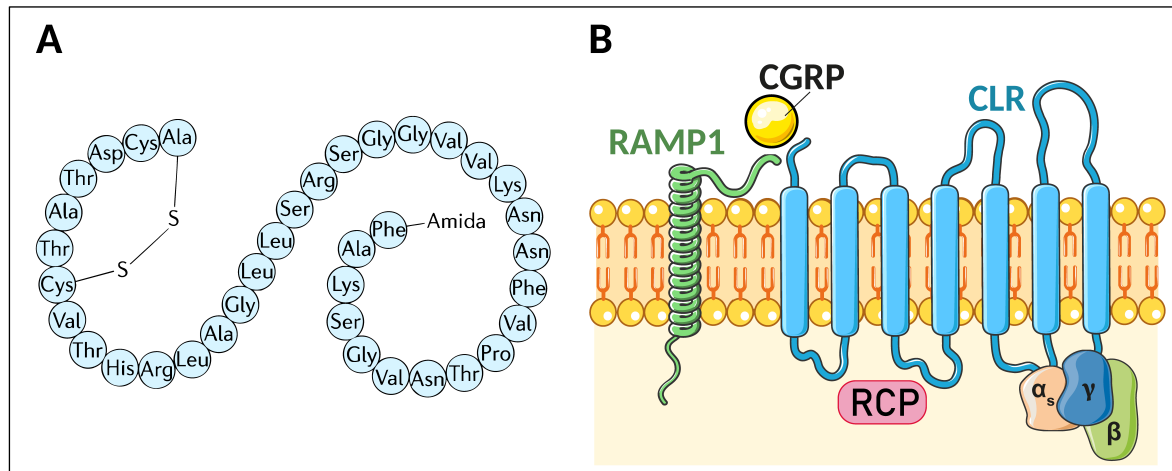


Figura 10. A | Secuencia de aminoácidos correspondiente al péptido relacionado con el gen de la calcitonina humano, isoforma α (CGRP- α). **B** | Receptor de CGRP. El receptor funcional está compuesto por el receptor calcitonina-like (CLR), la proteína modificadora de la actividad de receptores tipo 1 (RAMP1) y proteína componente del receptor (RCP).

La liberación de CGRP desde los nervios sensoriales perivasculares está modulada por estímulos químicos, físicos y eléctricos. Entre los mecanismos mejor caracterizados se encuentra la activación de receptores de potencial transitorio vaniloide (TRPV1) por capsaicina, calor o descenso del pH, que favorece la entrada de Ca^{2+} y la exocitosis de CGRP. Asimismo, la estimulación eléctrica de los nervios sensoriales perivasculares induce su liberación desde las terminaciones periféricas. Además, distintos receptores presinápticos y/o postsinápticos expresados en dichas terminaciones, incluidos los receptores de 5-HT, α_2 adrenérgicos, opioides, factor de crecimiento nervioso y prostaglandinas, modulan la liberación del neuropéptido (Cuesta *et al.*, 2014; González-Hernández *et al.*, 2023; Kumar *et al.*, 2019).

CGRP actúa sobre receptores específicos situados en la membrana plasmática de células endoteliales, células musculares lisas, cardiomiocitos, neuronas, células epiteliales,

adipocitos y células del sistema inmune (Bonura *et al.*, 2023; Hu *et al.*, 2016; Russo & Hay, 2023). El receptor de CGRP está constituido por un receptor acoplado a proteínas G, conocido como receptor *calcitonina-like* (CLR), y una proteína modificadora de la actividad de receptores tipo 1 (RAMP1), necesaria para que CLR alcance la superficie celular y pueda unirse específicamente a CGRP. Adicionalmente, se ha identificado un tercer componente, denominado proteína componente del receptor (RCP), que parece facilitar el acoplamiento a proteínas G (Figura 10B). Tras la activación del complejo CLR-RAMP1-RCP, se inicia la cascada de señalización mediada por la proteína $G\alpha_s$, con el consiguiente aumento en la producción de AMPc como segundo mensajero, aunque también se ha sugerido la participación de la proteína $G\alpha_{q/11}$, que activa la PLC. La familia de receptores de CGRP comprende además otras dos proteínas RAMP (RAMP2 y RAMP3), que, al asociarse con CLR, forman los receptores de adrenomedulina tipo 1 y 2 (AM_1 y AM_2), respectivamente (Argunhan & Brain, 2022; González-Hernández *et al.*, 2016; Russo & Hay, 2023).

A través de sus receptores, CGRP regula diversos procesos biológicos, incluyendo la nocicepción, la vasodilatación, el metabolismo, la inflamación, la cicatrización y el vaciado gastrointestinal (Bonura *et al.*, 2023). Entre estas funciones, su participación en la regulación cardiovascular y cerebrovascular ha sido especialmente investigada, tanto en el control del tono vascular y la función cardíaca, como en la fisiopatología de la migraña. Hasta la fecha, CGRP es el vasodilatador más potente identificado en la microvasculatura, siendo 10 veces más potente que las prostaglandinas, y de 100 a 1000 veces más potente que otros vasodilatadores como la ACh o la SP, además de presentar una duración de acción prolongada (hasta 6 horas) en comparación con otros vasodilatadores (González-Hernández *et al.*, 2016; Russo & Hay, 2023).

Dado que el receptor de CGRP se expresa en células endoteliales y musculares lisas vasculares, este neuropéptido puede inducir vasodilatación por mecanismos dependientes o independientes de endotelio (Argunhan & Brain, 2022; González-Hernández *et al.*, 2016):

- Mecanismo endotelio-dependiente. En las células endoteliales, la activación del receptor de CGRP incrementa los niveles de AMPc y activa la PKA, que fosforila eNOS, promoviendo un aumento en la síntesis de NO. Este difunde a las células musculares lisas, donde activa la guanilato ciclasa soluble, incrementando la producción de guanosina monofosfato cíclico (GMPc) y la activación de la proteína cinasa G (PKG), lo que induce vasodilatación.
- Mecanismo independiente de endotelio y de NO. En las células musculares lisas vasculares, el aumento de AMPc y PKA conduce a (i) una reducción de Ca^{2+} intracelular, (ii) una disminución de la afinidad del complejo Ca^{2+} -calmodulina por la cinasa de la cadena ligera de miosina (MLCK), y (iii) la activación de canales K_{ATP} . Estos mecanismos contribuyen de manera conjunta a la relajación de la célula muscular lisa y, por tanto, a la vasodilatación. Este mecanismo predomina en la mayoría de los territorios vasculares estudiados, con la excepción de la aorta y la arteria pulmonar, donde la respuesta se atenúa en presencia de inhibidores de eNOS.

El papel del sistema nervioso sensorial CGRPérgico sobre el sistema cardiovascular se estableció experimentalmente en la primera década de 1990. Inicialmente, estudios en la vasculatura mesentérica de ratas demostraron que la estimulación de los nervios periarteriales, en presencia de metoxamina (agonista de receptores α_1 adrenérgicos) y un bloqueante ganglionar simpático, producía una vasodilatación de origen nervioso (bloqueada por tetrodotoxina), independiente de mecanismos colinérgicos y β -adrenérgicos, que podía ser mimetizada por CGRP exógeno e inhibida por el antagonista CGRP₈₋₃₇ (Kawasaki *et al.*, 1988). Unos años más tarde, empleando el modelo de rata *pithed* bajo condiciones farmacológicas similares, Taguchi y colaboradores establecieron que la estimulación eléctrica del cordón espinal inducía una vasodilatación sistémica con un perfil farmacológico equivalente y, además, sensible a capsaicina, lo que permitió atribuir el efecto a la liberación de CGRP desde los nervios sensoriales perivascuales (Taguchi *et al.*, 1992).

Estudios posteriores han confirmado su relevancia fisiológica en el control cardiovascular, al constatar que la denervación sensorial incrementa la presión arterial (Watts, 2014). Estos hallazgos han promovido el estudio de los mecanismos que modulan la actividad vascular del sistema CGRPérgico, entre los que destaca el sistema serotoninérgico.

En ratas macho *pithed*, la serotonina inhibe la neurotransmisión CGRPérgica perivascular mediante la activación de receptores presinápticos 5-HT_{1B/1F}, mientras que los receptores 5-HT_{1A/1D} no parecen participar en esta regulación periférica (González-Hernández *et al.*, 2010, 2011). Además, los receptores 5-HT₇, tradicionalmente asociados a efectos vasodilatadores por su localización postsináptica en el músculo liso vascular, también actúan a nivel presináptico inhibiendo la liberación de CGRP desde los nervios sensoriales perivasculares. Este efecto parece implicar la activación de la vía de la endotelina, cuya activación atenúa la vasodilatación inducida por CGRP (Cuesta *et al.*, 2014).

A nivel molecular, se ha descrito la expresión de los receptores 5-HT_{1B/1F} y 5-HT₇ en neuronas del DRG, así como de otros subtipos como 5-HT_{2A/2C}, 5-HT₃ y 5-HT₅. No obstante, la implicación funcional de estos últimos en la neurotransmisión sensorial cardiovascular no es conocida, con la excepción de los receptores 5-HT₃, que favorecen la liberación de CGRP en el tejido cardíaco de cobaya, induciendo una respuesta inotrópica positiva (Tramontana *et al.*, 1993).

En los vasos sanguíneos craneales, CGRP desempeña un papel fundamental en la fisiopatología de la migraña, enfermedad neurovascular que afecta aproximadamente al 15 % de la población mundial (Ferrari *et al.*, 2022). La migraña se caracteriza por episodios recurrentes de cefalea intensa y pulsátil, de duración variable de entre 4 y 72 horas, frecuentemente acompañados de náuseas, vómitos, fotofobia y fonofobia (Russo & Hay, 2023; Shankar Kikkeri & Nagalli, 2024). Aproximadamente un tercio de los pacientes presentan migraña con aura, en la que la cefalea está precedida de síntomas neurológicos

transitorios, principalmente alteraciones visuales como luces centelleantes en forma de líneas en zigzag, aunque también pueden presentarse síntomas somatosensoriales (parestesia), motores o auditivos (Goadsby, 2025; Shankar Kikkeri & Nagalli, 2024). CGRP se expresa de forma destacada en el sistema trigeminovascular, que inerva las arterias craneales. Durante un ataque de migraña, se produce la activación de las neuronas del TG y un aumento de la liberación de CGRP (Figura 11), lo que desencadena vasodilatación de las arterias meníngeas y cerebrales, inflamación neurogénica perivascular y potenciación de la transmisión nociceptiva en el sistema trigeminovascular (Bonura *et al.*, 2023; Pleş *et al.*, 2023). Este incremento en la liberación de CGRP se refleja en concentraciones elevadas del neuropéptido en diversos fluidos biológicos, incluyendo plasma, saliva y lágrimas, durante los ataques agudos de migraña (Russo & Hay, 2023).

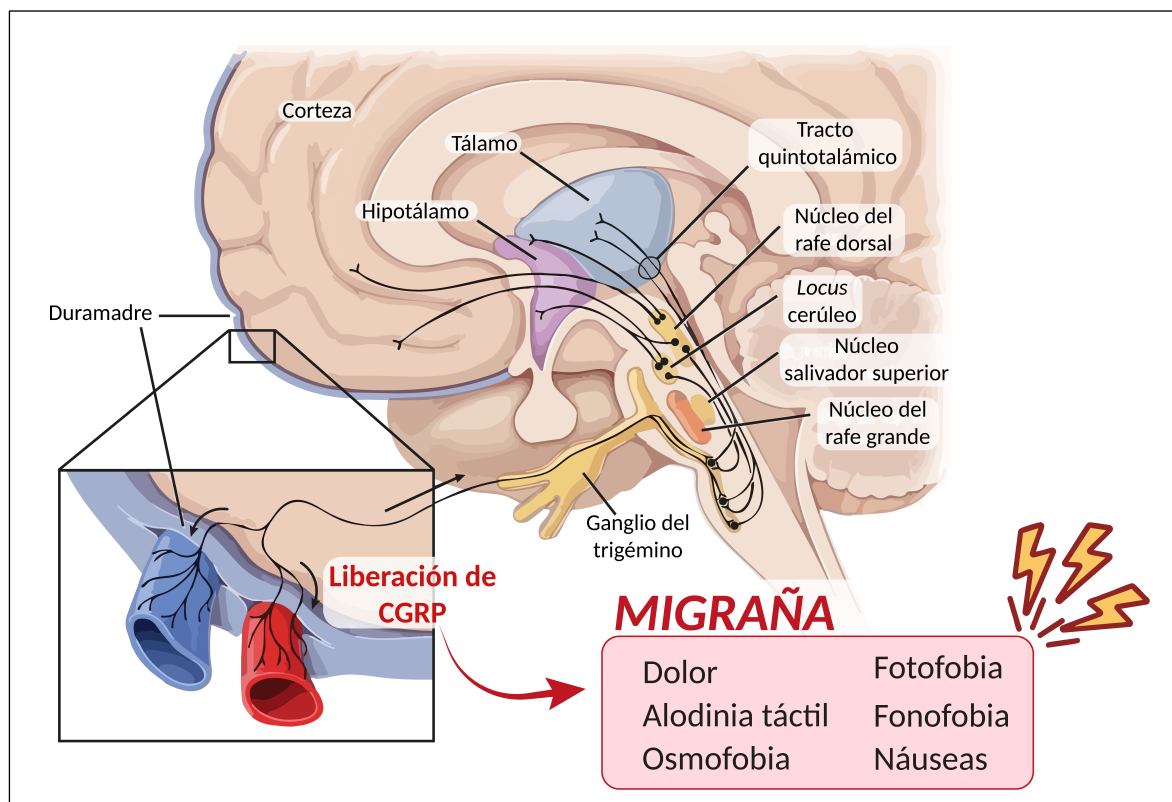


Figura 11. Implicación del sistema trigeminovascular en la fisiopatología de la migraña (adaptado de Goadsby, 2025). CGRP, péptido relacionado con el gen de la calcitonina.

Dada la implicación de CGRP en la fisiopatología de la migraña, la modulación de su actividad constituye una estrategia terapéutica relevante (Ferrari *et al.*, 2022; Lewter *et al.*, 2025). Los fármacos agonistas de los receptores 5-HT_{1B/1D} (triptanes) y 5-HT_{1F} (ditanes) representan el grupo de elección en migrañas moderadas o graves, debido en gran parte a su capacidad de inhibir la neurotransmisión CGRPérgica en el sistema trigeminovascular, además de producir vasoconstricción craneal a través de los receptores 5-HT_{1B} (Flórez *et al.*, 2025; González-Hernández *et al.*, 2023). Más recientemente, se han desarrollado dos grandes grupos de fármacos dirigidos frente al sistema CGRP: los antagonistas orales o gepantes, y los anticuerpos monoclonales (erenumab, galcanezumab, fremanezumab y eptinezumab), indicados en la prevención de la migraña, aunque también actúan como fármacos sintomáticos; de hecho, rimegepant ha sido comercializado para ambas indicaciones (Flórez *et al.*, 2025).

3. Influencia del sexo en el sistema serotoninérgico, cardiovascular y migraña

El sexo y el género son variables determinantes de la salud física y mental (Figura 12). El sexo se define como el conjunto de características biológicas (cromosómicas, hormonales y anatómicas), que determinan diferencias moleculares y celulares en prácticamente todos los tejidos del organismo. Las diferencias asociadas al sexo afectan a la fisiopatología, la sintomatología y la respuesta a tratamientos farmacológicos en múltiples enfermedades. Por su parte, el género se refiere a un conjunto de diferencias sociales aprendidas, que varían con el tiempo y entre culturas, y que pueden tener un impacto sobre la salud y la evolución de la enfermedad (Clayton & Gaugh, 2022; Heidari *et al.*, 2016).

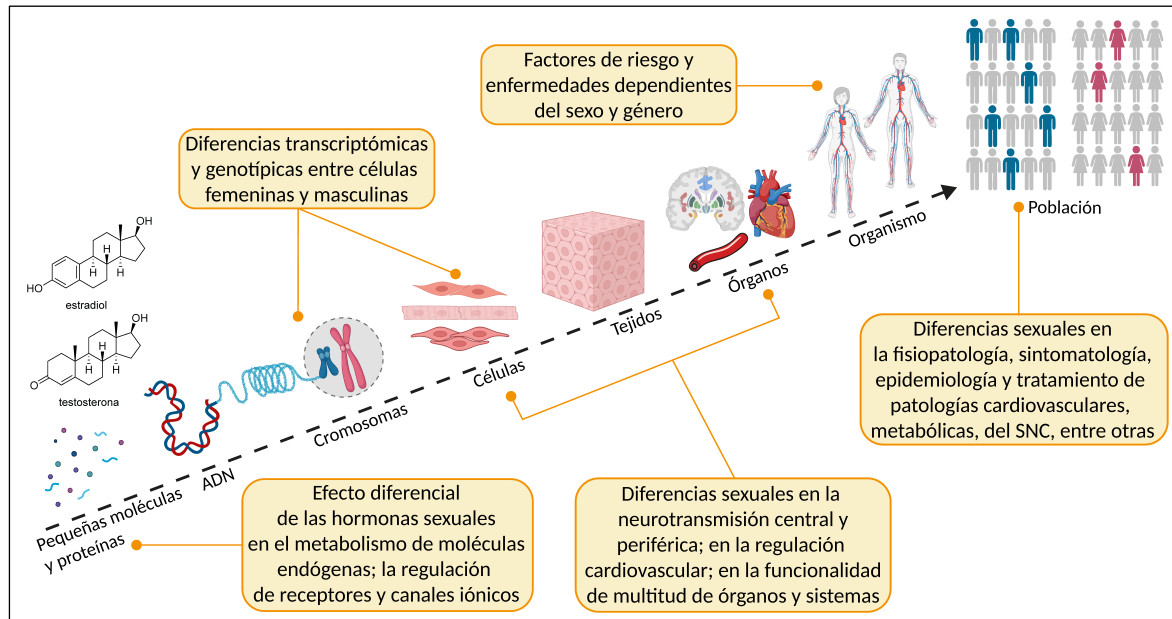


Figura 12. Representación esquemática de la influencia del sexo biológico desde el nivel molecular hasta el poblacional (adaptado de McClain *et al.*, 2024). ADN, ácido desoxirribonucleico; SNC, sistema nervioso central.

Históricamente, la investigación biomédica, tanto en humanos como en modelos animales, así como en estudios celulares y tisulares, se ha basado predominantemente en el sexo masculino, bajo la premisa de reducir la variabilidad atribuida al ciclo hormonal femenino y asumiendo erróneamente que los resultados eran extrapolables a ambos sexos. Este sesgo ha generado lagunas diagnósticas y terapéuticas, especialmente para mujeres, y ha motivado la implementación de políticas internacionales que instan a considerar la influencia del sexo y el género en la investigación biomédica, como las guías *Sex and Gender Equity in Research* (SAGER; Heidari *et al.*, 2016) y las iniciativas de la Comisión Europea (J. White *et al.*, 2021) o los *National Institutes of Health* (NIH) (Clayton, 2018). En el ámbito de la farmacología, el sesgo de sexo se ha evidenciado en tratamientos que han resultado inefectivos o inseguros en mujeres a pesar de haber sido prometedores en muestras principalmente masculinas. Entre los años 1997 y 2000, diez tratamientos farmacológicos fueron suspendidos por la *Food and Drug Administration* (FDA) de los Estados Unidos, ocho de los cuales suponían un mayor riesgo para mujeres (Lee, 2018).

3.1. Sistema serotoninérgico

Las hormonas sexuales modulan el sistema serotoninérgico a múltiples niveles, incluyendo la síntesis del neurotransmisor, su señalización a través de receptores específicos y su degradación. Los estrógenos promueven la síntesis de 5-HT al incrementar la expresión de la TPH1 y TPH2 (Bendis et al., 2024; Hernández-Hernández et al., 2019; Morris et al., 2021; K. White et al., 2011). En paralelo, reducen su degradación al disminuir la actividad de la MAO-A y MAO-B, lo que se ha demostrado en modelos animales y en humanos (Hernández-Hernández et al., 2019; Lokuge et al., 2011). Asimismo, los estrógenos modulan la expresión y funcionalidad de SERT tanto en regiones del SNC como en tejidos periféricos, incluyendo la vasculatura pulmonar (Bendis et al., 2024; K. White et al., 2011).

Además de modular el metabolismo de 5-HT, los estrógenos ejercen efectos epigenéticos y postraduccionales sobre diversos subtipos de receptores serotoninérgicos. En este sentido, los estrógenos disminuyen la señalización de los receptores 5-HT_{1A} presinápticos al favorecer el desacoplamiento de las proteínas G (Bendis et al., 2024; Hernández-Hernández et al., 2019). En el caso del receptor 5-HT_{1B}, se han descrito diferencias sexuales tanto en el desarrollo de hipertensión pulmonar (K. White et al., 2011), como en la hiperalgesia inducida por los triptanes en modelos animales, donde este receptor presenta una menor participación en hembras (Araldi et al., 2017). Por otro lado, los estrógenos incrementan la densidad de los receptores 5-HT_{2A} en la corteza cerebral, el núcleo accumbens y los núcleos del rafe del SNC, efecto demostrado mediante estudios de unión a radioligando en ratas hembra ovariectomizadas tratadas con estradiol (Cyr et al., 1998; Sumner & Fink, 1995).

La influencia del sexo biológico sobre el sistema serotoninérgico se ha estudiado principalmente a nivel central, en el contexto de patologías neuropsiquiátricas con marcada diferencia sexual, como la depresión y los trastornos de la conducta alimentaria (Dalla et al., 2010; Hernández-Hernández et al., 2019; Spies et al., 2020). Sin embargo, el

conocimiento sobre la influencia sexual en el sistema serotoninérgico periférico continúa siendo limitado.

3.2. *Enfermedades cardiovasculares*

El término ECV engloba a todas las patologías que afectan al corazón y a los vasos sanguíneos, y constituyen la primera causa de mortalidad a nivel mundial (Organización Mundial de la Salud [OMS], 2025). En España, según los datos más recientes (2024), los tumores han superado en número total de defunciones a las ECV; sin embargo, al analizar los datos nacionales estratificados por sexo, las ECV continúan siendo la principal causa de muerte en mujeres (GBD 2023 Causes of Death Collaborators, 2025; Instituto Nacional de Estadística, 2025; Palaniappan *et al.*, 2026).

Aunque mujeres y hombres comparten los factores de riesgo clásicos en el desarrollo de ECV (hipertensión, diabetes, obesidad y tabaquismo), el sexo modula de manera diferencial su impacto y la evolución de la enfermedad. Las mujeres en edad fértil presentan un menor riesgo cardiovascular en comparación con hombres de la misma edad. Esta diferencia se ha atribuido tradicionalmente al efecto protector de los estrógenos, que modulan múltiples vías implicadas en la regulación cardiovascular, incluyendo la actividad del sistema nervioso simpático, la señalización de calcio intracelular en células vasculares, cardíacas y renales, así como la función endotelial y la modulación de mediadores vasodilatadores y vasoconstrictores (Clayton & Gaugh, 2022; Hart *et al.*, 2012; Mauvais-Jarvis *et al.*, 2020; McClain *et al.*, 2024). En mujeres en edad fértil, la presión arterial basal es significativamente menor en comparación con hombres de la misma edad, una diferencia que no puede atribuirse únicamente a las acciones vasculares directas de los estrógenos, sino también a su modulación del SNS. En este sentido, las mujeres jóvenes muestran una mayor sensibilidad a la activación de los receptores β adrenérgicos vasculares, favoreciendo la vasodilatación y atenúa la vasoconstricción α -adrenérgica (Briant *et al.*, 2016; Hart *et al.*, 2012). No obstante, este mecanismo protector β adrenérgico se pierde tras la menopausia

(Baker *et al.*, 2018; Hart *et al.*, 2012), momento a partir del cual el riesgo cardiovascular en mujeres aumenta significativamente, igualándose o incluso superando al de los hombres (Stanhewicz *et al.*, 2018).

A nivel experimental, diversos estudios en modelos animales han confirmado el efecto protector de los estrógenos sobre el sistema cardiovascular. La administración de estradiol en ratones hembra en edad fértil con y sin ovariectomía induce vasodilatación dependiente de NO (Guo *et al.*, 2005). Adicionalmente, en ratones con delección conjunta de eNOS y COX-1 se ha demostrado una marcada diferencia sexual en los mecanismos de relajación vascular dependiente de endotelio, donde NO y prostaciclina son los principales mediadores vasodilatadores en machos, mientras que en hembras predomina EDH (Scotland *et al.*, 2005). Sin embargo, el efecto protector de los estrógenos disminuye con la edad. Así, se ha descrito una pérdida del efecto vasoprotector de 17 β -estradiol sobre las células musculares vasculares lisas en ratones hembra de edad avanzada (52 semanas) en comparación con hembras jóvenes (10 semanas) (Bowling *et al.*, 2014).

Aunque en condiciones fisiológicas los estrógenos confieren protección cardiovascular, recientes estudios muestran que el sexo femenino presenta mayor sensibilidad al sistema renina-angiotensina-aldosterona (SRAA). En células endoteliales vasculares humanas de mujeres tratadas con Ang II se han observado cambios en la expresión génica relacionados con procesos inflamatorios y oxidativos, mientras que las células endoteliales masculinas presentaron cambios mínimos (Weber *et al.*, 2023). Del mismo modo, en modelos animales, la sobreexpresión de receptores de Ang II tipo 1 (AT₁) induce mayor riesgo de miocardiopatía y paro cardíaco en ratones hembra que en machos (Mathieu *et al.*, 2018).

Cabe destacar que la terapia hormonal sustitutiva en mujeres posmenopáusicas no reproduce el perfil protector observado en mujeres premenopáusicas, lo que sugiere que las diferencias sexuales en las ECV no dependen exclusivamente de la acción de los estrógenos (Clayton & Gaugh, 2022). En conjunto, las diferencias sexuales en la función endotelial, el

control simpático vascular y el SRAA determinan una fisiopatología de las ECV dependiente del sexo, con patrones distintos en la prevalencia, evolución clínica y respuesta terapéutica, poniendo de manifiesto la necesidad de un abordaje clínico específico según el sexo (Vogel *et al.*, 2021).

3.3. Migraña

La migraña afecta predominantemente a las mujeres, con una prevalencia de tres a cuatro veces superior a la de los hombres durante la edad reproductiva. Esta marcada diferencia sexual se establece tras la menarquía y se atenúa con la menopausia, lo que sugiere un papel relevante de las hormonas sexuales femeninas en la fisiopatología de la migraña (Russo & Hay, 2023; Vetvik & MacGregor, 2017). Además, las mujeres experimentan crisis migrañosas de mayor intensidad, duración y frecuencia que los hombres, con sintomatología más grave (de Vries Lentsch *et al.*, 2021; Elgendy *et al.*, 2019), y presentan con mayor frecuencia migraña con aura, asociada a su vez con mayor riesgo cardiovascular (Elgendy *et al.*, 2019; Vetvik & MacGregor, 2017). Dada esta mayor prevalencia y gravedad, la migraña constituye la primera causa de años vividos con discapacidad en mujeres menores de 50 años a nivel mundial, según los análisis más recientes del estudio *Global Burden of Disease* (GBD 2023 Headache Collaborators, 2025; Krause *et al.*, 2021; Steiner *et al.*, 2020).

A nivel fisiopatológico, las hormonas sexuales femeninas modulan la actividad del sistema sensorial CGRPérgico, cuya activación es determinante en la génesis del dolor migrañoso. Se ha demostrado que las concentraciones plasmáticas basales de CGRP en mujeres son significativamente superiores a las de los hombres (de Vries Lentsch *et al.*, 2021), y que las fluctuaciones hormonales del ciclo menstrual condicionan su actividad tanto en la periferia como en el SNC (Favoni *et al.*, 2019). En este sentido, estudios de inmunohistoquímica en ratas muestran que las hembras expresan más receptores de estrógeno en el TG en comparación con los machos; específicamente, la isoforma α (ER α)

se localiza en neuronas que expresan CGRP o su receptor, lo que sugiere una mayor capacidad de modulación hormonal sobre el CGRP en el sexo femenino (Warfvinge *et al.*, 2020).

En cuanto a la terapia antimigrañosa, tanto los fármacos serotoninérgicos como los dirigidos frente al sistema CGRPérgicos han demostrado ser eficaces en ambos sexos, aunque las mujeres tienden a consumir más medicación y presentan mayor discapacidad (Vetvik & MacGregor, 2017). Sin embargo, la evidencia sobre diferencias sexuales en la respuesta terapéutica es limitada, ya que la mayoría de los ensayos preclínicos y clínicos no han sido diseñados para evaluar estas diferencias (Lewter *et al.*, 2025).

HIPÓTESIS Y OBJETIVOS

Las ECV y neurológicas con componente vascular, como la migraña, presentan una marcada influencia del sexo en su presentación clínica, evolución, prevalencia y tratamiento farmacológico. Tradicionalmente, la menor incidencia de ECV observada en mujeres premenopáusicas se ha atribuido a los efectos protectores de los estrógenos. Sin embargo, estudios recientes muestran que las diferencias sexuales en la regulación cardiovascular también involucran mecanismos neuronales, incluyendo el control autonómico y la participación de la inervación sensorial. A pesar de ello, la mayor parte de la investigación preclínica en farmacología cardiovascular se ha realizado en modelos animales macho, lo que limita la comprensión de los mecanismos específicos dependientes del sexo.

El sistema serotoninérgico periférico desempeña un papel relevante en la regulación cardiovascular, ya que la 5-HT ejerce acciones directas sobre los vasos sanguíneos y el corazón, y al mismo tiempo modula diferentes sistemas de neurotransmisión periférica, entre los que destacan la inervación simpática y la sensorial CGRPérgica. Asimismo, se ha puesto de manifiesto que el sistema serotoninérgico está influenciado por el sexo biológico, observándose diferencias en su metabolismo y funcionalidad, particularmente a nivel del SNC y en determinadas patologías neuropsiquiátricas.

Nuestro grupo de investigación, junto con otros investigadores, cuentan con una amplia experiencia en el estudio de la participación periférica de la serotonina y sus mecanismos en la regulación cardiovascular en ratas macho, intentando comprender el papel funcional de los distintos tipos y subtipos de receptores serotoninérgicos implicados en la regulación cardiovascular mediante técnicas *in vivo* en ratas control (sin patología cardiovascular), con alteraciones cardiometabólicas, o con tratamientos farmacológicos que afectan al sistema serotoninérgico (Tabla 3).

Tabla 3. Estudios previos realizados por nuestro grupo de investigación y otros autores sobre las acciones indirectas de serotonina en la vasculatura sistémica de ratas macho *pithed* en diferentes condiciones experimentales.

Modelos experimentales		(Acción) Receptor 5-HT [vías]	Referencia
Neurotransmisión simpática vascular			
Control	Sin tratamiento	(-) 5-HT _{1A/1D} presináp. (+) 5-HT ₃ presináp.	Morán <i>et al.</i> , 1994, 1998
	Tratadas con fluoxetina	(-) 5-HT _{1A/1D} presináp. [NO/COX] (+) 5-HT _{2A} pre/postsináp. [COX]	García-Pedraza <i>et al.</i> , 2021
	Tratadas con sarpogrelato	(-) 5-HT _{1D} presináp. [COX] (-) 5-HT ₇ presináp. [Canales K _{ATP}]	García-Pedraza <i>et al.</i> , 2013, 2014
Diabéticas (4 semanas)	Control	(-) 5-HT _{1A} presináp. [NO]	García <i>et al.</i> , 2005, 2006
	Tratadas con fluoxetina	(-) 5-HT _{1A/1D} pre/postsináp. [NO/COX] (+) 5-HT _{2A} pre/postsináp. [COX]	García-Pedraza <i>et al.</i> , 2022
	Tratadas con sarpogrelato	(-) 5-HT _{1D/7} pre/postsináp.	Fernández-González <i>et al.</i> , 2022
Diabéticas (8 semanas)		(-) 5-HT _{1A/2A} presináp. [NO/COX]	Morán <i>et al.</i> , 2010; Restrepo <i>et al.</i> , 2012
Hipertensas (SHR)		(-) 5-HT _{1B} presináp.	Fernández, 1999
Neurotransmisión sensorial CGRPérgica perivascular			
Control		(-) 5-HT _{1B/1F} presináp.	González-Hernández <i>et al.</i> , 2010, 2011
		(-) 5-HT ₇ presináp. [ET]	Cuesta <i>et al.</i> , 2014

(-), inhibición; (+), potenciación; COX, ciclooxigenasa; ET, endotelina; K_{ATP}, canales de K⁺ dependientes de ATP; NO, óxido nítrico; postsináp., postsináptico; presináp., presináptico; SHR, ratas espontáneamente hipertensas.

Dados los antecedentes previamente expuestos en ratas macho, y considerando las diferencias sexuales descritas tanto en el sistema serotoninérgico como en los mecanismos neuronales de regulación cardiovascular, planteamos la hipótesis de que el sexo biológico influye en las respuestas serotoninérgicas que modulan la neurotransmisión simpática y sensorial CGRPérgica en la vasculatura sistémica.

Por tanto, este trabajo de Tesis Doctoral plantea como objetivo general determinar si los mecanismos serotoninérgicos involucrados en la regulación cardiovascular presentan diferencias dependientes del sexo biológico en ratas.

En consecuencia, se plantean los siguientes objetivos específicos:

1. Analizar, en ratas hembra, los parámetros cardiovasculares basales (presión arterial y frecuencia cardíaca) y las respuestas vasculares obtenidas por (i) estimulación simpática total (vasoconstricción), y (ii) estimulación de las fibras sensoriales CGRPérgicas (vasodilatación), comparando estos resultados con los obtenidos en ratas macho.
2. Evaluar la modulación que ejerce el sistema serotoninérgico sobre la neurotransmisión simpática vascular en ratas hembra, determinando los tipos y subtipos de receptores implicados en las respuestas vasculares observadas, así como la naturaleza de estos.
3. Estudiar la posible participación de mediadores vasoactivos en la modulación serotoninérgica de las respuestas vasopresoras obtenidas por liberación de NA endógena en ratas hembra.
4. Investigar la implicación de los receptores 5-HT_{1/5/7} en la regulación las respuestas CGRPérgicas perivasculares en ratas hembra, comparando estos resultados con los obtenidos en ratas macho.

ARTÍCULOS DE INVESTIGACIÓN**Artículo 1*****Sex influence on serotonergic modulation of the vascular noradrenergic drive in rats***

Terol-Úbeda, A. C., Fernández-González, J. F., Roldán-Hernández, C. A., Martín, M. L., Morán, A., García-Domingo, M., & García-Pedraza, J. Á.

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Resumen

El presente estudio tuvo como objetivo analizar el papel simpato-modulador de 5-HT en ratas hembra, caracterizando los receptores implicados. Se utilizaron ratas Wistar hembra de 14 a 16 semanas de edad, que fueron preparadas para la estimulación simpática. La presión arterial media y la frecuencia cardíaca se monitorizaron durante todo el experimento, y las respuestas vasopresoras se obtuvieron mediante estimulación eléctrica de los nervios simpáticos (0.1-5 Hz) o por administración intravenosa de noradrenalina (0.01-0.5 µg/kg). Se determinaron los efectos de diferentes fármacos serotoninérgicos sobre dichas respuestas vasopresoras, empleándose ratas macho de la misma edad como control. La presión arterial media basal en hembras fue menor que la observada en machos, mientras que los incrementos de presión arterial media obtenidos por estimulación eléctrica fueron similares en ambos sexos. En hembras, la 5-HT provocó una inhibición dosis-dependiente de las vasoconstricciones inducidas por estimulación simpática; este efecto fue reproducido por los agonistas 5-CT (5-HT_{1/5/7}) y L-694,247 (5-HT_{1D}). Por otro lado, el agonista selectivo 5-HT_{2A/2B/2C} (α-metil-5-HT) y el agonista 5-HT₃ (1-PBG)

potenciaron las respuestas vasopresoras producidas por estimulación eléctrica. Ninguno de los otros agonistas/antagonistas de los receptores serotoninérgicos estudiados (5-HT_{1A/1B/1F}, 5-HT_{2B/2C}, 5-HT₄, 5-HT_{5A} o 5-HT₇) modificó dichas respuestas vasoconstrictoras. Únicamente 1-PBG (agonista 5-HT₃) modificó los incrementos de presión arterial media inducidos por dosis crecientes de noradrenalina exógena. En conclusión, en ratas hembra, los efectos simpatolíticos vasculares de la 5-HT se deben a la activación presináptica del receptor 5-HT_{1D}, mientras que la activación pre y/o postsináptica del receptor 5-HT₃, así como la activación presináptica del receptor 5-HT_{2A}, están implicadas en la potenciación de la neurotransmisión simpática vascular.

RESEARCH ARTICLE



Sex influence on serotonergic modulation of the vascular noradrenergic drive in rats

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Background and Purpose: In male rats, the serotonergic system modulates sympathetic outflow at vascular levels, causing sympatho-inhibition and sympatho-excitation, mainly via 5-HT_{1D/1A} and 5-HT₃ receptors, respectively. However, sex influence on vascular serotonergic regulation has not yet been elucidated. This study aimed to analyse the 5-HT sympatho-modulatory role in female rats, characterising the 5-HT receptors involved.

Experimental Approach: Female Wistar (14- to 16-week-old) rats were prepared for sympathetic stimulation. Mean blood pressure (MBP) and heart rate (HR) were continuously measured. Vasopressor responses were obtained by electrical stimulation of the sympathetic outflow (0.1–5 Hz) or i.v. noradrenaline (0.01–0.5 µg·kg⁻¹). 5-HT-related drug effects on adrenergic system were determined. Age-matched male rats were used as control.

Key Results: Basal MBP in females was lower than in male rats, whereas electrical-induced increases in MBP were similar. In females, 5-HT exerted a dose-dependent inhibition on the sympathetic-evoked vasoconstrictions, that was reproduced by some agonists; 5-CT (5-HT_{1/5/7}) and L-694,247 (5-HT_{1D}), whereas the selective 5-HT_{2A/2B/2C} (α-methyl-5-HT) and 5-HT₃ agonist (1-PBG) increased the electrically-produced vasopressor responses. None of the other drugs tested (targeting 5-HT_{1A/1B/1F}, 5-HT_{2B/2C}, 5-HT₄, 5-HT_{5A} or 5-HT₇) modified these vasoconstrictions. Only 1-PBG (5-HT₃) modified the vasoconstrictions induced by exogenous noradrenaline.

Conclusions and Implications: In female rats, vascular serotonergic sympatholytic effects are due to prejunctional 5-HT_{1D} receptor activation, whereas pre and/or postjunctional 5-HT₃ and prejunctional 5-HT_{2A} receptor activation is involved in the potentiating effect of vascular sympathetic neurotransmission. These findings may

Abbreviations: CVD, cardiovascular diseases; D-R, dose-response; MBP, mean blood pressure; SNS, sympathetic nervous system; S-R, stimulus-response; 1-PBG, 1-phenylbiguanide; 5-CT, 5-carboxamidotryptamine.

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open novel sex-differential therapeutic strategies for treating cardiovascular conditions.

KEYWORDS

5-HT, 5-HT_{1D} receptor, 5-HT_{2A} receptor, 5-HT₃ receptor, sex differences, sympathetic modulation, vasoconstriction

1 | INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death globally (Vaduganathan et al., 2022). For many years, CVD research has been primarily focused on men, and female sex has been under-appreciated from etiological, diagnostic, and therapeutic perspectives, in part due to the well-described protective role of hormones in the premenopausal period. Currently, it is suggested that the effectiveness of specific therapies may depend on sex or levels of sex-biasing factors such as **oestrogens** (Murphy & Steenbergen, 2014). Although sex variations in CVD are caused in part by environmental/social differences between men and women, such as occupational hazards, habits or social stress, there are also biological factors that contribute to the development of CVD and must be studied in animal models (Puckrein et al., 2015; Shi et al., 2016). One of these factors is the autonomic nervous system which is essential in the control of cardiovascular homeostasis. Moreover, imbalance of the sympathetic nervous system (SNS) is related to the development of hypertension or heart failure, contributing to vascular damage, endothelial dysfunction and vasoconstriction in target organs (Heradien et al., 2019; Malpas, 2010; Quarti-Trevano et al., 2021). There are sex-based variations in the contribution of the SNS to resting blood pressure (BP) regulation and the sympathetic mechanisms involved in the development of hypertension (Briant et al., 2016).

5-HT (serotonin) is an endogenous neurohormone involved in distinct functions at central (anxiety, depression or eating disorders) and peripheral (platelet aggregation, vascular tone or heart rhythm) levels due to its great variety of receptors (Barnes et al., 2021). Sexual differences in the serotonergic system is widely discussed because oestrogens (1) alter gene expression of the **serotonin transporter (SERT)** or **monoamine oxidase A** (Lasiuk & Hegadoren, 2007; Lokuge et al., 2010), (2) also up-regulate levels of **tryptophan hydroxylase**, which makes males and females exhibit different rates of serotonin synthesis (Pais et al., 2023) and (3) modify the expression of several **5-HT receptors**, such as 5-HT₂ or 5-HT_{1A} (Lasiuk & Hegadoren, 2007; Lokuge et al., 2010). Thus, within the serotonergic system, sex may account for differences in the genetic risk of pathological conditions, where 5-HT receptors are known to be involved (Perry et al., 2017).

The serotonergic system is a modulator of the SNS and thus contributes to physiological control of the cardiovascular system. In male rats, our research group has already shown that the serotonergic system causes mesenteric and renal sympatho-inhibition mainly by activation of **5-HT_{1D}** receptors (García-Pedraza et al., 2015, 2017), whereas at vascular level the sympatholytic effect of the serotonergic

What is already known?

- The serotonergic system, via 5-HT receptors, modulates sympathetic neurotransmission at vascular level in male rats.
- 5-HT_{1D/1A} receptors are mainly sympatholytic, whereas 5-HT₃ receptors potentiate vascular noradrenergic out-flow in males.

What does this study add?

- In female rats, the serotonergic system is also involved in the regulation of sympathetic-induced vasoconstrictions.
- 5-HT_{1D} receptors have sympatholytic effect, whereas 5-HT₃ and 5-HT_{2A} receptors are vascular noradrenergic-enhancers in females.

What is the clinical significance?

- In female and male rats, the serotonergic system contributes differently to cardiovascular homeostasis.
- This study opens new sex-differential pharmacological strategies in cardiovascular pathologies with marked sympathetic hyperactivity.

system is mediated by 5-HT_{1D} and 5-HT_{1A} receptors (García-Pedraza et al., 2013; Morán et al., 1998), but **5-HT₃** receptor activation results in a vascular sympatho-excitatory effect (García-Pedraza et al., 2021; Morán et al., 1994). The induction of pathologies (e.g., diabetes) or the pharmacological modulation of serotonergic system in males change the serotonergic effect on vascular sympathetic discharge (Fernández-González et al., 2022; García et al., 2005; García-Pedraza et al., 2013, 2021, 2022; Morán et al., 2010), maintaining its main sympatholytic effect. However, serotonergic sexual divergence has not been yet elucidated at this level.

Considering that (1) CVD are the main cause of death and affect women and men differently (Vaduganathan et al., 2022), (2) noradrenergic contribution to vascular homeostasis is influenced by sex and SNS disequilibrium is related to the development of CVD (Briant

et al., 2016), (3) the serotonergic system modulates noradrenergic vasopressor responses in male rats (García-Pedraza et al., 2013, 2021; Morán et al., 1994, 1998) and (4) central 5-HT deficiency leads to hypertension, related to increased sympathetic vascular tone, in both male and female rats (Spinieli et al., 2022), the aim of this work was to establish the sympatho-regulatory profile of 5-HT in female rats, characterising the 5-HT receptors that modulate the vascular sympathetic outflow. These findings may open new differential therapeutic strategies in the treatment of vascular pathologies between men and women, involving serotonergic mechanisms.

2 | METHODS

2.1 | Animals

All animal care and experimental protocols were approved by the Research Ethics Committee of the University of Salamanca (ID number 973) and comply with the ARRIVE guidelines (Percie du Sert et al., 2020), the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020) as well as with the current European and Spanish guidelines (Directive 2010/63/EU; R.D. 53/2013).

Female and male Wistar rats aged 14–16 weeks ($n = 155$ and $n = 10$, respectively) were supplied by the animal facility of the University of Salamanca. All animals were housed in open-topped

polycarbonate cages, with 4–5 animals per cage with ad libitum access to food and water, on a light–dark cycle of 12–12 h, 50% humidity and controlled temperature ($22 \pm 2^\circ\text{C}$).

2.2 | Surgical procedure and general methods

The animals were anaesthetised with pentobarbital ($60 \text{ mg}\cdot\text{kg}^{-1}$; i.p.) and a tracheal cannula was placed for artificial respiration ($1\text{-ml air}\cdot 100 \text{ g}^{-1}$, $50 \text{ strokes}\cdot\text{min}^{-1}$). A pituiting rod was inserted through the orbit and foramen magnum into the spinal cord. Both jugular veins were cannulated for the continuous infusion of agonists and for the bolus administration of antagonists. The left carotid artery was cannulated for recording MBP and HR (using a pressure transducer connected to an e-corder 410 amplifier [Model ED410, Cibertec, Spain], using Chart™ and Scope™ software). The animals received i.v. bolus injections of heparin ($1000 \text{ UI}\cdot\text{kg}^{-1}$) and atropine ($1 \text{ mg}\cdot\text{kg}^{-1}$) to avoid the development of thrombi and cholinergic effects, respectively (García-Pedraza et al., 2013, 2021; Morán et al., 1994, 1998).

At this point, animals were randomly divided in two main groups (Figure 1) in order to study the role of several serotonergic agents (or their vehicles) on the vasopressor responses induced by electrical sympathetic stimulation ($n = 145$) or by i.v. administration of exogenous **noradrenaline** ($n = 20$).

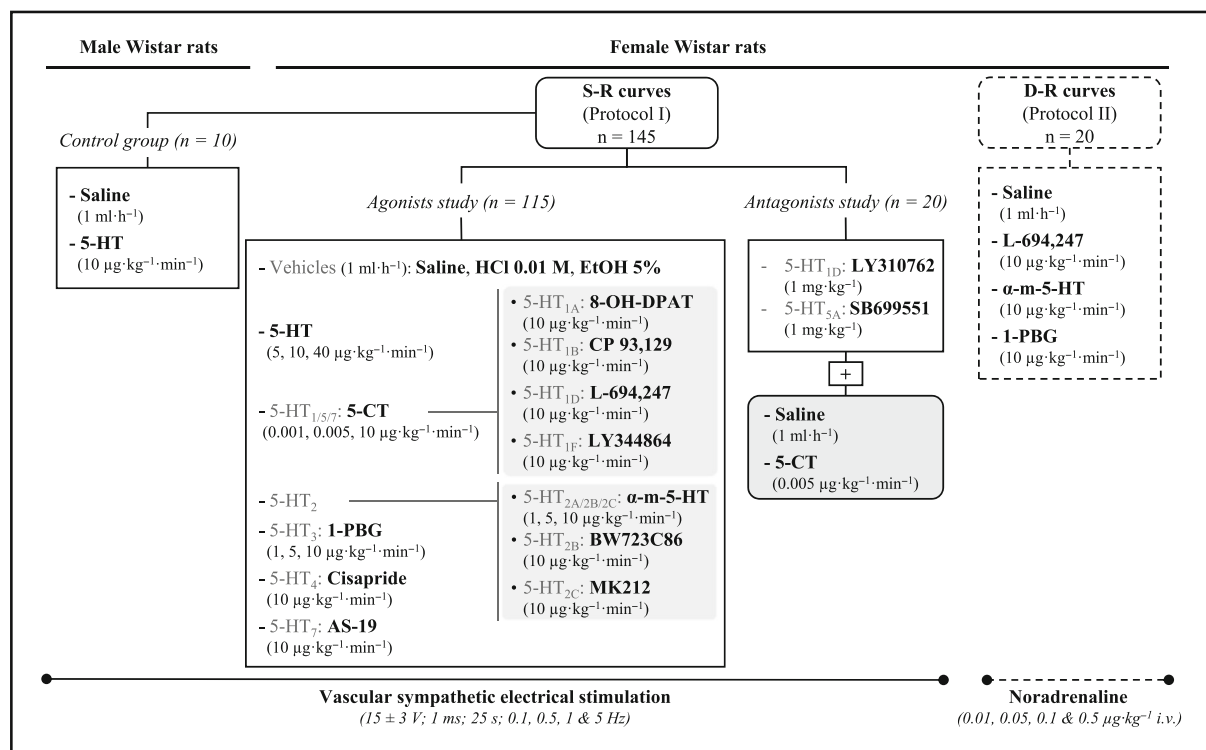


FIGURE 1 Scheme of experimental protocols, showing the number of animals used. Experimental protocols showing the two main sets and the different groups used in these experiments, in which vasopressor responses are obtained by electrical sympathetic stimulation (S–R curves) or i.v. bolus of noradrenaline (D–R curves). α-m-5-HT: α-methyl-5-HT; D–R: dose–response (to exogenous i.v. noradrenaline); EtOH: ethanol; S–R: (electrical) stimulus–response.

The vasopressor stimulus-response (S-R) and dose-response (D-R) curves induced by electrical stimulation and exogenous noradrenaline, respectively, were completed in about 20 min with no significant changes in baseline MBP and HR. The time lapse between the different stimulation frequencies or noradrenaline doses was approximately 5 min, when MBP had returned to baseline values after vasopressor responses. The doses of all drugs used were based on results from our previous studies, and the infusion rate for the serotonergic agonists (and their vehicles) was $1 \text{ ml} \cdot \text{h}^{-1}$ (Fernández-González et al., 2022; García et al., 2005; García-Pedraza et al., 2021). Drugs were dissolved in physiological saline at the time of experimentation, excluding cisapride (dissolved in 0.01 M HCl) and AS-19 (dissolved in ethanol [EtOH] 5%). These vehicles had no effect on the baseline mean blood pressure (MBP) or heart rate (HR).

2.3 | Experimental protocols

After achieving a stable haemodynamic condition for at least 10 min, baseline values for MBP and HR were determined and the animals were randomly separated to perform Protocol i and Protocol ii (Figure 1).

2.3.1 | Protocol i- electrical stimulation of the vascular sympathetic innervation

Prior to electrical stimulation, the animals were treated with d-tubocurarine ($2 \text{ mg} \cdot \text{kg}^{-1}$, i.v.) to prevent electrically-induced muscular spasms. The entire sympathetic outflow from the spinal cord was stimulated as previously stated: $15 \pm 3 \text{ V}$, 1 ms, for 25 s at increasing

frequencies (0.1, 0.5, 1 and 5 Hz), obtaining the control S-R curve (E0; Figure 2) (Morán et al., 1998; García-Pedraza et al., 2013, 2021). This main group of animals was divided into two sets (Figure 1).

The first set, used as male control group to confirm previous results ($n = 10$; $n = 5$ each), received i.v. infusion of saline ($1 \text{ ml} \cdot \text{h}^{-1}$, control group) or 5-HT ($10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) (García-Pedraza et al., 2021; Morán et al., 1994, 1998). After 10 min of starting the corresponding infusion, two new S-R curves (E1 and E2) were obtained as previously described for E0.

The second set in female rats ($n = 135$) was subdivided in two subgroups. The first subgroup of female rats ($n = 115$; $n = 5$ each dose and drug tested) received a continuous i.v. infusion of (a) vehicles (EtOH 5%; HCl 0.01 M or saline; $1 \text{ ml} \cdot \text{h}^{-1}$), (b) 5-HT (endogenous ligand; 5, 10 and $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or selective 5-HT receptor (sub)type agonists: (c) 5-CT (5-HT_{1/5/7}; 0.001, 0.005 and $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (d) 8-OH-DPAT (5-HT_{1A}; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (e) CP 93,129 (5-HT_{1B}; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (f) L-694,247 (5-HT_{1D}; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (g) LY344864 (5-HT_{1F}; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (h) α -methyl-5-HT (5-HT₂; 1, 5 and $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (i) BW723C86 (5-HT_{2B}; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (j) MK212 (5-HT_{2C}; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (k) 1-PBG (5-HT₃; 1, 5 and $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), (l) cisapride (5-HT₄; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or (m) AS-19 (5-HT₇; $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) (Figure 1). The S-R curves (E1 and E2) were constructed as previously indicated for the control group of male rats.

The second subgroup of female rats ($n = 20$) was used to confirm the 5-HT receptor (sub)type involved in the 5-HT modulatory effect on the vasopressor responses induced by electrical stimulation. To do so, animals received $1 \text{ mg} \cdot \text{kg}^{-1}$ (i.v. bolus) of the antagonists LY310762 (5-HT_{1D}) or SB699551 (5-HT_{5A}) and the corresponding curves (E0_{LY310762} and E0_{SB699551}) were completed after 10 min, respectively. Then, each of these pretreatments was subdivided in

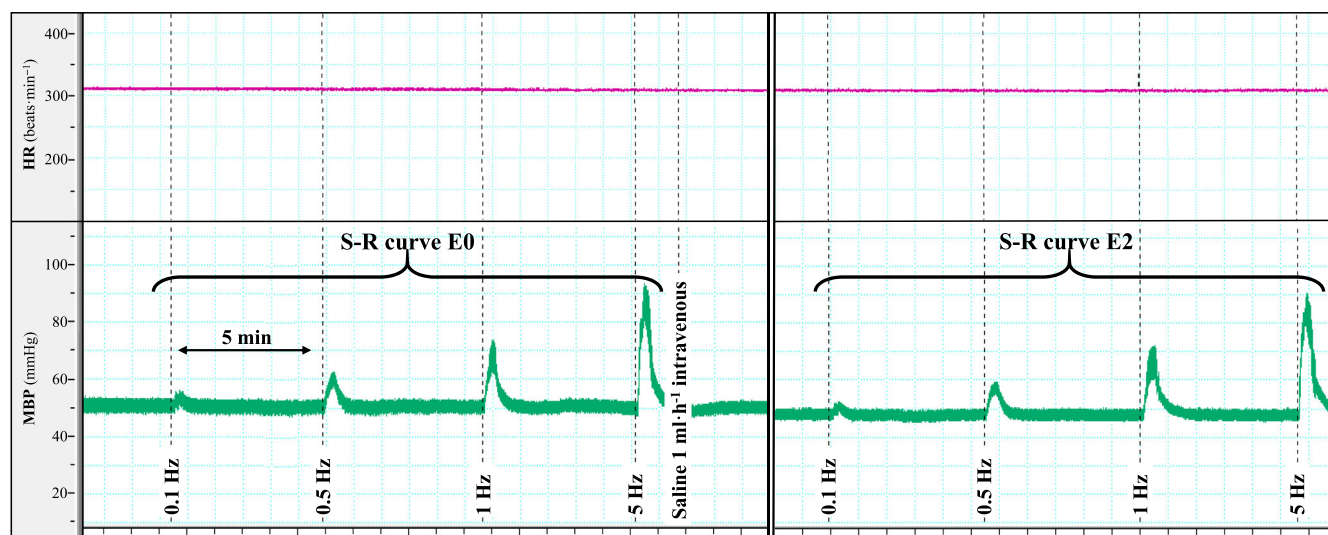


FIGURE 2 Original experimental tracing illustrating the vasopressor responses induced by electrical sympathetic stimulation before and during perfusion of saline ($1 \text{ ml} \cdot \text{h}^{-1}$) in female pithed rats. Heart rate (HR; $\text{beats} \cdot \text{min}^{-1}$) and mean blood pressure (MBP; mmHg) are shown in the figure. S-R curves prior (E0) and during i.v. perfusion of saline (E2) are represented. Note that heart rate did not change significantly throughout the experiment. The vasopressor responses returned to baseline levels immediately after electrical stimulation. S-R: (electrical) stimulus-response.

two sets that received an i.v. infusion of saline ($1 \text{ ml} \cdot \text{h}^{-1}$; $n = 5$ each) or 5-CT ($0.005 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $n = 5$ each). After 10 min of the corresponding infusion, E1 and E2 curves were obtained.

2.3.2 | Protocol ii- administration of exogenous noradrenaline

To determine, in female rats ($n = 20$), the pre- and/or post-junctional nature of the receptors involved, increasing doses of noradrenaline (0.01 , 0.05 , 0.1 and $0.5 \mu\text{g} \cdot \text{kg}^{-1}$; i.v. bolus) were administered to induce vasopressor responses (Figure 1). D-R curves to the action of noradrenaline were obtained before (E'0) and during (E'1 and E'2) the continuous infusion of saline ($1 \text{ ml} \cdot \text{h}^{-1}$; $n = 5$), L-694,247, α -methyl-5-HT or 1-PBG ($10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $n = 5$ each). The perfusions were started after E'0 was completed and were maintained during E'1 and E'2, which were constructed in the same conditions.

2.4 | Data and statistical analysis

All experimental protocols and data analysis were randomised and blinded. Data and statistical procedures complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). All results are expressed as mean $\pm$ SEM of five experiments ($n = 5$). The variations on MBP produced by electrical sympathetic stimulation or exogenous noradrenaline are represented as increases in mmHg from the baseline value. Statistical analyses were carried out using GraphPad Prism 8.0 (GraphPad, USA). Normal distribution was determined using the Saphiro-Wilk test and homogeneity of variances was assessed by Brown-Forsythe test. Differences between the means of two unpaired groups were analysed by one-tailed Student's test. Changes in basal MBP and HR before and (a) during the infusion of serotonergic agonists or (b) after i.v. administration of serotonergic antagonists was evaluated with one-way ANOVA with Dunnett's post hoc test. When a time course had two variables, such as treatment and frequencies of stimulation or treatment and doses of noradrenaline, differences between means of multiple unpaired groups were assessed using two-way ANOVA, followed by Dunnett's (compared with saline group) post hoc test. Post hoc tests were conducted only if F in ANOVA achieved $P < 0.05$. Statistical significance was accepted at $P < 0.05$. As the data obtained for E1 and E2 (or E'1 and E'2) curves were essentially identical, only E2 (or E'2) curves are shown in the figures. Because the electrical- and noradrenaline-induced increases in MBP in the presence of i.v. vehicles (saline, 0.01 M HCl and $\text{EtOH } 5\%$) were similar to those produced in the E0 curve, the statistical analysis was only performed versus saline.

2.5 | Materials

The compounds used in this study and their sources are listed as follows: pentobarbital sodium (Dolethal[®]; Vetoquinol; Madrid, Spain); heparin

sodium (Rovi; Madrid, Spain); atropine sulphate (Scharlab; Barcelona, Spain); 5-HT hydrochloride, 1-phenylbiguanide (1-PBG), d-tubocurarine hydrochloride, α -methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine monohydrochloride (BW723C86), noradrenaline bitartrate (Merck Life Science S.L.U.; Madrid, Spain); 5-carboxamidotryptamine maleate (5-CT), 8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrol[3,2-b]pyridin-5-one dihydrochloride (CP 93,129), 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine (L-694,247), 1-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-1,3-dihydro-3,3-dimethyl-2H-indol-2-one hydrochloride (LY310762), N-[(3R)-3-(dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide hydrochloride (LY344864), α -methyl-5-hydroxytryptamine maleate (α -methyl-5-HT), 6-chloro-2-(1-piperazinyl)pyrazine hydrochloride (MK212), ($\pm$)-4-amino-5-chloro-N-[1-[(3R,4S)-3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide (cisapride), N-[2-(dimethylamino)ethyl]-N-[[4'-[[2-(phenylethyl)amino]methyl][1,1'-biphenyl]-4-yl]methyl]cyclopentanepropanamide dihydrochloride (SB699551) and (2S)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS-19) (Tocris Bioscience; Bristol, UK).

2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Christopoulos, Davenport, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Amarosi, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Annett, et al., 2023; Alexander, Mathie, Peters, Veale, Striessnig, Kelly, Armstrong, Faccenda, Harding, Davies, Aldrich, et al., 2023).

3 | RESULTS

3.1 | Systemic haemodynamic variables

In anaesthetised pithed animals, basal MBP was lower in female than in male rats (48 ± 1 and $58 \pm 2 \text{ mmHg}$, respectively). Basal HR was similar in female and male animals (313 ± 4 and $306 \pm 16 \text{ beats} \cdot \text{min}^{-1}$, respectively). Neither MBP nor HR were altered after the i.v. bolus injections of atropine and d-tubocurarine. In female rats, these variables were not significantly altered by the i.v. perfusion of vehicles, 8-OH-DPAT, CP 93,129, L-694,247, LY344864, BW723C86, cisapride and AS-19, or i.v. bolus of LY310762 or SB699551 (Table S1). Only α -methyl-5-HT increased both MBP and HR at higher doses (Table 1). However, 5-CT (0.005 and $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) reduced MBP, and 5-HT, MK212, 1-PBG and the higher dose of 5-CT increased only the HR (Table 1). The vasodilator effect induced by $0.005 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ of 5-CT was reversed by pretreatment with LY310762 ($46 \pm 3 \text{ mmHg}$).

TABLE 1 Variations in baseline values of mean blood pressure (Δ MBP) and heart rate (Δ HR) immediately after the beginning of the i.v. infusion of 5-HT receptor agonists in female and male rats.

	5-HT receptor agonists	Dose ($\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	Δ MBP (mmHg)	Δ HR (beats $\cdot\text{min}^{-1}$)
Female	5-HT	5	-4.8 ± 1.2	$61.7 \pm 27.5^*$
		10	-9.2 ± 0.8	$62.1 \pm 16.8^*$
		40	-5.6 ± 3.7	$101.8 \pm 28.6^*$
	5-CT	0.001	-6.1 ± 2.6	19.8 ± 6.4
		0.005	$-13.1 \pm 2.1^*$	17.5 ± 7.7
		10	$-13.2 \pm 1.5^*$	$52.1 \pm 8.4^*$
	α -methyl-5-HT	1	0.2 ± 2.0	$131.8 \pm 14.7^*$
		5	$11.8 \pm 2.4^*$	$216.8 \pm 20.4^*$
		10	$58.1 \pm 4.4^*$	$151.4 \pm 11.5^*$
	MK212	10	4.9 ± 2.8	$56.5 \pm 15.4^*$
Male	1-PBG	1	1.3 ± 2.2	$124.3 \pm 26.2^*$
		5	1.7 ± 2.1	$125.0 \pm 12.0^*$
		10	3.1 ± 4.1	$78.8 \pm 16.9^*$
	5-HT	10	-9.2 ± 3.1	$72.0 \pm 14.4^*$

Note: All values are expressed as means $\pm$ SEM ($n = 5$). Data were analysed by one-way ANOVA followed by Dunnett's post hoc test.

* $P < 0.05$, significantly different from baseline.

3.2 | Effect of vehicles or 5-HT on the vasoconstrictions induced by electrical stimulation in male and female rats

Electrical stimulation of the sympathetic outflow from the spinal cord (0.1, 0.5, 1 and 5 Hz) in male and female rats resulted in frequency-dependent increments in MBP (2.2 ± 0.3 , 11.2 ± 1.8 , 24.7 ± 2.5 and 47.6 ± 3.9 , S-R curve E0 in female rats), with minor variations in HR (Figure 2). These increases in MBP were reproducible, since they were basically unaltered after i.v. saline (Figure 2), EtOH 5% or HCl 0.01 M (Table S2).

In male rats, continuous infusion of 5-HT ($10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) inhibited the sympathetic-induced vasopressor responses at all frequencies tested, except for 5 Hz (Table 2). Similarly, in female rats, 5-HT infusion (10 and $40 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) produced a significant dose-dependent inhibition of the sympathetic-provoked vasoconstrictions, more pronounced at lower stimulation frequencies (Table 2).

3.3 | Effects of 5-HT receptor agonists on the vasoconstrictions induced by electrical stimulation in female rats

Continuous infusion of 5-CT, a selective 5-HT_{1/5/7} receptor agonist, reduced the sympathetic-evoked vasopressor responses (Figures 3 and 4). Increasing doses of 5-CT (0.001, 0.005 and $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) decreased the vasopressor responses in a frequency- and dose-dependent way (Figure 4). In contrast, 5-HT₂ and 5-HT₃ receptor agonists, α -methyl-5-HT and 1-PBG ($10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ each), respectively, significantly increased the vasoconstrictions induced by electrical stimulation at all frequencies studied (Figure 3). Both

α -methyl-5-HT and 1-PBG enhancing effects were produced in a frequency- and dose-dependent manner (Figures S1 and S2). However, neither cisapride nor AS-19 (5-HT₄ and 5-HT₇ receptor agonists, respectively), at a dose of $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ each, modified the sympathetic-induced vasoconstrictions (Figure 3).

3.4 | Role of 5-HT₁ or 5-HT₂ receptor subtype agonists on sympathetic vasopressor responses in female rats

The inhibitory effect of 5-CT was mimicked by i.v. infusion of $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ of L-694,247, a selective 5-HT_{1D} receptor agonist (Figure 5); whereas the same dose of 8-OH-DPAT, CP 93,129 and LY344864 (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1F} receptor agonists, respectively) did not modify the vasopressor responses (Figure 5).

In relation to 5-HT₂ receptor subtype involvement in the enhancing effect of electrical-induced vasopressor responses, only $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ of α -methyl-5-HT, a non-selective 5-HT₂ receptor subtype agonist, significantly increased the vasopressor responses evoked by sympathetic stimulation (Figure 6); but neither BW723C86 (5-HT_{2B} agonist) nor MK212 (5-HT_{2C} agonist) at $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ each, reproduced the potentiating serotonergic effect (Figure 6).

3.5 | Influence of 5-HT receptor antagonists on the 5-CT sympathetic inhibition in female rats

The i.v. pretreatment with the selective 5-HT_{1D} receptor subtype antagonist, LY310762 ($1 \text{ mg}\cdot\text{kg}^{-1}$), or the selective 5-HT_{5A} antagonist, SB699551 ($1 \text{ mg}\cdot\text{kg}^{-1}$), did not modify per se the vasopressor

TABLE 2 Effect of saline and 5-HT on the increases in mean blood pressure (Δ MBP) induced by sympathetic electrical stimulation in male and female rats.

		Frequency of stimulation (Hz)			
		0.1	0.5	1	5
Male	Saline	2.4 $\pm$ 0.3	8.4 $\pm$ 0.8	18.5 $\pm$ 1.8	43.0 $\pm$ 3.8
	5-HT (10)	1.0 $\pm$ 0.1*	4.8 $\pm$ 0.4*	11.5 $\pm$ 1.2*	35.6 $\pm$ 3.5
Female	Saline	2.1 $\pm$ 0.1	9.8 $\pm$ 0.3	21.1 $\pm$ 0.7	46.3 $\pm$ 1.1
	5-HT (5)	1.8 $\pm$ 0.3	8.1 $\pm$ 1.6	16.4 $\pm$ 2.4	44.1 $\pm$ 2.7
	5-HT (10)	1.2 $\pm$ 0.2*	4.9 $\pm$ 1.4*	10.5 $\pm$ 2.2*	27.8 $\pm$ 2.7*
	5-HT (40)	0.5 $\pm$ 0.1*	2.6 $\pm$ 0.4*	4.1 $\pm$ 0.9*	11.3 $\pm$ 1.6*

Δ MBP (mmHg)

Note: All values are expressed as mean $\pm$ SEM (n = 5). Data were analysed by two-way ANOVA followed by Dunnett's post hoc test.

*P < 0.05, significantly different from saline.

FIGURE 3 Effect of 5-HT receptor type agonists on the sympathetic vasopressor responses in female pithed rats. Increases in mean blood pressure (Δ MBP; mmHg) induced by electrical sympathetic stimulation during i.v. continuous perfusions (n = 5 each) of saline (control; 1 ml·h⁻¹) or 10 μ g·kg⁻¹·min⁻¹ of the following 5-HT receptor type agonists: 5-CT (5-HT_{1/5/7}), α -methyl-5-HT (α -m-5-HT; 5-HT₂), 1-PBG (5-HT₃), cisapride (5-HT₄) or AS-19 (5-HT₇) in female pithed rats. Data shown are individual values with means $\pm$ SEM. *P < 0.05, significantly different from saline; two-way ANOVA followed by Dunnett's post hoc test.

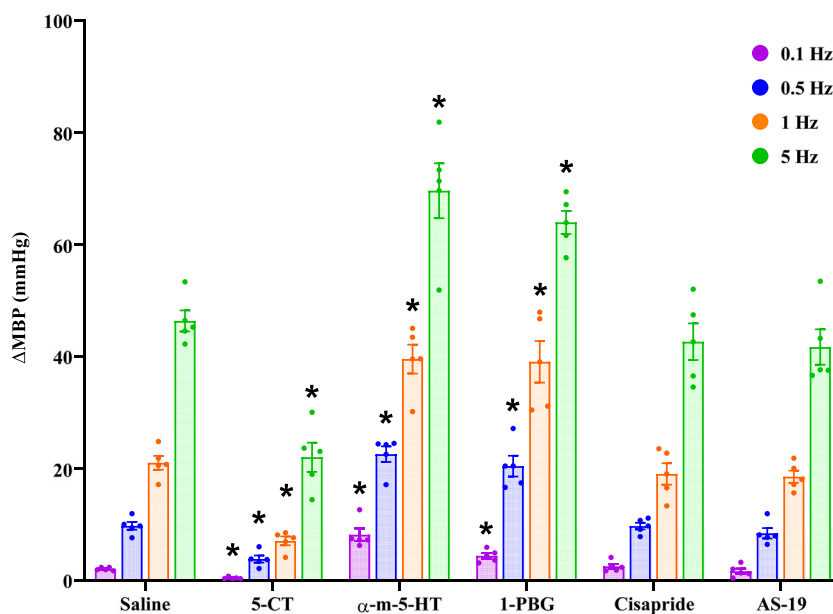
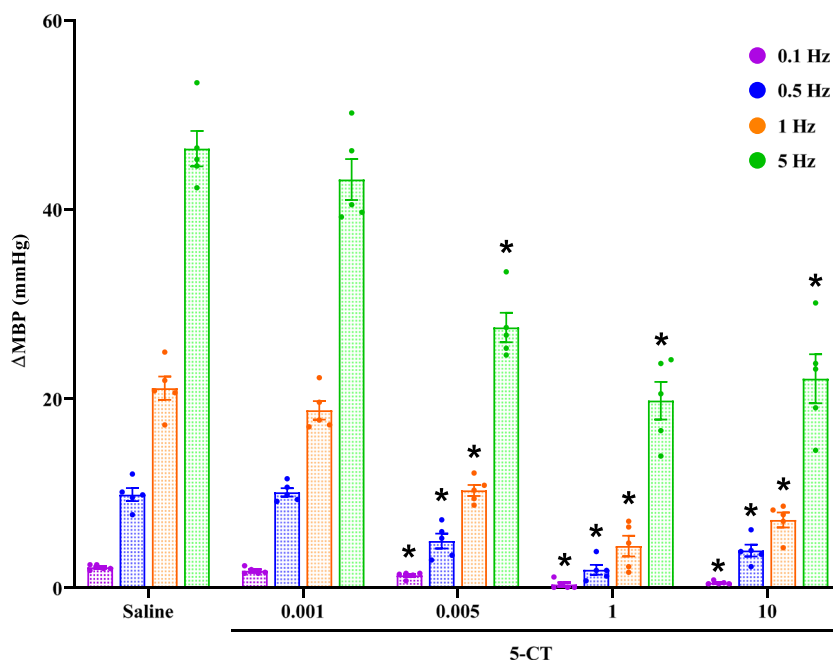


FIGURE 4 Effect of 5-CT on the sympathetic vasopressor responses in female pithed rats. Effect of the i.v. perfusion of saline (control; 1 ml·h⁻¹) or 5-CT (0.001, 0.005, 1 and 10 μ g·kg⁻¹·min⁻¹; n = 5 for each dose) on the increases in mean blood pressure (Δ MBP; mmHg) induced by electrical sympathetic stimulation in female pithed rats. Data shown are individual values with means $\pm$ SEM. *P < 0.05, significantly different from saline; two-way ANOVA followed by Dunnett's post hoc test.



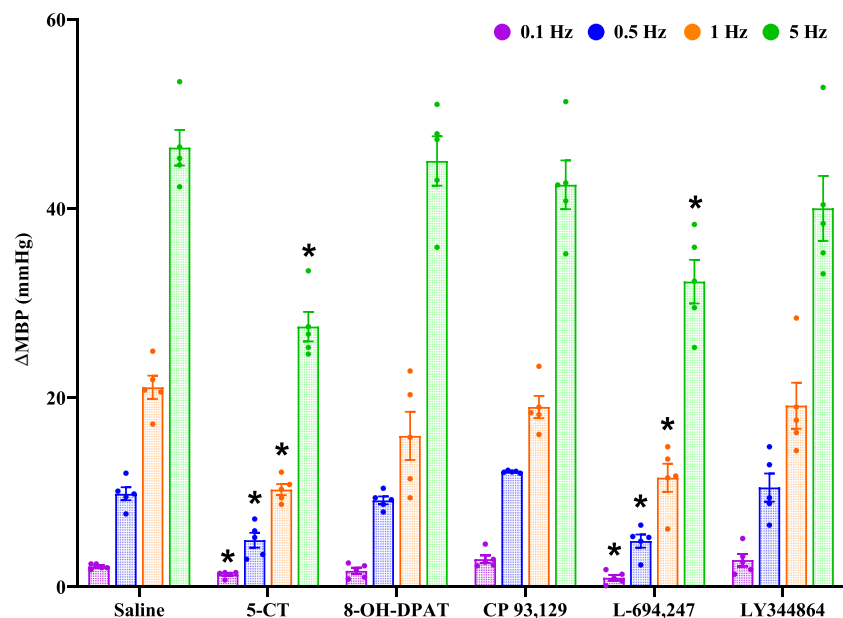


FIGURE 5 Effect of 5-HT₁ receptor (sub)type agonists on the sympathetic vasopressor responses in female pithed rats. Effect of i.v. continuous perfusion ($n = 5$ each) of saline (control; $1 \text{ ml} \cdot \text{h}^{-1}$), $0.005 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ of 5-CT and $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ of 8-OH-DPAT, CP 93,129, L-694,247 or LY344864 on the increases in mean blood pressure (ΔMBP ; mmHg) induced by electrical sympathetic stimulation in female pithed rats. Data shown are individual values with means $\pm$ SEM. * $P < 0.05$, significantly different from saline; two-way ANOVA followed by Dunnett's post hoc test.

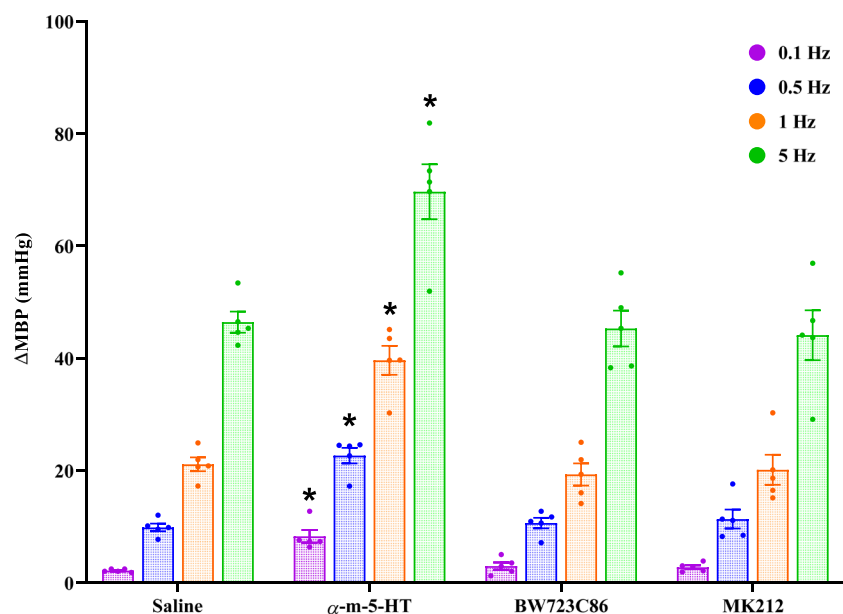


FIGURE 6 Effect of 5-HT₂ receptor subtype agonists on the sympathetic vasopressor responses in female pithed rats. Effect of i.v. continuous perfusion of saline (control; $1 \text{ ml} \cdot \text{h}^{-1}$), α -m-5-HT (α -methyl-5-HT), BW723C86 or MK212 ($10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ each; $n = 5$ for each treatment) on the increases in mean blood pressure (ΔMBP ; mmHg) induced by electrical sympathetic stimulation in female pithed rats. Data shown are individual values with means $\pm$ SEM. * $P < 0.05$, significantly different from saline; two-way ANOVA followed by Dunnett's post hoc test.

responses induced by electrical sympathetic stimulation in female rats (Table S2). The inhibitory effect of 5-CT ($0.005 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was completely blocked after i.v. administration of LY310762 but was not modified by pretreatment with SB699551 (Figure 7).

3.6 | Effects of L-694,247, α -methyl-5-HT or 1-PBG on exogenous noradrenaline-induced vasoconstriction in female rats

The i.v. administration of noradrenaline (0.01 , 0.05 , 0.1 and $0.5 \mu\text{g} \cdot \text{kg}^{-1}$) caused dose-dependent increments in MBP (16.2 ± 3.0 , 21.3 ± 2.3 , 30.1 ± 2.8 and 50.7 ± 4.1 , D-R curve E'0 in female rats). The vasopressor responses to noradrenaline remained stable after the

perfusion of saline (Table 3). Continuous infusion of L-694,247 or α -methyl-5-HT ($10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ each) did not modify the vasoconstrictor responses to exogenous noradrenaline (Table 3), whereas the i.v. perfusion of 1-PBG ($10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) augmented the noradrenaline-induced vasopressor responses compared to saline (Table 3).

4 | DISCUSSION

Our study shows, for the first time in female rats, that the peripheral 5-HT receptors modulate the vascular sympathetic innervation. The prejunctional 5-HT_{1D} receptors were the main mediators of this sympatholytic effect, while prejunctional 5-HT_{2A} and pre and/or

FIGURE 7 Effect of 5-HT_{1D} (LY310762) or 5-HT_{5A} (SB699551) receptor subtypes blockade on 5-CT inhibitory action on electrical-induced vasoconstrictions in female pithed rats. Effect of i.v. bolus injections of LY310762 or SB699551 (1 mg·kg⁻¹ each) in the inhibitory effect of 5-CT (0.005 µg·kg⁻¹·min⁻¹) on the increases in mean blood pressure (ΔMBP) induced by electrical sympathetic stimulation in female pithed rats. Data shown are individual values with means ± SEM. * *P* < 0.05, significantly different from saline; # *P* < 0.05, significantly different from 5-CT; two-way ANOVA followed by Dunnett's post hoc test.

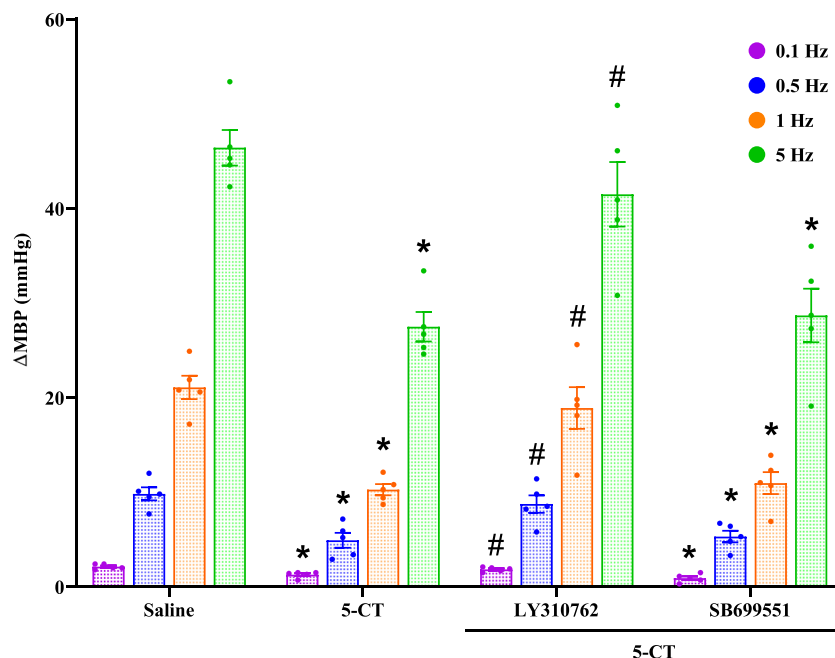


TABLE 3 Effects of 5-HT_{1D}, 5-HT₂ or 5-HT₃ receptor agonists on the vasoconstrictions induced by exogenous noradrenaline i.v. administration in female animals.

i.v. infusions (µg·kg ⁻¹ ·min ⁻¹)	Noradrenaline (µg·kg ⁻¹ ; i.v. bolus)			
	0.01	0.05	0.1	0.5
Saline	15.3 ± 3.1	24.7 ± 3.3	35.5 ± 4.8	61.4 ± 7.3
L-694,247 (10)	15.4 ± 2.5	26.0 ± 4.4	40.6 ± 2.5	67.5 ± 2.5
α-methyl-5-HT (10)	19.0 ± 1.6	32.3 ± 3.0	44.0 ± 5.4	62.8 ± 6.4
1-PBG (10)	25.8 ± 5.8*	38.1 ± 8.1*	54.7 ± 9.0*	81.1 ± 10.3*

ΔMBP (mmHg)

Note: All values are expressed as mean ± SEM (n = 5). Data were analysed by two-way ANOVA followed by Dunnett's post hoc test.

**P* < 0.05, significantly different from saline.

postjunctional 5-HT₃ receptors were involved in the sympatho-excitatory effect in female pithed rats. This data support previous scientific evidence of sex differential cardiovascular regulation in animal models (Gohar et al., 2016; Mahmoodzadeh et al., 2012) and humans (Farrell et al., 2020; Gerdtts et al., 2022). In fact, our earlier results had shown that, in male rats, 5-HT_{1D/1A} receptor subtypes mediated the vascular sympatho-inhibition, whereas only 5-HT₃ receptor activation was responsible for the potentiation of noradrenergic vasoconstrictions (García-Pedraza et al., 2013, 2021; Morán et al., 1994, 1998). The induction of cardiovascular pathologies in males, such as diabetes, also modified the receptors involved in serotonergic modulation of vasopressor outflow (Fernández-González et al., 2022; García et al., 2005; Morán et al., 2010).

Regulation of the BP is influenced by biological effects of sex chromosomes, hormones and reproductive events (Bartz et al., 2020). Thus, over the fertile ages, BP in women is lower than in men, mainly due to female reproductive hormones, which down-regulate, among others, autonomic control of vascular tone (Charkoudian et al., 2017; Colafella & Denton, 2018). In accordance with this fact, in our experimental model of 14–16 week old female (cycling period)

anaesthetised pithed (with no central influence on BP) rats, MBP was significantly lessened (around 10 mmHg) compared with male rats, whereas HR values were similar in both sexes. In females, the i.v. infusion of the different 5-HT doses tested did not modify the MBP while significantly increasing HR. These data are in contrast with other results previously obtained in naïve male rats where 5-HT did not modify any of the parameters or, in a chronic fluoxetine-treatment experimental model, where higher doses of 5-HT produced both increases in MBP and HR in normoglycaemic and diabetic male rats (García-Pedraza et al., 2021, 2022). As already described by our group in other experimental male models, the i.v. perfusion of a selective 5-HT₂ receptor agonist (α-methyl-5-HT) increased both parameters (MBP and HR), whereas higher doses of 5-CT, 5-HT_{1/5/7} receptor agonist, reduced MBP (García-Pedraza et al., 2021). The 5-CT (0.005 µg·kg⁻¹·min⁻¹) vasodilator effect was abolished by pretreatment with LY310762 in female rats, which demonstrates that 5-HT_{1D} is involved in the 5-CT vasodilator effect as occurred in normoglycaemic sarpogrelate-treated male rats (García-Pedraza et al., 2013). The selective 5-HT_{2C} (MK212) and 5-HT₃ (1-PBG) receptor agonists, as well as the higher dose of 5-CT (10 µg·kg⁻¹·min⁻¹) produced

tachycardia (see results section), already described for 1-PBG in males (García-Pedraza et al., 2021).

CVD are the leading cause of death in the world for men and women (Vaduganathan et al., 2022). However, CVD seem to affect females in a different way (Murphy & Steenbergen, 2014). In this context, the contribution of the SNS to vascular homeostasis has been strongly proposed as being sex-dependent (Farrell et al., 2020). Peripheral vasoconstrictor responses to sympathetic activity are less marked in young women than in young men, which may contribute to the increased prevalence of orthostatic intolerance in females (Baker et al., 2016). Although some research have described that, in female rats, oestrogens can act at multiple sites centrally to influence sympathetic drive (Hay et al., 2014), in our experimental model where the CNS is effectively silenced, we have shown that vasoconstrictor responses induced by sympathetic electrical stimulation are similar in males and females (see results section; Morán et al., 1994, 1998; García-Pedraza et al., 2013, 2021) and, in both cases, are frequency-dependent. The increases in MBP induced by administration of exogenous noradrenaline are also similar to those previously obtained in male rats (García-Pedraza et al., 2013; Morán et al., 1998). The induction of experimental diabetes in male rats provoked higher increases in MBP than in normoglycaemic rats (Fernández-González et al., 2022; García et al., 2005; Morán et al., 2010), probably due to a dysfunction of the autonomic nervous system (Bjerre-Christensen et al., 2021; Jia & Sowers, 2021; Vinik et al., 2003), but this fact has not yet been studied in female rats. Anyway, other authors have demonstrated in a streptozotocin-induced diabetic model of female pithed rats that vasopressor responses to adrenergic stimulation are diminished after the chronic administration of oestrogens, specifically 17 β -oestradiol (Acosta-Cota et al., 2014).

Sexual variance in the serotonergic system has also been extensively studied, mainly at the CNS level, because some pathologies related to 5-HT alterations, such as mood and/or eating disorders, affect males and females to different extents (Dalla et al., 2010; Spies et al., 2020), or women and men (Gougoulaki et al., 2021). In this context, oestrogens have direct effects on 5-HT biosynthesis and its degradation, but also they also affect the expression of 5-HT receptor types (Spies et al., 2020). However, in tryptophan hydroxylase-2 knockout animals, the loss of central inhibitory serotonergic drive leads to hypertension in both male and female rats, due to augmented sympathetic vascular tone (Spinieli et al., 2022).

Our results showed that the sympatholytic effect mediated by 5-HT receptors, was greater in females than in male rats at the same doses. Consistent with these results, some authors have already shown that, although in platelet-rich fractions, 5-HT concentrations were lower in female compared with male rats, the free 5-HT concentrations tended to be higher in female than male rats (Linder et al., 2011), which may account for this greater effect. In fact, at peripheral level, it is already known that oestrogens up-regulate peripheral 5-HT activity and concentration through increases in tryptophan hydroxylase as well as 5-HT transporter expression (White et al., 2011), which may contribute to this sex-difference in serotonergic sympatho-inhibition.

The 5-HT_{1/5/7} and 5-HT_{1D} receptor type/subtype agonists (5-CT and L-694,247, respectively) reproduced the inhibitory action of 5-HT on the vasoconstrictions induced by sympathetic stimulation in females. The 5-HT₇, 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1F} receptors were not involved in this sympatholytic action, as their specific agonists (AS-19, 8-OH-DPAT, CP 93,129 and LY344864, respectively) did not modify electrical-produced vasoconstrictions. The 5-HT_{5A} receptor subtype was ruled out of any sympatholytic action since 5-CT maintained its inhibitory effect in the presence of SB699551, a 5-HT_{5A} receptor antagonist. The participation of 5-HT_{1D} receptors in female rats was confirmed using the antagonist LY310762, which totally reversed 5-CT-produced sympathetic inhibition. Thus, 5-HT_{1D} is the sole 5-HT receptor subtype that mediates inhibition of the vascular noradrenergic responses in female rats. These results bring to light the sexual difference in the serotonergic inhibition of vascular sympathetic innervation because, in male rats, the 5-HT_{1D}, but also 5-HT_{1A}, receptor subtypes were unmasked as sympatho-inhibitors (García-Pedraza et al., 2013; Morán et al., 1998), or even the 5-HT₇ receptor appear to be involved in other experimental models (Fernández-González et al., 2022; García-Pedraza et al., 2013). In male Wistar rats (García-Pedraza et al., 2013; Morán et al., 1994), this inhibitory effect is prejunctional in nature, as L-694,247 was not able to modify the vasopressor responses induced by exogenous noradrenaline.

Although 5-HT acts as a sympatholytic agent, the activation of 5-HT₂ and 5-HT₃ receptors using selective agonists, α -methyl-5-HT and 1-PBG respectively, enhanced the electrical-generated vasoconstrictions in females. We discounted the involvement of 5-HT_{2B} and 5-HT_{2C} receptor subtypes on the enhancing effect of electrical-induced vasopressor responses, because neither BW723C86 (5-HT_{2B} agonist) nor MK212 (5-HT_{2C} agonist) reproduced the potentiating effect of 5-HT. Consequently, the increases on electrical-produced vasoconstrictor responses produced by α -methyl-5-HT were due to 5-HT_{2A} activation in female rats. In male rats, we demonstrated that the 5-HT₃ receptor activation potentiated the noradrenergic-induced vasoconstrictions, whereas the 5-HT₂ receptor, in males, was not involved (García-Pedraza et al., 2021; Morán et al., 1994, 1998). However, the increase in 5-HT concentrations by a selective inhibitor of SERT, fluoxetine, in males unmasked 5-HT_{2A} receptor as vascular sympatho-enhancer (García-Pedraza et al., 2021, 2022), as we currently described in naïve females.

To clarify the nature of the 5-HT receptors involved in the increased noradrenergic vascular responses, we determined whether α -methyl-5-HT or 1-PBG modified the vasoconstrictor responses to exogenous noradrenaline, and we concluded that the sympatho-excitatory 5-HT_{2A} receptors were prejunctional in nature, whereas 5-HT₃ receptors were pre and/or postjunctional in nature, as 1-PBG also enhanced the vasoconstrictor responses induced by exogenous noradrenaline. The latter differs from results in male rats where 5-HT₃ receptors implicated in serotonergic sympatho-stimulation were prejunctional (Morán et al., 1994, 1998).

Our current results in female rats demonstrate the complexity of the serotonergic system, coming across with a newly described

variant at peripheral level, as serotonergic modulation of vascular noradrenergic responses clearly depended on the sex of the animal. Sexual characteristics modify not only the effect (inhibition or potentiation of vascular noradrenergic input) but also the receptors involved and their nature.

Our present results adds to the evidence that has emerged for the last decades indicating that cardiovascular homeostasis strongly depends on the sex (Farrell et al., 2020; Gerdtz et al., 2022; Gohar et al., 2016; Mahmoodzadeh et al., 2012). Thus, our research reinforces the need of preclinical pharmacological studies in both female and male animal models, emphasising the knowledge of cardiovascular physiopathology and how treatments for CVD must be implemented in patients depending on sex.

Our study has some clear constraints. For instance, we utilised the invasive model of pithed rats, thus excluding any effects of the CNS on the cardiovascular system and we measured vasopressor responses provoked by electrically-induced noradrenaline release in the systemic vasculature but we did not directly assess vascular sympathetic nerve activity. Furthermore, our female experimental model was naïve (with no cardiovascular pathology). Nevertheless we were able to show, for the first time, that 5-HT receptors modulated sympathetic vascular tone in female rats differently from its effects in males, by either increasing or reducing electrically-evoked vasoconstrictions (García-Pedraza et al., 2013, 2021; Morán et al., 1994, 1998). Thus, although further analysis in females with cardiovascular pathologies should be performed, our results demonstrate that differential activation of 5-HT receptors up-regulates or down-regulates sympathetic activity at vascular levels and, thus, modulation of the 5-HT receptor system may become a novel strategy to treat cardiovascular disturbances prevalent in females, such as arterial hypertension or diabetes, which are clearly linked to alterations in the SNS (Baker et al., 2016; Briant et al., 2016; Farrell et al., 2020; Hay et al., 2014).

In conclusion, our results show clearly that, in cycling-age female rats, 5-HT maintains its vascular sympatho-inhibitory effect. The pre-junctional 5-HT_{1D} receptors are the sole receptor type responsible for the vascular sympathetic inhibition, whereas prejunctional 5-HT_{2A} and pre and/or postjunctional 5-HT₃ receptor activation increases sympathetic-induced vasoconstrictions. These results provide evidence that the modulation, by 5-HT receptor activity, of vascular sympathetic outflow differs between sexes, and are the first steps in the investigation of new, sex-dependent, pharmacological strategies in the treatment of CVD.

AUTHOR CONTRIBUTIONS

A. C. Terol-Úbeda: Data curation, methodology, writing—original draft and writing—review and editing. A. Morán: Data curation, formal analysis, methodology, writing—original draft and writing—review and editing. C. A. Roldán-Hernández: Methodology. J. A. García-Pedraza: Conceptualization, data curation, formal analysis, funding acquisition, methodology, supervision, writing—original draft and writing—review and editing. J. F. Fernández-González: Methodology. M. L. Martín: Investigation and methodology. M. García-Domingo: Conceptualization,

formal analysis, funding acquisition, project administration, supervision, writing—original draft and Writing—review and editing. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design and Analysis](#) and [Animal Experimentation](#) and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

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Artículo 2

Angiotensin II and EDH pathways underlie the vascular sympatho-modulation by 5-HT in female rats

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Resumen

El objetivo de este trabajo fue estudiar la participación de vías indirectas en la inhibición serotoninérgica mediada por los receptores 5-HT_{1D} y en la potenciación mediada por los receptores 5-HT_{2A/3} de la neurotransmisión noradrenérgica vascular en ratas hembra. Para ello, se administró un bolo intravenoso de distintos inhibidores/bloqueantes de mediadores vasoactivos (NO, canales de K⁺, prostanoides, Ang II o endotelina) previo a la perfusión de los agonistas L-694,247 (5-HT_{1D}), TCB-2 (5-HT_{2A}) o 1-PBG (5-HT₃) en ratas hembra *pithed*. En estas condiciones, los nervios simpáticos vasculares fueron estimulados eléctricamente para obtener las respuestas vasopresoras. La simpato-inhibición vascular inducida por L-694,247 fue bloqueada por un antagonista no selectivo de los canales de K⁺ (tetraetilamonio). El efecto simpato-potenciador de 1-PBG no se modificó en presencia de los bloqueantes/inhibidores de los mediadores estudiados, mientras que la simpato-potenciación producida por TCB-2 fue bloqueada únicamente por losartán (antagonista de los receptores AT₁). Además, los niveles de Ang II aumentaron tras la perfusión de TCB-2 en ratas hembra. Estos resultados indican que la vía de EDH está implicada en la simpato-inhibición inducida por los receptores 5-HT_{1D}, mientras que el efecto simpato-potenciador

mediado por los receptores 5-HT_{2A} se asocia con la Ang II. Por el contrario, la simpato-potenciación mediada por los receptores 5-HT₃ no involucra ninguna vía indirecta.



Article

Angiotensin II and EDH Pathways Underlie the Vascular Sympatho-Modulation by 5-HT in Female Rats

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Abstract

The vascular 5-HT sympatho-modulation may involve inhibitory or potentiating pathways: nitric oxide (NO), endothelium-dependent hyperpolarization (EDH)-K⁺ channels, prostanoids, angiotensin II (Ang-II), or endothelin. Compared to males, female rats show differences in the serotonergic sympatho-regulation; therefore, we aimed to study the involvement of indirect pathways via 5-HT_{1D}-mediated inhibition and 5-HT_{2A/3}-mediated potentiation of vascular noradrenergic neurotransmission in females. An i.v. bolus of different inhibitors/blockers of modulators/mediators (NO, K⁺ channels, prostanoids, Ang-II, or endothelin) was administered prior to the infusion of the agonists, L-694,247 (5-HT_{1D}), TCB-2 (5-HT_{2A}), or 1-PBG (5-HT₃), in female pithed rats. In these conditions, the vascular sympathetic outflow was electrically stimulated to assess the vasopressor responses. The L-694,247 vascular sympatho-inhibition was abolished by a non-selective K⁺ channel blocker, tetraethylammonium. The 1-PBG sympatho-excitatory vascular effect was not modified by any of the inhibitors tested, whereas TCB-2 sympatho-potentiation was blocked solely by losartan (Ang-II type 1 receptor antagonist). Moreover, Ang-II levels were increased after TCB-2 infusion in females. The EDH pathway mediates the 5-HT_{1D}-induced sympatho-inhibition, while the 5-HT_{2A}-evoked sympatho-excitatory effect is associated with Ang-II. In contrast, the 5-HT₃ sympatho-potentiation does not involve any indirect pathway. These findings advance current understanding of the complex interactions between 5-HT and vascular homeostasis in female rats.

Keywords: 5-HT_{1D} and 5-HT_{2A} receptors; angiotensin II; female rats; K⁺ channels; sympatho-modulation; vasopressor responses



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1. Introduction

The serotonergic system is well known as a cardiovascular modulator, acting not only directly on blood vessels [1–4] but also indirectly, by regulating other systems, such as the sympathetic system, the cholinergic system, or the non-adrenergic-non-cholinergic system in male rats [5–10].

Recent evidence suggests that sex differences in the serotonergic axis may contribute to different 5-hydroxytryptamine (5-HT, serotonin) pathophysiological regulation at the central and peripheral level [11–13], possibly due to different rates of serotonin synthesis [14] or 5-HT receptor expression [15,16]. Although 5-HT within the central nervous system (CNS) helps prevent arterial blood pressure increases in both sexes, this effect is

more pronounced in males than in females, possibly due to ovarian hormones [17]. At the peripheral level, our group has recently shown that 5-HT contribution to cardiovascular homeostasis is also influenced by sex. In female rats, we have demonstrated that 5-HT_{1D} activation exerts a vascular sympatho-inhibitory action, as it does in males [6,7,18]; in contrast, while in male rats the sympatho-enhancing effect at the vascular level is mediated exclusively by 5-HT₃ receptors [7,19,20], in females, the 5-HT_{2A} receptor also contributes to the increase in sympathetic-induced vasoconstrictions [18].

Cardiovascular disease (CVD) is the leading cause of mortality and premature death in women worldwide. Traditional cardiovascular risk factors manifest differently in women compared to men, and these differences have implications for management and clinical outcomes [21]. The mechanisms responsible for these sex differences are likely to be multifactorial, including clinical (e.g., blood vessels size), social (e.g., occupational hazards or habits), and biological aspects (which can be approached in animal models), such as estrogen or endothelial function/dysfunction.

The female sex hormone regulates vascular physiological features and function by modulating ion fluxes on smooth muscle cells and regulating endothelial-dependent vasodilator production and activity [22,23]. In fact, a previous report showed that stimulation of endothelial cells of female arteries results in significant endothelium-dependent hyperpolarization (EDH), which regulates blood pressure and may contribute to the lower incidence of CVD in premenopausal women [24]. In animal models, specifically in stroke-prone spontaneously hypertensive rats, female vessels are able to relax significantly more than male's, probably due to higher expression or preserved function of ATP-sensitive potassium (K_{ATP}) channels in female vessels [25].

The contribution of vasoconstrictor agents, such as endothelin or angiotensin II (Ang-II), to blood pressure regulation also seems to be sex-dependent. In this line, there are controversial results, as several authors reported that female rats had a greater blood pressure-lowering response to angiotensin-converting enzyme inhibition and a greater response to chronic Ang-II infusion than male rats [26,27], while others indicated that chronic Ang-II increases blood pressure to a higher extent in male than in female mice [28].

In male rats, our group has already shown in different experimental models that direct 5-HT effect on renal vasculature is linked to the Ang-II or the cyclooxygenase (COX) pathway [2,3] while the chronic 5-HT₂ receptor blockade induces a vasodilator effect mediated by 5-HT_{1D}, 5-HT_{1B}, and 5-HT₇ receptors, involving COX-derived prostacyclin, nitric oxide (NO) synthesis/release, and K_{ATP} channels, respectively [4]. Moreover, in males, serotonergic modulation of sympathetic outflow at the vascular level is also regulated by different vasoactive agents depending on the experimental model and the 5-HT receptor subtype involved such as NO, COX, or EDH [29–31]. However, to date, no evidence exists on the possible contribution of vasoactive agents (either vasorelaxants or vasoconstrictors) to the serotonergic modulation of vascular sympathetic outflow in females. Against this background, our hypothesis was that in female rats, vasoactive agents involved in serotonergic sympatho-modulation differ from males. Thus, the objective of this work was to determine the possible mechanisms involved in the sympatho-modulatory effect of the serotonergic system at the vascular level in female rats, evaluating different indirect vasoactive pathways (NO, COX, EDH pathways via K⁺ channels, endothelin, and Ang-II) that may be implicated in the vascular 5-HT_{1D}-induced sympatho-inhibition and 5-HT₃- and 5-HT_{2A}-provoked sympatho-potentialiation in females.

2. Results

2.1. Systemic Hemodynamic Parameters

The resting mean blood pressure (MBP) and heart rate (HR) in female pithed rats were 58 ± 1 mm Hg and 307 ± 4 beats/min (bpm), respectively. These values were not significantly altered following i.v. bolus administration of the vehicles (saline, Figure 1; or mixture of 33% polyethylene glycol, 33% ethanol, and 34% NaOH 0.2 M (PEN)), indomethacin or sulfoxazole (Table 1), as previously reported in males [4,5,31]. Losartan induced a significant transient decrease in MBP (-12.4 ± 1.2 mm Hg) which returned to baseline within 10 min. In contrast, i.v. bolus administration of N(ω)-nitro-L-arginine methyl ester (L-NAME) resulted in a sustained increase in MBP, while tetraethylammonium (TEA) reduced HR (Table 1).

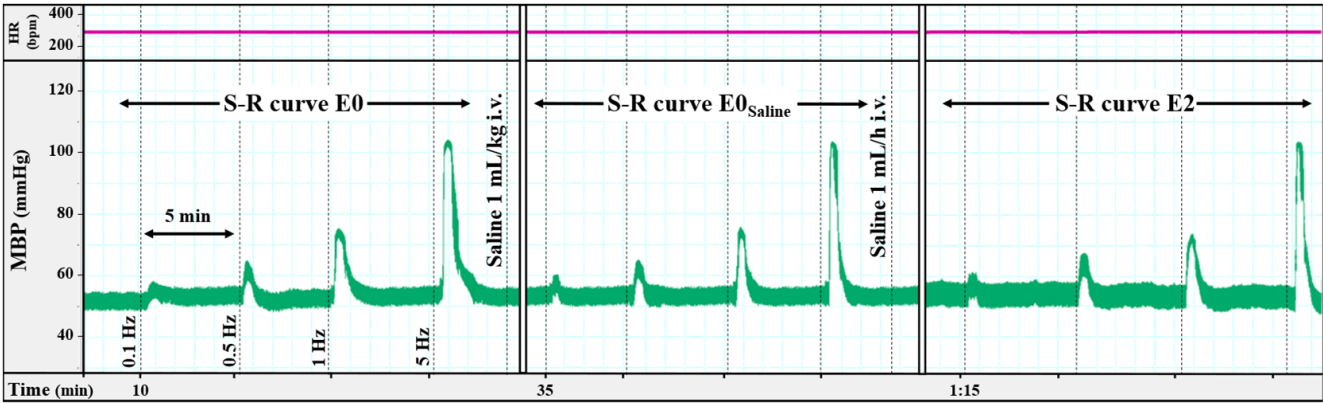


Figure 1. Original experimental tracing showing the vasopressor responses induced by electrical sympathetic stimulation. Electrically induced vasopressor responses and the effect of saline (1 mL/kg i.v. bolus followed by 1 mL/h i.v. infusion) in female pithed rats. Heart rate (HR; bpm) and mean blood pressure (MBP; mm Hg) are shown in the figure. S-R curves before saline administration (E0), after i.v. bolus of saline (E0_{saline}) and during i.v. saline perfusion (E2) are represented. Notably, heart rate did not change significantly throughout the experiment. The vasopressor responses returned to baseline levels immediately after electrical stimulation. bpm: beats per minute; S-R: (electrical) stimulus-response.

Table 1. Increases in baseline values of mean blood pressure (Δ MBP) and heart rate (Δ HR) after 10 min i.v. bolus of antagonists (or 30 min after i.v. bolus of L-NAME) and i.v. infusion of 5-HT receptor agonists in female rats.

		Drug	Dose (mg/kg)	Δ MBP (mm Hg)	Δ HR (bpm)
i.v. bolus Antagonists		Indomethacin	2	0.7 ± 1.3	7.5 ± 9.9
		L-NAME	10	$42.8 \pm 12 *$	0.3 ± 10.1
		TEA	16.5	-1.8 ± 1.2	$-44.5 \pm 6.7 *$
		Sulfoxazole	0.5	-3.6 ± 1.0	17.9 ± 13.3
		Losartan	1	2.0 ± 1.1	-5.7 ± 7.0
		Drug	Dose (μ g/kg/min)	Δ MBP (mm Hg)	Δ HR (bpm)
i.v. perfusion 5-HT receptor agonists		L-694,247	10	-1.0 ± 1.9	16.0 ± 7.9
		TCB-2	1	$30.2 \pm 7.1 *$	$59.0 \pm 11.7 *$
		1-PBG	10	6.9 ± 1.3	$90.0 \pm 16.9 *$

All values are expressed as mean $\pm$ SEM. Data were analyzed by *t*-test with Welch correction. * *p* < 0.05 vs. baseline; bpm: beats per minute. 1-PBG: 1-phenylbiguanide; L-NAME: N(ω)-nitro-L-arginine methyl ester; TEA: tetraethylammonium.

Intravenous infusion of saline (Figure 1) or L-694,247 (Table 1) did not affect baseline hemodynamic parameters. However, TCB-2 increased both MBP and HR, whereas 1-phenylbiguanide (1-PBG) selectively increased HR without altering MBP (Table 1). None of the antagonists modified the baseline hemodynamic values of the serotonergic agonists.

2.2. Effect of Saline or 5-HT Receptor Agonists: L-694,247, TCB-2, and 1-PBG on the Vasopressor Responses Induced by Electrical Stimulation in Female Rats

Electrical stimulation of vascular sympathetic nerves induced frequency-dependent increases in MBP (2.2 ± 0.3 , 10.5 ± 0.5 , 24.8 ± 2.3 , and 54.1 ± 3.8 mm Hg for 0.1, 0.5, 1, and 5 Hz, respectively; control stimulation–response curve (S-R curve E0)). These vasopressor responses were not altered after i.v. infusion of saline (2.2 ± 0.2 , 10.2 ± 0.3 , 22.7 ± 0.5 , and 52.4 ± 1.0 , respectively; Figures 2–4).

Continuous infusion of the selective 5-HT_{1D} agonist, L-694,247 (10 µg/kg/min), inhibited the sympathetic-induced vasopressor responses (Figure 2) [18]. In contrast, i.v. perfusion of the 5-HT_{2A} and 5-HT₃ receptor agonists, TCB-2 (1 µg/kg/min) and 1-PBG (10 µg/kg/min), respectively, potentiated the electrically induced vasoconstrictions at all frequencies tested (Figures 3 and 4).

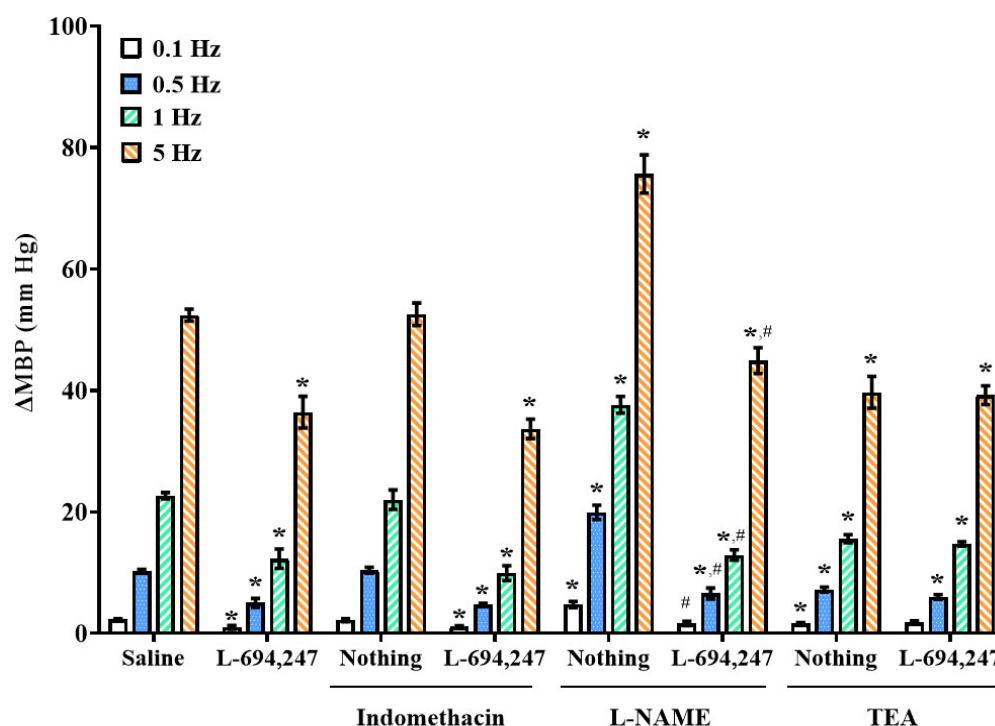


Figure 2. Effect of the i.v. administration of different inhibitors/blockers of vasoactive mediators on the vascular sympatholytic effect induced by 5-HT_{1D} activation. Increases in mean blood pressure (ΔMBP; mm Hg) induced by electrical sympathetic stimulation during i.v. perfusion of saline (control; 1 mL/h) and i.v. perfusion of L-694,247 (10 µg/kg/min) in the absence or presence of i.v. bolus of indomethacin (2 mg/kg), TEA (16.5 mg/kg), or L-NAME (10 mg/kg) in female rats. Data were analyzed by two-way ANOVA followed by Dunnett's post hoc test. All values are expressed as mean ± SEM. * $p < 0.05$ vs. saline. # $p < 0.05$ vs. L-694,247 alone. L-NAME: N(ω)-nitro-L-arginine methyl ester; TEA: tetraethylammonium.

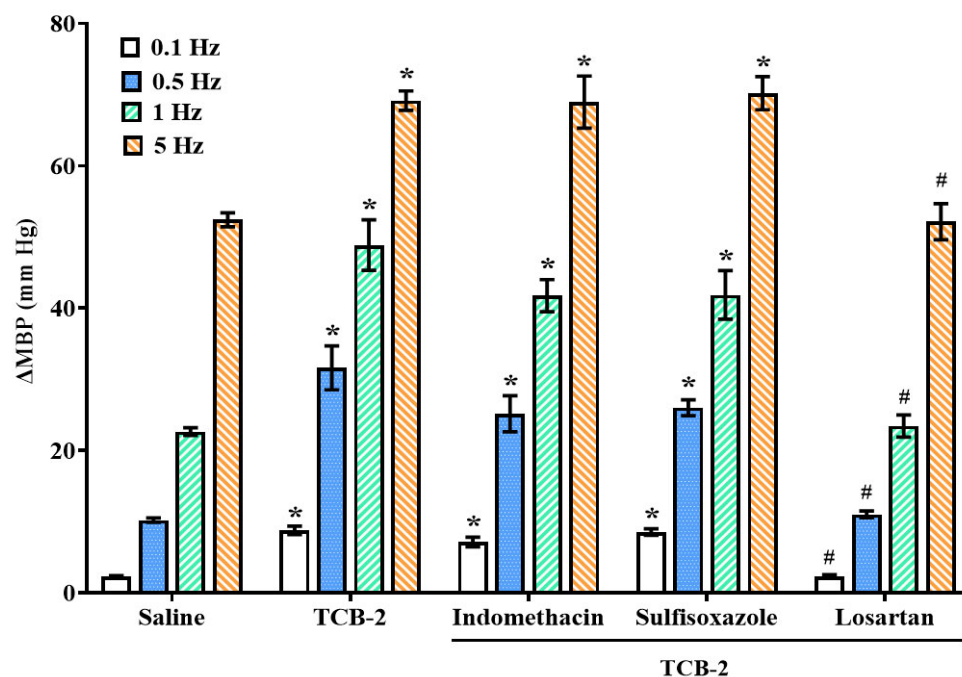


Figure 3. Effect of the i.v. administration of different inhibitors/blockers of vasoactive agents on the vascular sympatho-enhancement triggered by 5-HT_{2A} activation. Effect of the i.v. bolus of indomethacin (2 mg/kg), sulfisoxazole (0.5 mg/kg), or losartan (1 mg/kg) on the potentiating effect of TCB-2 (1 µg/kg/min) on the electrically induced vasopressor responses in female rats. Data were analyzed by two-way ANOVA followed by Dunnett's post hoc test. All values are expressed as mean ± SEM. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. TCB-2 alone. ΔMBP: increases in mean blood pressure.

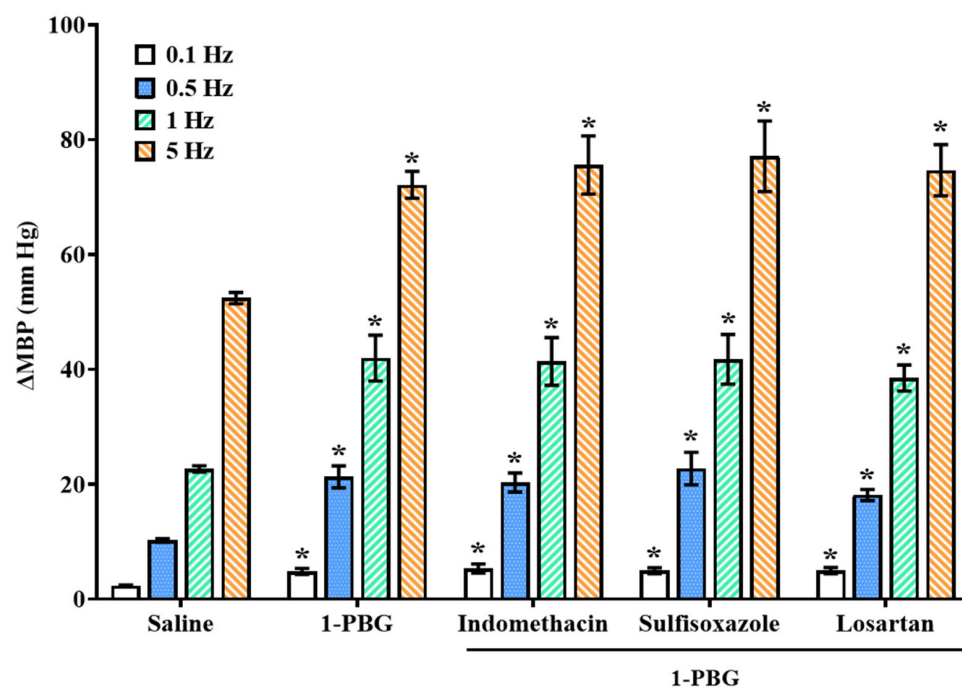


Figure 4. Effect of the i.v. administration of different inhibitors/blockers of vasoactive substances on the vascular sympatho-potential evoked by 5-HT₃ activation. Effect of the i.v. bolus of indomethacin (2 mg/kg), sulfisoxazole (0.5 mg/kg), or losartan (1 mg/kg) on electrically induced vasoconstrictions in the presence of 1-PBG infusion (10 µg/kg/min) in female rats. Data were analyzed by two-way ANOVA followed by Dunnett's post hoc test. All values are expressed as mean ± SEM. * $p < 0.05$ vs. saline. No statistical significance vs. 1-PBG alone. 1-PBG: 1-phenylbiguanide. ΔMBP: increases in mean blood pressure.

2.3. Influence of i.v. Bolus of Vehicles or Antagonists (Indomethacin, L-NAME, TEA, Sulfisoxazole, or Losartan) per se on the Vasopressor Responses Induced by Electrical Stimulation in Female Rats

Intravenous bolus administration of vehicles (saline and PEN, 1 mL/kg each; Figure 1), indomethacin (a non-selective COX inhibitor; 2 mg/kg), sulfisoxazole (an endothelin ET_A receptor antagonist; 0.5 mg/kg), or losartan (an Ang-II type 1 (AT₁) receptor antagonist; 1 mg/kg) did not significantly modify the S-R curve E0 (Table 2). However, i.v. administration of L-NAME (a non-selective NO synthase (NOS) inhibitor; 10 mg/kg) enhanced the vasopressor responses induced by electrical stimulation, whereas i.v. administration of TEA (a non-selective K⁺ channel blocker; 16.5 mg/kg) reduced the sympathetic-induced vasopressor responses (Table 2, Figure 2).

Table 2. Effect of i.v. bolus of antagonists/inhibitors on the increases in mean blood pressure (Δ MBP) induced by sympathetic electrical stimulation in female rats.

i.v. Bolus Administration (mg/kg)	Frequency of Stimulation (Hz)			
	0.1	0.5	1	5
Nothing (control)	2.2 $\pm$ 0.3	10.5 $\pm$ 0.5	24.8 $\pm$ 2.3	54.1 $\pm$ 3.8
Indomethacin (2)	2.2 $\pm$ 0.2	10.3 $\pm$ 0.5	22.0 $\pm$ 1.5	52.4 $\pm$ 2.0
L-NAME (10)	4.8 $\pm$ 0.5 *	19.9 $\pm$ 1.2 *	37.6 $\pm$ 1.4 *	75.7 $\pm$ 3.1 *
TEA (16.5)	1.7 $\pm$ 0.1 *	7.2 $\pm$ 0.4 *	15.6 $\pm$ 0.7 *	39.7 $\pm$ 2.6 *
Sulfisoxazole (0.5)	2.2 $\pm$ 0.2	10.5 $\pm$ 0.8	21.9 $\pm$ 1.2	52.9 $\pm$ 2.8
Losartan (1)	2.4 $\pm$ 0.1	10.7 $\pm$ 0.4	22.5 $\pm$ 0.7	53.8 $\pm$ 2.1
ΔMBP (mm Hg)				

All values are expressed as mean $\pm$ SEM. Data were analyzed by two-way ANOVA followed by Dunnett's post hoc test. * $p < 0.05$ vs. control. L-NAME: N(ω)-nitro-L-arginine methyl ester; TEA: tetraethylammonium.

2.4. Impact of i.v. Administration of Indomethacin, L-NAME, or TEA on the L-694,247-Induced Serotonergic Sympatho-Inhibitory Effect in Female Rats

The inhibitory effect of L-694,247 (5-HT_{1D} receptor agonist) on the electrically induced vasoconstrictions was exclusively abolished by the i.v. administration of TEA (Figure 2). In contrast, the i.v. administration of indomethacin or L-NAME failed to reverse the inhibitory action of L-694,247 on the vasopressor responses induced by sympathetic stimulation in female rats (Figure 2).

2.5. Influence of i.v. Administration of Indomethacin, Sulfisoxazole, or Losartan on the TCB-2 or 1-PBG Sympathetic Potentiation in Female Rats

The i.v. pretreatment with indomethacin or sulfisoxazole did not alter the sympatho-potentiation induced by the 5-HT_{2A} receptor agonist, TCB-2; but, i.v. administration of losartan completely blocked the sympatho-potentiation induced by TCB-2 in female rats (Figure 3). The enhancing effect of 1-PBG (5-HT₃ receptor agonist) on the vasopressor responses evoked by sympathetic stimulation was not modified by pretreatment with either indomethacin, sulfisoxazole, or losartan (Figure 4).

2.6. Plasma Concentration of Ang-II

Basal plasma concentration of Ang-II was similar between male and female pithed rats (29.5 $\pm$ 2.9 and 25.1 $\pm$ 2.5 pg/mL, respectively). After 25 min of TCB-2 (5-HT_{2A} receptor agonist) perfusion in female pithed rats, Ang-II levels significantly increased (38.6 $\pm$ 4.3 pg/mL; * $p < 0.05$ vs. basal Ang-II levels in female pithed rats).

3. Discussion

Our study put in relevance, for the first time, that 5-HT_{1D} sympatho-inhibition at the vascular level is mediated by the K⁺ channels opening and the 5-HT_{2A} sympatho-

excitatory effect is produced via AT_1 activation in female rats. On the contrary, the 5-HT₃ sympatho-modulatory effect seems to be direct in females (Figure 5).

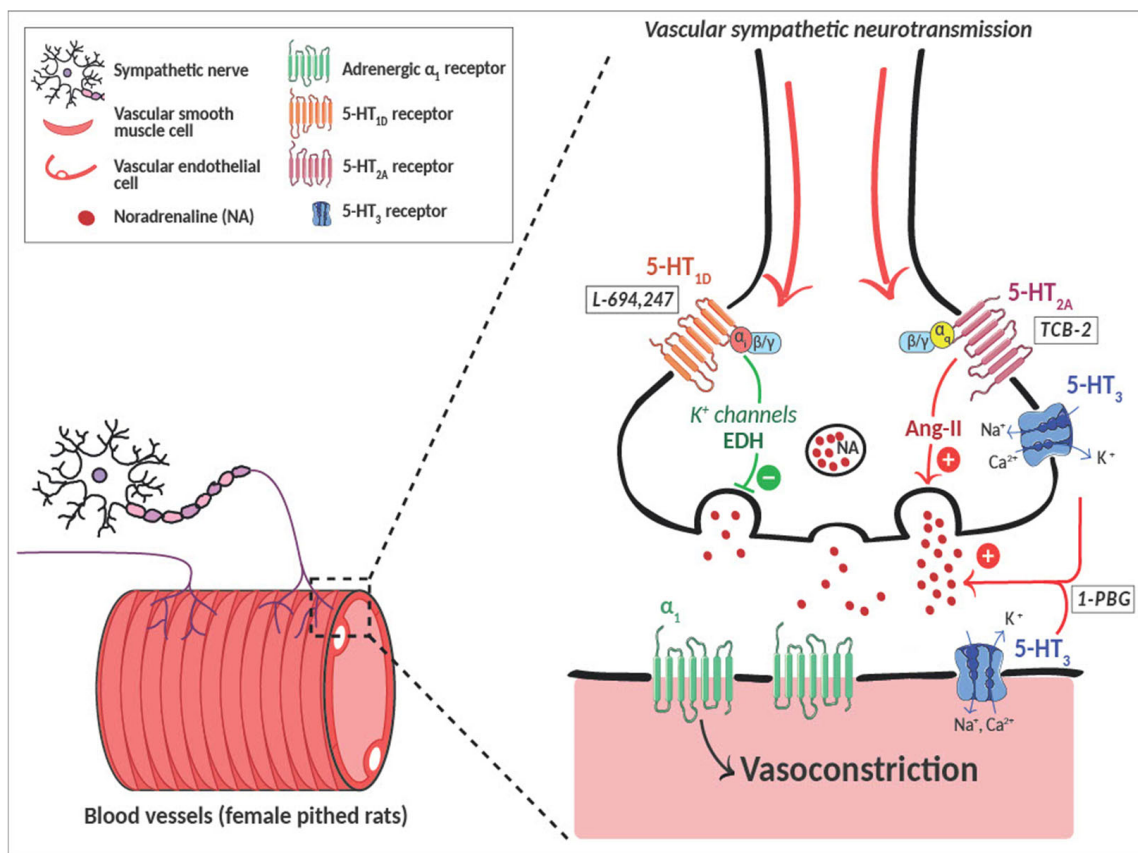


Figure 5. Scheme summarizing the main outcomes in 5-HT modulation of the vascular sympathetic neurotransmission in female rats. At the vascular level, L-694,247 (5-HT_{1D} agonist) sympatho-inhibition is mediated by the K⁺ channels opening, TCB-2 (5-HT_{2A} agonist) sympatho-excitatory action is produced via AT_1 activation and 1-PBG (5-HT₃ agonist) sympatho-potentialiation is a direct effect in females. 1-PBG: 1-phenylbiguanide; Ang-II: angiotensin II; EDH: endothelium-dependent hyperpolarization.

3.1. Basal Hemodynamic Parameters in Female Rats

In our experiments, we used 14–16-week-old female rats, as representative of animals in fertile age [18]. Moreover, and continuing with our previous work, we utilized the model of the pithed rat [8,9,18,32], where MBP was around 58 mm Hg and HR was approximately 307 bpm. Most drugs administered, either in i.v. bolus (indomethacin, sulfisoxazole, TEA) or in i.v. perfusion (L-694,247, 1-PBG), did not modify MBP. However, and probably due to its mechanism of action, i.v. bolus administration of losartan (a blocker of AT_1 receptors) [33] transiently decreased MBP and i.v. administration of L-NAME (a non-selective NOS inhibitor) induced a persistent elevation of MBP [29]. In this line, i.v. infusion of TCB-2, a 5-HT_{2A} receptor agonist, significantly increased MBP and HR as previously reported for other 5-HT₂ receptor agonists [7,18]. In fact, 5-HT_{2A} receptor activation is associated with vascular smooth muscle contraction, platelet aggregation, and tachycardia [7,34], which may account for alteration in MBP and HR after i.v. perfusion of TCB-2. TEA and 1-PBG modified HR by reducing or increasing it, respectively, as previously reported by us [18].

3.2. Indirect Mechanisms Involved in 5-HT Vascular Sympatho-Inhibition in Female Rats

The electrical stimulation of sympathetic outflow induced frequency-dependent increases in MBP, without variations in HR, as previously shown by us [18]. In males, the direct 5-HT effect at the vascular and renal level and also the indirect vascular/renal effects of serotonergic system modulating the sympathetic or non-adrenergic-non-cholinergic systems are mediated by either vasoconstrictor or vasodilator agents, such as Ang-II, NO or EDH, in different experimental models, regardless of whether their involvement varies depending on the vascular territory studied as well as the pathophysiological conditions [29–31]. The serotonergic sympatho-inhibitory action is mediated, both in female and male pithed rats, by 5-HT₁ receptors; while in males the 5-HT_{1D}/5-HT_{1A} receptor subtypes seem to be involved in the serotonergic reduction in sympathetic discharge [6,7,19], in female rats 5-HT_{1A} receptor activation is devoid of this action and only the 5-HT_{1D} receptors are responsible of this inhibitory effect, since L-694,247 is able to reproduce 5-HT inhibitory effects on electrically induced vasoconstrictions (current data [18]).

Considering that 5-HT_{1D} receptor activation is coupled to G_{i/o} family proteins, which leads to (1) the decrease in cAMP levels, acting as a neurotransmitter inhibitor and (2) the stimulation of other signaling pathways, suggesting a role in the modulation of various cellular functions [35], we tried to determine the involvement of several vascular relaxing factors such as (i) NO, which is produced through the enzymatic conversion of L-arginine by NOS, (ii) prostacyclin via the COX pathway, and (iii) EDH, which usually promotes relaxation by hyperpolarizing vascular smooth muscle cells, involving the activation of K⁺ channels [36,37]. To do so, we intravenously pretreated the female pithed rats with either L-NAME [29,30], indomethacin [9,30,31], or TEA [38], prior to the i.v. perfusion of L-694,247 to evaluate if the presence of these mediators were implicated in the 5-HT_{1D} receptor-mediated vascular sympatho-inhibition. It is important to remark that in our study, in female pithed rats, TEA decreased per se the vasopressor responses induced by stimulation of sympathetic outflow, which is in contrast with data reported in male pithed rats where the non-selective K⁺ channel blocker TEA had no effect on the vasopressor responses elicited by sympathetic stimulation [38]. On the contrary, L-NAME, as already reported [29,39], a non-selective NOS inhibitor, enhanced the MBP increases evoked by electrical stimulation, which may result from the inhibition of relaxation normally caused by NO. Moreover, it has been demonstrated that the absence of NO leads to greater NA release from sympathetic nerves [40].

Intravenous administration of TEA completely blocks the L-694,247 vascular sympatholytic effect. The K⁺ channels are integral to the function of EDH [41] and the blockade of these channels by TEA inhibits EDH-mediated responses, underscoring their critical role in vascular tone regulation [42]. Thus, in female rats, the EDH pathway appears to mediate the inhibition of sympathetic neurotransmission via 5-HT_{1D} receptors (current data). These findings are partially in agreement with previous works in male rats treated with sarpogrelate, a 5-HT₂ receptor antagonist, where the sympatholytic effect of 5-HT_{1/7} receptors was also mediated by endothelial factors. However, the sex, experimental model, and pathophysiological conditions seem to be critical in the involvement of specific endothelial pathways. For instance, a chronic 5-HT₂ receptor blockade in male rats unmasked a COX-dependent mechanism (mainly COX-2) in the 5-HT_{1D} effect, whereas the 5-HT₇-mediated sympathetic inhibition was produced through EDH [31]. Similarly, the induction of a diabetic state in male rats revealed the NO pathway as the main mediator of 5-HT_{1A} sympatho-inhibitory effects [29]. Moreover, our current results are consistent with previous studies suggesting that the EDH pathway plays a more prominent role in female rats [43,44]. Estrogen activity may underlie this sex difference in the prevalence of EDH responses. Indeed, an estrogen deficit in animals, achieved by ovariectomy, reduces EDH

responses induced by several different stimuli and this effect is reversed by treatment with 17 β -estradiol [43,45,46].

3.3. Indirect Mechanisms Involved in 5-HT Vascular Sympatho-Potentialiation in Female Rats

We have also shown in female rats that the serotonergic system is also responsible for a sympathetic potentiation at the vascular level, through 5-HT_{2A} and 5-HT₃ receptor activation (current data [18]). Taking into account that (1) direct serotonergic vasoconstrictor effects, through 5-HT₂ receptor activation, at the renal level, are mediated through Ang-II in male rats [2] and (2) the induction of diabetes in males modifies the mediators involved in 5-HT_{2A}-induced renal vasoconstriction, unmasking the COX pathway as the main actor [3]; we decided to evaluate in female rats the possible role of endothelium-derived contracting factors (Ang-II, prostanoids or endothelin) in the 5-HT_{2A} and 5-HT₃ sympatho-enhancing action at the vascular level, administering i.v. losartan, indomethacin, or sulfisoxazole prior to intravenous infusion of TCB-2 (5-HT_{2A} agonist) or 1-PBG (5-HT₃ agonist). None of these antagonists modified per se the vasoconstrictions obtained by electrical stimulation of sympathetic outflow.

Regarding the sympatho-excitatory effect induced by 1-PBG, this action was found to be independent of indirect pathways, as the blockade of COX, ET_A, and Ang-II pathways did not modify the potentiation of vasopressor responses evoked by electrical stimulation during 1-PBG perfusion. It is important to remark that the 5-HT₃ receptor is a ligand-gated ion channel, distinct from other serotonin receptors, which are typically G protein-coupled. Upon binding of serotonin to the 5-HT₃ receptor, the channel opens to allow the influx of Na⁺ and Ca²⁺ ions, and the efflux of K⁺ ions, leading to membrane depolarization and rapid excitatory neurotransmission [47], which seems to form the basis of the direct vascular sympatho-excitatory effect in female rats (current data).

Concerning the sympatho-excitatory action induced by the activation of 5-HT_{2A} receptors, indomethacin and the ET_A receptor antagonist, sulfisoxazole, did not modify this effect. Unlike the blockade of AT₁ receptors by losartan, it completely reversed the sympatho-potentiation induced by the 5-HT_{2A} receptor agonist TCB-2, exhibiting an increase in blood Ang-II concentration in females. The 5-HT_{2A} receptor is coupled to the G_{q/11} family, whose activation increases cytoplasmic calcium and induces phosphorylation of various downstream targets involved in cellular signaling [48]. It is probable that this cascade leads to a wide range of responses, including Ang-II release at the vascular level; in this context, our work shows that Ang-II levels are augmented following 5-HT_{2A} receptor activation in female rats. Although in male rats, 5-HT_{2A} is devoid of any vascular sympatho-excitatory effect [18], several studies have previously underscored the interplay between serotonergic and Ang-II systems at the cardiovascular and renal levels. In fact, at the cardiac level, it has been demonstrated that co-administration of 5-HT and Ang-II led to significant alterations in contractility and extracellular matrix remodeling of cardiac valve cells, enhanced cellular contractility, cytoskeletal reorganization, and increased collagen remodeling, that can contribute to valvular heart diseases [49,50]. Furthermore, at the renal level, we have demonstrated that direct 5-HT₂ vasoconstrictor action is related to the AT₁ receptor activation [2], confirming this synergistic effect. However, this work shows for the first time that the 5-HT_{2A} sympatho-excitatory effect at the vascular level is mediated by Ang-II in female rats.

3.4. Limitations and Clinical Perspectives

Our study has certain limitations that must be taken into account, as our pithe rat model eliminates all central nervous system control over blood pressure. Furthermore, our experimental design involving electrical stimulation does not allow for the direct measure-

ment of norepinephrine release; instead, we rely on an indirect assessment based on the increases in blood pressure following stimulation. In addition, since NO and prostanoids also act via K^+ channels, EDH-specific mechanisms could have been determined by dual NO/prostanoid inhibition (L-NAME + indomethacin) or selective EDH-pathway blockers (e.g., apamin/charybdotoxin). Nevertheless, as neither NO nor prostanoids separately alter the 5-HT_{1D} receptor-mediated inhibitory response, and only TEA (non-selective K^+ channels inhibitor) reversed this serotonergic effect, we linked this sympatholytic effect to EDH.

Although CVD is the leading cause of death in the world, the cardiovascular risk factors seem to be quite different in women and men [21]. Thus, biological sex-based aspects appear to form the base of the clinical management pyramid for these disorders. In the 21st century, sex differences are extensively being studied in animal models, and mechanistic investigations should be undertaken to analyze these sex disparities, which in the end could lead to a sex-specific approach that may open a novel therapeutic avenue for CVD. The contribution of the sympathetic nervous system (SNS) to cardiovascular regulation has been widely studied and seems to be sex-dependent [51,52]; furthermore, currently there are commercialized pharmacological treatments that modulate SNS to reduce CVD, but with no sex or gender differentiation. Hence, there is still a great need to look for new sex-related therapeutic approaches to diminish morbimortality associated with CVD. Our findings provide a new insight into the intricate interplay between the serotonergic and sympathetic nervous systems at the cardiovascular level. The involvement of different mediators in this crosstalk (EDH or Ang-II) between males and females suggests a multifactorial mechanism that may contribute to sex-specific differences in cardiovascular homeostasis. Understanding these interactions could pave the way for the development of targeted and sex-tailored therapeutic strategies in the management of cardiovascular diseases.

4. Materials and Methods

4.1. Drugs Used

The drugs and the sources used in the research were sodium pentobarbital (Dolethal®; Vetoquinol; Madrid, Spain), sodium heparin (Rovi; Madrid, Spain), atropine sulphate (Scharlab; Barcelona, Spain), L-NAME hydrochloride, TEA chloride, d-tubocurarine hydrochloride, and 1-PBG (Merck Life Science S.L.U.; Madrid, Spain); L-694,247, TCB-2, sulfoxazole, and losartan potassium (Tocris Bioscience; Bristol, UK); and indomethacin (Acofarma; Barcelona, Spain). The doses of all the drugs used were based on results from previous studies, and the infusion rate for the serotonergic agonists was 1 mL/h [4,5,18,31,38]. All the compounds were dissolved in saline at the time of experimentation, except indomethacin (dissolved in PEN).

4.2. Animals

Female and male Wistar rats ($n = 75$ and $n = 5$, respectively) aged 14–16 weeks with a body weight of 250 ± 25 g were obtained from the animal facility of the University of Salamanca. The animals were housed under controlled conditions in open-topped polycarbonate cages (4–5 animals per cage), at a constant temperature of 22 ± 2 °C, 50% humidity, 12 light/dark cycle, and received food and water ad libitum.

4.3. Surgical Procedure and Animal Preparation

The animals were anaesthetized with pentobarbital (60 mg/kg; i.p.) and a tracheal cannula was placed for artificial ventilation (1 mL air/100 g, 50 strokes/min; Harvard Rodent Ventilator, Model 683). A stainless pithing rod was inserted through the orbit and foramen magnum into the spinal cord. Jugular veins were cannulated for the continuous perfusion

of agonists and for the bolus administration of antagonists/inhibitors (or their vehicles). The left carotid artery was cannulated and coupled to a pressure transducer, connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain) to record MBP and HR using ChartTM (v5.5.11; eDAQ) and LabChartTM (v7.2; ADInstruments) software. The stimulation of the entire sympathetic outflow was conducted using Cibertec Stimulator CS-9. One electrode was connected to the pithing rod, and the other electrode was inserted subcutaneously into a leg. To prevent thrombus, heparin (1000 UI/kg) was intravenously administered. The animals were treated with atropine (1 mg/kg) and d-tubocurarine (2 mg/kg) to avoid cholinergic effects and electrically induced muscular spasms, respectively [7,18,31].

4.4. Vascular Sympathetic Stimulation in Female Rats

When the animals were in a stable hemodynamic condition for at least 10 min, baseline values of MBP and HR were recorded. Afterwards, vascular sympathetic nerves were electrically stimulated as previously stated: 15 ± 3 V, monophasic pulses of 1 ms, for 25 s at increasing frequencies (0.1, 0.5, 1, and 5 Hz). An interval of approximately 5 min was maintained between each frequency. The responses obtained showed increases in MBP (Δ MBP), without significant changes in HR, and basal MBP was restored immediately after interruption of the stimulation. The S-R curve E0 was achieved in 20 min [6–8,18,32].

At this point, the animals were randomly divided into five sets (Figure 6). The first set (Figure 6) was performed to confirm the previous results [18]. The animals were administered a continuous i.v. perfusion of the selective 5-HT_{1D} (L-694,247; 10 μ g/kg/min), 5-HT_{2A} (TCB-2; 1 μ g/kg/min), or 5-HT₃ (1-PBG; 10 μ g/kg/min) receptor (sub)type agonist (n = 5 for each agonist). Then, two new E1 and E2 S-R curves were carried out equally to E0.

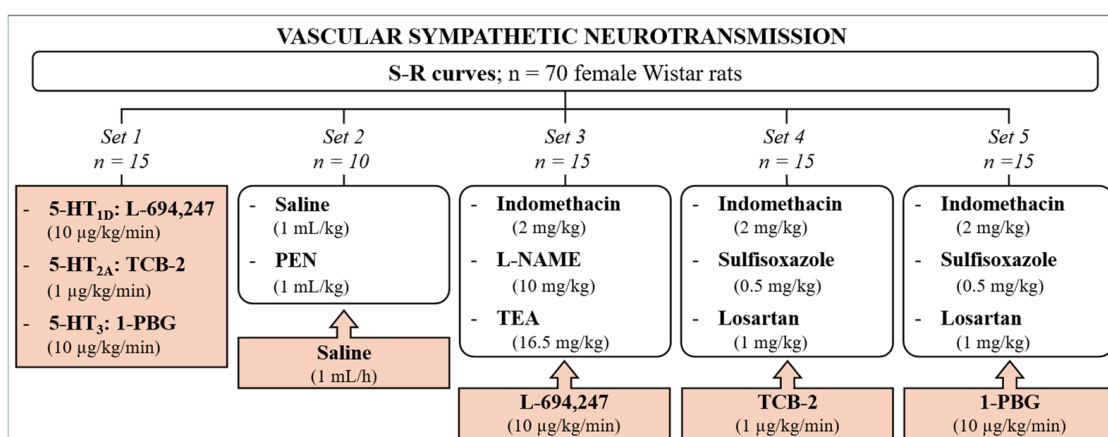


Figure 6. Schematic representation of the in vivo experimental protocols and the number of animals used. The scheme illustrates the five main experimental sets used in the vascular sympathetic neurotransmission study, where vasopressor responses were elicited by electrical sympathetic stimulation (S-R curves). 1-PBG: 1-phenylbiguanide; L-NAME: N(ω)-nitro-L-arginine methyl ester; PEN: mixture of 33% polyethylene glycol, 33% ethanol, and 34% NaOH 0.2 M; TEA: tetraethylammonium.

In the second set, the animals were administered i.v. saline (1 mL/kg) or PEN (1 mL/kg) (Figure 6; n = 5 for each vehicle). The corresponding curve (E0_{saline} and E0_{PEN}) was completed after 10 min. Then, the animals received an i.v. infusion of saline (1 mL/h). After 10 min of starting the i.v. infusion, two new curves S-R (E1 and E2) were obtained as previously described for the S-R curve E0 (Figure 1).

In the third set (Figure 6; n = 15, using n = 5 for each antagonist tested) the animals were administered an i.v. bolus injection of, respectively, (a) a non-selective COX inhibitor, indomethacin (2 mg/kg), (b) a non-selective NOS inhibitor, L-NAME (10 mg/kg),

or (c) a non-selective K^+ channel blocker, TEA (16.5 mg/kg). The corresponding curve ($E0_{\text{Indomethacin}}$, $E0_{\text{L-NAME}}$ or $E0_{\text{TEA}}$) was completed after 10 min, or 30 min in the case of L-NAME. Then, the animals received an i.v. perfusion of L-694,247 (5-HT_{1D} agonist; 10 $\mu\text{g/kg/min}$), and after 5 min, two new curves S-R (E1 and E2) were obtained.

In the fourth and fifth sets (Figure 6; $n = 15$ per set; $n = 5$ each antagonist tested) the rats received the following antagonists intravenously: (a) indomethacin (2 mg/kg), (b) an endothelin ET_A receptor antagonist, sulfisoxazole (0.5 mg/kg), or (c) an AT₁ receptor antagonist, losartan (1 mg/kg), 10 min before its corresponding S-R curve ($E0_{\text{Indomethacin}}$, $E0_{\text{Sulfisoxazole}}$ or $E0_{\text{Losartan}}$). After that, the animals received an i.v. perfusion of TCB-2 (fourth set; 5-HT_{2A} agonist; 1 $\mu\text{g/kg/min}$) or 1-PBG (fifth set; 5-HT₃ agonist; 10 $\mu\text{g/kg/min}$), and two new curves S-R were obtained (E1 and E2).

4.5. Plasma Ang-II Determination

After the surgical procedure, in a group of $n = 5$, female rats' blood samples were collected from the carotid artery before and 25 min after i.v. perfusion of the 5-HT_{2A} agonist, TCB-2. A group of $n = 5$ male rats was used to obtain blood samples after reaching a stable hemodynamic condition. Blood samples were collected into tubes containing heparin as an anticoagulant for plasma preparation and immediately centrifuged to separate the plasma ($1000 \times g$ for 15 min at 4 °C) and stored at -80°C until use. Plasma concentrations of Ang-II were measured using a rat Ang-II ELISA kit according to the manufacturer's instructions, based on the ELISA sandwich technique. The ELISA kit for rat Ang-II was acquired from Cusabio (Catalog No. CSB-E04494r; Houston, TX, USA).

4.6. Data Presentation and Statistical Analysis

All experimental protocols and data analysis were randomized and blinded. Results are presented as mean $\pm$ SEM of at least five experiments ($n = 5$). The variations in MBP produced by electrical sympathetic stimulation are represented as increases in mm Hg from the baseline value. Statistical analyses were performed using GraphPad Prism 9.3.0 (GraphPad, USA). Normal distribution was determined using the Shapiro–Wilk test and homogeneity of variances was assessed by the Brown–Forsythe test. Changes in basal MBP and HR before and (i) during the infusion of serotonergic agonists or (ii) after i.v. administration of antagonists (or their vehicles) were evaluated by a *t*-test with Welch correction. Group differences using two variables (treatment and frequencies of stimulation) were assessed by two-way ANOVA, followed by Dunnett's (compared to control group) post hoc test. For the biochemical assay, one-way ANOVA and Dunnett's post hoc test (when comparing with female baseline levels of Ang-II) were employed.

Post hoc tests were conducted only if *F* in ANOVA achieved $p < 0.05$. Statistical significance was accepted at $p < 0.05$. Since the data obtained from the E2 curves onwards were practically identical, only the E2 curves are shown in the figures. In *in vivo* experiments (pithed rats), given that electrically induced increases in MBP in the presence of saline were similar to those produced in the E0 curve, the statistical analysis was performed versus saline.

5. Conclusions

This study demonstrates the involvement of indirect mechanisms in the serotonergic modulation of vascular sympathetic neurotransmission in female pithed rats. Our results suggest that the EDH pathway, via K^+ -channel activation, mediates the inhibition of sympathetic neurotransmission via 5-HT_{1D} receptors, while the sympatho-excitatory effect of 5-HT_{2A} receptors is associated with the Ang-II pathway. In contrast, the 5-HT₃ sympatho-excitatory effect does not involve indirect pathways.

Author Contributions: A.C.T.-Ú.: Performed in vivo and ELISA experiments, analyzed the data, and writing—reviewing and editing. A.M.: Performed the in vivo experiments, analyzed the data, and writing—reviewing and editing. J.Á.G.-P.: Designed in vivo and ELISA experiments, analyzed the data, and writing—reviewing and editing. J.F.F.-G.: Performed in vivo experiments. M.G.-D.: Designed the in vivo and ELISA experiments, analyzed the data, and writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by University of Salamanca Institutional Bioethics committee (ID number 973; PI: M. García-Domingo; date of approval: 5 May 2023; Responsible authority: Junta de Castilla y León) and complies with current European and Spanish guidelines (Directive 2010/63/EU; R.D. 53/2013), as well as with the ARRIVE guidelines [53].

Data Availability Statement: The main data are included in this manuscript. All data are available from the corresponding author on reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

ΔMBP	Increases in MBP
1-PBG	1- phenylbiguanide
5-HT	5-hydroxytryptamine, Serotonin
Ang-II	Angiotensin II
AT ₁ receptor	Ang-II type 1 receptor
CNS	Central nervous system
COX	Cyclooxygenase
CVD	Cardiovascular diseases
EDH	Endothelium-dependent hyperpolarization
HR	Heart rate
L-NAME	N(ω)-nitro-L-arginine methyl ester
MBP	Mean blood pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
PEN	Polyethylene glycol (33%), ethanol (33%), NaOH 0.2 M (34%)
SNS	Sympathetic nervous system
S-R curve	Stimulation–response curve
TEA	Tetraethylammonium

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Artículo 3

***Sex-dependent modulation of CGRPergic neurovascular activity
by 5-CT in rats***

Terol-Úbeda, A. C., Morán, A., García-Domingo, M., & García-Pedraza, J. Á.

En: *Frontiers in Pharmacology* 2026 (Aceptado para su publicación)

Resumen

El objetivo de este trabajo fue investigar los (sub)tipos de receptores serotoninérgicos implicados en la acción de 5-CT (agonista 5-HT_{1/5/7}) sobre la modulación de la neurotransmisión CGRPérgica perivascular en ratas, analizando las diferencias dependientes del sexo. Se utilizaron ratas Wistar macho y hembra de 14 a 16 semanas de edad que fueron desmeduladas y pretratadas con una perfusión intravenosa continua de hexametonio y metoxamina; posteriormente, se administraron distintos agonistas/antagonistas de receptores serotoninérgicos. La presión arterial media y la frecuencia cardíaca se registraron de forma continua a lo largo de los experimentos, y las respuestas vasodilatadoras CGRPérgicas se obtuvieron mediante estimulación eléctrica de los nervios sensoriales (0.1-5 Hz) o mediante administración intravenosa de α -CGRP (0.1-1 μ g/kg). La presión arterial media y la frecuencia cardíaca basales fueron menores en hembras que en machos, mientras que el incremento de presión arterial media inducido por la metoxamina fue mayor en hembras. Las respuestas vasodilatadoras obtenidas por estimulación eléctrica, así como su inhibición por 5-CT, fueron similares en ambos sexos. En machos, el efecto inhibitorio de 5-CT fue reproducido por los agonistas de los receptores 5-HT_{1B}, 5-HT_{1F} y 5-HT₇ (CP-93,129, LY344864 y AS-19, respectivamente) y se mantuvo en presencia del antagonista del receptor 5-HT_{5A} SB699551. En cambio, en hembras, la

inhibición inducida por 5-CT fue mimetizada por los agonistas de los receptores 5-HT_{1F} y 5-HT₇, y no se vio afectada por la administración de SB699551. Ninguno de los otros agonistas de receptores serotoninérgicos estudiados (5-HT_{1A/1B/1D}) modificó las respuestas vasodilatadoras CGRPérgicas en hembras. Únicamente AS-19 redujo las respuestas vasodepresoras inducidas por la administración de α -CGRP exógeno en hembras. En conclusión, 5-CT inhibe la neurotransmisión sensorial CGRPérgica perivascular tanto en ratas macho como hembra. A diferencia de los machos, donde el efecto de 5-CT está mediado por los receptores 5-HT_{1B/1F/7} presinápticos, en las hembras este efecto inhibitor está mediado por los receptores 5-HT_{1F} presinápticos y los receptores 5-HT₇ pre y/o postsinápticos.

Lausanne, 10 April 2026

Certificate of acceptance

To whom it may concern,

I hereby certify that the following article

Sex-dependent modulation of CGRPergic neurovascular activity by 5-CT in rats

by Anaïs Clara Terol-Úbeda, Asunción Morán, Mónica García-Domingo and José Ángel García-Pedraza, 1766406

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Sex-dependent modulation of CGRPergic neurovascular activity by 5-CT in rats

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Keywords: 5-HT_{1F} receptor, 5-HT₇ receptor, 5-CT, CGRP, migraine, vasodepressor sensory outflow, sex differences.

Abstract

Background and purpose: Serotonin modulates vascular tone both directly and indirectly through autonomic and sensory nerves innervating blood vessels. Perivascular sensory nerves release calcitonin gene-related peptide (CGRP), a potent vasodilator strongly implicated in migraine pathophysiology. In male rats, the serotonergic system inhibits CGRPergic vasodepressor responses via 5-HT_{1B/1F} and 5-HT₇ receptors. Since both serotonergic and CGRPergic pathways exhibit marked sex differences, the present study investigated the 5-HT receptor (sub)types involved in the 5-carboxamidotryptamine (5-CT, 5-HT_{1/5/7} receptor agonist) modulation of vascular CGRPergic neurotransmission in rats, focusing on sex-dependent differences.

Methods: Male and female Wistar rats (14–16 weeks old) were pithed and pretreated with an i.v. continuous infusion of hexamethonium and methoxamine, followed by administration of 5-HT-related drugs. Mean blood pressure (MBP) and heart rate (HR) were continuously recorded throughout the experiments. Vasodepressor CGRPergic responses were elicited by electrical stimulation of the sensory outflow (0.1–5 Hz) or i.v. α -CGRP (0.1–1 μ g/kg).

Results: Basal MBP and HR were lower in females than in males, whereas the methoxamine-induced increase in MBP was greater in females. The electrically evoked vasodepressor responses, as well as their inhibition by 5-CT, were similar in both sexes. In males, the inhibitory effect of 5-CT was reproduced by 5-HT_{1B}, 5-HT_{1F}, and 5-HT₇ receptor agonists (CP-93,129, LY344864, and AS-19, respectively) and persisted in the presence of the 5-HT_{5A} receptor antagonist SB699551. In contrast, in females, 5-CT-induced inhibition was mimicked by 5-HT_{1F} and 5-HT₇ receptor agonists and was not affected by administration of SB699551. None of the other 5-HT receptor agonists (5-HT_{1A/1B/1D}) modified the CGRPergic vasodilator responses in females. Only AS-19 reduced the vasodepressor responses elicited by exogenous α -CGRP in females.

Conclusion: 5-CT inhibits perivascular sensory CGRPergic neurotransmission in both male and female rats. Unlike males, where the 5-CT effect is mediated by prejunctional 5-HT_{1B/1F/7} receptors,

in females, this inhibitory effect is mediated by prejunctional 5-HT_{1F} and pre and/or postjunctional 5-HT₇ receptors. These findings provide novel insights into sex-specific serotonergic modulation of neurovascular function.

1 Introduction

In addition to its prominent role in the central nervous system (CNS), serotonin (5-hydroxytryptamine, 5-HT) participates in the regulation of gastrointestinal motility, endocrine function and cardiovascular homeostasis (Nichols et al., 2008), in which 5-HT exerts multifaceted and even biphasic effects on vascular function (vasoconstriction/vasodilation). This complexity is due to (i) the ability of 5-HT to act at multiple levels—including the heart, vascular endothelial and smooth muscle cells, and peripheral autonomic and sensory nervous systems; and (ii) the involvement of its multiple receptor types and subtypes (González-Hernández et al., 2023), since seven major classes of 5-HT receptors have been identified, namely from 5-HT₁ to 5-HT₇ receptors, with a total of 14 receptor subtypes (Barnes et al., 2021; Feng et al., 2025; Zhang et al., 2025).

Perivascular sensory nerves, mainly C and Aδ fibers, play an important role in the regulation of vascular tone and the maintenance of blood pressure (BP) (Cuesta et al., 2014; González-Hernández et al., 2016; Thakore et al., 2017). These nerves release potent vasodilatory peptides, with substance P and calcitonin gene-related peptide (CGRP) as the principal mediators (González-Hernández et al., 2016; Thakore et al., 2017). CGRP receptors are expressed throughout the central and peripheral nervous systems, including the trigeminovascular pathways, where their activation is strongly linked to migraine pathophysiology (Hay et al., 2017). In male pithed rats, electrical stimulation of perivascular sensory nerves induces vasodilation *via* CGRP release (Lozano-Cuenca et al., 2009; González-Hernández et al., 2010, 2011; Cuesta et al., 2014; Miguel-Martínez et al., 2023). Interestingly, these non-adrenergic non-cholinergic (NANC) responses can be inhibited prejunctionally by activation of 5-HT_{1B/1F} (González-Hernández et al., 2010, 2011) and 5-HT₇ (Cuesta et al., 2014) receptors.

Migraine, a highly prevalent neurovascular disorder affecting approximately 15% of the global population (Ferrari et al., 2022), is the main contributor to headache disorders, which rank as the third leading cause of years lived with disability globally (GBD 2021 Headache Collaborators, 2025). During a migraine attack, increased CGRP release from trigeminal sensory neurons promotes vasodilation, neurogenic inflammation and nociceptive transmission (Bonura et al., 2023; Pleş et al., 2023). Accordingly, pharmacological modulation of CGRP signaling represents a major therapeutic strategy in migraine management (Lewter et al., 2025).

Sex-related differences have been reported in several neurovascular (Maddahi et al., 2023; de Vries Lentsch et al., 2021) and autonomic pathways (Dearing et al., 2022; Terol-Úbeda et al., 2025b). CGRP-mediated vasodilation appears to be influenced by female sex hormones. Plasma levels of CGRP are higher in women than in men, and cyclic fluctuations of ovarian hormones modulate CGRP both in peripheral and CNS (Favoni et al., 2019). Indeed, starting during puberty, migraine occurs in women three to four times more often than in men, and this bias decreases after menopause (Russo et al., 2023). Accordingly, the Global Burden of Disease Study (2019) ranked migraine as the leading cause of disability among women in reproductive age (15-49 years) worldwide (Steiner et al., 2020; Krause et al., 2021).

Our group has recently shown that serotonergic contribution to cardiovascular homeostasis is also sex dependent, as demonstrated in its modulation of vascular sympathetic neurotransmission (Terol-

Úbeda et al., 2025a, 2025b). Considering that 5-HT₁ and 5-HT₇ receptor families play a predominant role in the serotonergic inhibition of vasodepressor sensory CGRPergic outflow in male rats, the present study aimed to investigate the 5-HT receptor (sub)types involved in the 5-carboxamidotryptamine (5-CT, 5-HT_{1/5/7} receptor agonist)-induced modulation of vascular CGRPergic neurotransmission in rats, with particular emphasis on sex-dependent differences.

2 Material and Methods

2.1 Compounds

Each drug and its respective supplier used in the study were: sodium pentobarbital (Dolethal®; Vetoquinol; Madrid, Spain); sodium heparin (Rovi; Madrid, Spain); atropine sulphate (Scharlab; Barcelona, Spain); d-tubocurarine hydrochloride, hexamethonium bromide and methoxamine hydrochloride (Merck Life Science S.L.U.; Madrid, Spain); 5-CT maleate, 8-OH-DPAT, AS-19, α -CGRP, CP-93,129 dihydrochloride, GR127935 hydrochloride, LY344864 hydrochloride, SB258719 hydrochloride, SB699551 and sumatriptan succinate (Tocris Bioscience; Bristol, UK).

All these compounds were dissolved in saline at the experimentation time, except AS-19 (dissolved in ethanol 5% (EtOH)).

2.2 Animal preparation and study design

Male (n = 40) and female (n = 75) Wistar rats (14 - 16 weeks old, 250 $\pm$ 25 g) were obtained and housed in the animal facility of the University of Salamanca until the time of experimentation. Animals were co-housed in groups of up to five per cage under controlled environmental conditions (22 $\pm$ 2°C, 50% humidity, 12-hour light/dark cycle) and had unrestricted access to food and water.

Rats were anaesthetised with pentobarbital (60 mg/kg) via intraperitoneal injection. Once withdrawal reflexes were no longer present, a cannula was placed in the trachea and the animals were pithed by inserting a stainless-steel rod through the right orbital sinus and foramen magnum and down into the spinal cord, as previously established (Cuesta et al., 2014; Terol-Úbeda et al., 2025a, 2025b). Then, rats were artificially ventilated with room air (50 strokes/min, 100 mL air/100 g) and catheters were placed in (i) jugular and femoral veins, for the continuous perfusion of agonists (methoxamine followed by 5-HT receptor agonists) and i.v. administration of 5-HT receptors antagonists; and (ii) the left carotid artery, coupled to a pressure transducer and connected to an e-corder 410 amplifier, for recording mean BP (MBP; mmHg) and heart rate (HR; beats/min, bpm) using ChartTM (v5.5.11; eDAQ) and LabChartTM (v7.2; ADInstruments) software. Coagulation of the blood was avoided by the i.v. injection of heparin (1000 IU/kg) and cholinergic effects were blocked with atropine (1 mg/kg i.v.).

At this point, rats were divided into two main sets (Figure 1), in order to study the effect produced by the continuous infusion of 5-HT agonists on the vasodepressor responses induced by (i) electrical stimulation of perivascular sensory outflow (set 1; n = 95) or (ii) i.v. administration of exogenous α -CGRP (set 2; n = 20). The vasodepressor stimulus-response curves (S-R curves) and dose-response curves (D-R curves) induced by electrical stimulation and exogenous α -CGRP, respectively, were completed in about 50 min with no significant changes in baseline MBP and HR. As previously reported (Cuesta et al., 2014; Miguel-Martínez et al., 2023), only one S-R curve or D-R curve was performed per animal as tachyphylaxis of the CGRPergic vasodepressor responses was observed when eliciting a second S-R or D-R curve, and the time lapse between the different stimulation

frequencies (0.1, 0.5, 1 and 5 Hz) or α -CGRP doses (0.1, 0.3 and 1 $\mu\text{g/kg}$) was approximately 5 min, when MBP had returned to baseline values after vasodepressor responses.

2.3 Experimental protocols

Once a stable haemodynamic condition had been maintained for at least 10 minutes, baseline MBP and HR values were recorded, and the animals were randomly assigned to either Protocol I or Protocol II (Figure 1).

2.3.1 Protocol I. Electrical stimulation of the perivascular sensory outflow

Prior to electrical stimulation, the first set of animals received the following i.v. treatments: (i) a bolus administration of d-tubocurarine (2 mg/kg) to prevent skeletal muscle contraction during electrical stimulation of the spinal cord; (ii) 10 min later, a continuous perfusion of hexamethonium (2 mg/kg/min) to block vasopressor responses induced by stimulation of the preganglionic sympathetic vasopressor outflow; and (iii) after an additional 15 min, a continuous infusion of methoxamine (20 $\mu\text{g/kg/min}$) to increase MBP (Villalon et al., 2008; Cuesta et al., 2014; Miguel-Martínez et al., 2023). After 25 min, baseline values of MBP and HR were assessed. Then, animals were distributed into four experimental groups by sex (groups 1-2: males; groups 3-4: females; Figure 1)

The first group (Group 1; male rats; $n = 30$) was divided into six subgroups ($n = 5$ each) that received the following continuous i.v. perfusions: (a) saline, (b) EtOH 5% (vehicles; 1 mL/h) or selective 5-HT receptor (sub)type agonists: (c) 5-CT (5-HT_{1/5/7}; 0.001 $\mu\text{g/kg/min}$), (d) CP-93,129 (5-HT_{1B}; 20 $\mu\text{g/kg/min}$), (e) LY344864 (5-HT_{1F}; 20 $\mu\text{g/kg/min}$), or (f) AS-19 (5-HT₇; 20 $\mu\text{g/kg/min}$). After 15 minutes of starting the corresponding infusion, MBP and HR were determined again, and the sensory CGRPergic outflow was electrically stimulated to elicit vasodepressor responses by applying 25-s trains of monophasic pulses (1 ms, 50 V) at increasing stimulation frequencies (0.1, 0.5, 1, and 5 Hz), as previously described (Cuesta et al., 2014). Once MBP returned to baseline, the next frequency was applied to obtain the S-R curve.

The second group (Group 2; male rats; $n = 10$) was pretreated with an i.v. bolus injection of SB699551 (5-HT_{5A} antagonist; 1 mg/kg). Five minutes later, an infusion of saline (1 mL/h; $n = 5$) or 5-CT (0.001 $\mu\text{g/kg/min}$; $n = 5$) was given; after 15 min, a S-R curve was constructed as described above.

The third group (Group 3; female rats; $n = 35$) was subdivided into seven subgroups ($n = 5$ each) comprising i.v. perfusions of: (a) 1 mL/h saline; (b) 1 mL/h EtOH 5%; (c) 0.001 $\mu\text{g/kg/min}$ 5-CT; (d) 20 $\mu\text{g/kg/min}$ 8-OH-DPAT (5-HT_{1A} agonist); (e) 20 $\mu\text{g/kg/min}$ sumatriptan (5-HT_{1B/1D} agonist); (f) 20 $\mu\text{g/kg/min}$ LY344864 and (g) 20 $\mu\text{g/kg/min}$ AS-19. After 15 min, a S-R curve was obtained as described above.

The fourth group (Group 4; female rats; $n = 20$) received an i.v. bolus injection of SB699551 (5-HT_{5A} antagonist; 1 mg/kg) or a combination of GR127935 plus SB258719 (5-HT_{1B/1D/1F} antagonist; 310 $\mu\text{g/kg}$; and 5-HT₇ antagonist; 1 mg/kg, respectively). Five minutes later, an infusion of saline (1 mL/h; $n = 5$ each) or 5-CT (0.001 $\mu\text{g/kg/min}$; $n = 5$ each) was given; after 15 min, a S-R curve was obtained as described above.

2.3.2 Protocol II. Administration of exogenous α -CGRP

In the second set of rats (Figure 1; female group; $n = 20$) the pithing rod was left in place throughout the experiment, and neither d-tubocurarine nor hexamethonium was administered since no electrical stimulation was performed. All animals on this set received a continuous i.v. perfusion of methoxamine ($20 \mu\text{g/kg/min}$). After 25 min, when a stable haemodynamic condition was reached, this set was divided into four subgroups ($n = 5$ each) that received (i.v.): (i) saline (1 mL/h); (ii) EtOH 5% (1 mL/h); (iii) LY344864 ($20 \mu\text{g/kg/min}$); or (iv) AS-19 ($20 \mu\text{g/kg/min}$). Fifteen minutes later, MBP and HR were reassessed, and the vasodepressor responses induced by i.v. bolus administration of exogenous α -CGRP (0.1 , 0.3 and $1 \mu\text{g/kg}$) were examined during the infusion of vehicles or 5-HT agonists.

2.4 Statistical analysis and data presentation

All experimental protocols and data analysis were randomised and blinded. Results are presented as mean $\pm$ SEM of at least five experiments ($n = 5$). The changes in MBP (ΔMBP) by electrical stimulation or exogenous α -CGRP are represented as decreases in MBP from the baseline value. Statistical analyses were performed using GraphPad Prism 9.3.0 (GraphPad, USA). Normal distribution was determined using the Shapiro-Wilk test and homogeneity of variances was assessed by Brown-Forsythe test. Variations in basal MBP and HR before and after the corresponding treatment were evaluated by t test with Welch correction. Moreover, vasodepressor responses obtained by electrical stimulation or exogenous α -CGRP in the different subgroups of animals were compared with a two-way ANOVA, followed by Dunnett's (compared to control group) *post hoc* test. *Post hoc* tests were conducted only if F in ANOVA achieved $P < 0.05$. Statistical significance was accepted at $P < 0.05$. Since the decreases in MBP induced by electrical stimulation or i.v. α -CGRP in the presence of i.v. EtOH did not differ from those obtained with saline, statistical comparisons were performed only versus the saline group.

3 Results

3.1 Systemic haemodynamic variables

After anaesthesia, basal MBP and HR in male pithed rats ($n = 40$) were $64 \pm 2 \text{ mmHg}$ and $319 \pm 5 \text{ bpm}$, respectively, whereas in females ($n = 75$) these values were significantly lower compared with males: $53 \pm 1 \text{ mmHg}$ and $302 \pm 5 \text{ bpm}$, respectively ($* P < 0.05$ vs males). These variables were not modified after the i.v. bolus administration of atropine or d-tubocurarine (not shown), or during the continuous infusion of hexamethonium ($69 \pm 2 \text{ mmHg}$ and $327 \pm 6 \text{ bpm}$ in males; $54 \pm 1 \text{ mmHg}$ and $306 \pm 4 \text{ bpm}$ in females). By contrast, methoxamine infusion ($20 \mu\text{g/kg/min}$, after 25 min) substantially increased both parameters, reaching $184 \pm 4 \text{ mmHg}$ and $362 \pm 9 \text{ bpm}$ in males, and $183 \pm 2 \text{ mmHg}$ and $335 \pm 5 \text{ bpm}$ in females ($* P < 0.05$ vs the corresponding basal value) (Figure 2). Interestingly, the methoxamine-induced increase in MBP was significantly greater in females ($127.9 \pm 2.2 \text{ mmHg}$) compared with males ($118.7 \pm 3.5 \text{ mmHg}$) ($* P < 0.05$ vs males).

During methoxamine infusion, MBP and HR were not altered by i.v. perfusion/bolus of 5-HT agonists/antagonists (or their vehicles) in either male or female pithed rats (Figure 2; Table 1). The only exception, observed in both sexes, was a sustained decrease in MBP induced by the perfusion of 5-CT ($0.001 \mu\text{g/kg/min}$) and AS-19 ($20 \mu\text{g/kg/min}$) (Table 1). Moreover, in female pithed rats, the vasodilator effect of 5-CT was reversed by pretreatment with the mixture of GR127935 plus SB258719 (ΔMBP : $-13.2 \pm 1.8 \text{ mmHg}$; $* P < 0.05$ vs 5-CT, $P > 0.05$ vs saline).

3.2 Vascular responses by sensory nerves stimulation in male and female rats

During the continuous infusion of methoxamine, which produced a sustained increase in vascular tone throughout the experiment, electrical stimulation of the perivascular sensory outflow (0.1 - 5 Hz) resulted in frequency-dependent decreases in MBP in male and female pithed rats (Figure 2A) (Cuesta et al., 2014). In all cases, these vasodepressor responses were due to selective systemic vasodilation, since (i) only minor variations were observed in HR and (ii) appeared about 10 s after the stimulus and reached a maximum 1 min after the stimulus has ended (Cuesta et al., 2014, González-Hernández et al., 2010, 2011; Figure 2).

In male pithed rats, vasodepressor responses induced by electrical stimulation (S-R curve) during continuous i.v. saline perfusion (control; 1 mL/h) were -10.4 ± 1.0 , -22.0 ± 0.9 , -39.7 ± 2.0 and -81.1 ± 3.3 mmHg (Figure 3). In females, the corresponding values were -9.4 ± 0.8 , -19.0 ± 1.5 , -39.3 ± 3.9 and -84.1 ± 0.5 mmHg (Figures 4 - 6), with no significant differences between sexes.

3.3 Effect of ethanol and 5-HT receptor ligands on the vasodepressor responses induced by electrical stimulation in male rats

In male pithed rats, the decreases in MBP due to electrical stimulation were not modified by i.v. infusion of EtOH 5% (1 mL/h), as previously reported (Cuesta et al., 2014). In contrast, continuous infusion of 5-CT (0.001 μ g/kg/min), a selective 5-HT_{1/5/7} receptor agonist, reduced the electrically induced vasodepressor responses (Figure 3). This inhibitory effect was mimicked by i.v. infusion of CP-93,129 (5-HT_{1B} agonist), LY344864 (5-HT_{1F} agonist) and AS-19 (5-HT₇ agonist), each at dose of 20 μ g/kg/min (Figure 3).

The i.v. pretreatment with the selective 5-HT_{5A} receptor subtype antagonist, SB699551 (1 mg/kg), did not affect the vasodepressor responses induced by electrical stimulation in male rats (data not shown). Furthermore, the inhibitory effect of 5-CT (0.001 μ g/kg/min) on these responses was not altered by pretreatment with SB699551 (Figure 3).

3.4 Effect of ethanol and 5-HT receptor type/subtype agonists on the electrically induced vasodepressor responses in female rats

Continuous infusion of EtOH 5% (1 mL/h) did not affect the vasodepressor responses induced by electrical stimulation in female pithed rats (Figure 4). In contrast, i.v. perfusion of 5-CT (5-HT_{1/5/7} agonist; 0.001 μ g/kg/min) and AS-19 (5-HT₇ agonist; 20 μ g/kg/min) inhibited the vascular CGRPergic outflow (Figure 4).

To investigate the involvement of 5-HT₁ receptor subtypes in this inhibitory effect, the agonists tested (all at 20 μ g/kg/min) were: 8-OH-DPAT (5-HT_{1A} agonist), sumatriptan (5-HT_{1B/1D} agonist) and LY344864 (5-HT_{1F} agonist). Only LY344864 significantly reduced the electrically induced vasodepressor responses, whereas 8-OH-DPAT and sumatriptan had no effect (Figure 5).

3.5 Influence of 5-HT receptor antagonists on 5-CT-induced vascular sensory-inhibition in female rats

During an i.v. infusion of the vehicle of 5-CT (saline; 1 mL/h), the electrically induced vasodepressor responses in females receiving i.v. pretreatment with (i) SB699551 (5-HT_{5A} antagonist; 1 mg/kg) or (ii) a combination of GR127935 plus SB258719 (5-HT_{1B/1D/1F} antagonist; 310 μ g/kg; and 5-HT₇ antagonist; 1 mg/kg, respectively) remained unaltered (data not shown). The 5-CT-induced sensory

inhibition was (i) unaffected by SB699551 (1 mg/kg) and (ii) abolished by the mixture of GR127935 plus SB258719 (310 µg/kg and 1 mg/kg, respectively) (Figure 6).

3.6 Effect of ethanol and selective 5-HT agonists on the vasodepressor responses induced by i.v. administration of α -CGRP in female rats

The i.v. administration of exogenous α -CGRP (0.1 - 1 µg/kg) caused dose-dependent decreases in MBP with minor changes in HR (Figure 2B; Table 2). Neither EtOH 5% (1 mL/h) nor LY344864 (5-HT_{1F} agonist; 20 µg/kg/min) modified these vasodepressor responses (Table 2). In contrast, AS-19 (5-HT₇ agonist; 20 µg/kg/min) significantly reduced the i.v. CGRP-induced vasodepressor responses (Table 2).

4 Discussion

The maintenance of BP depends on the dynamic balance between peripheral vascular tone and cardiac output, which are regulated by neuronal, humoral and local mechanisms. In this sense, the serotonergic system modulates vascular sympathetic and sensory neurotransmission, and both are influenced by sex steroid hormones (Cuesta et al., 2014; Allais et al., 2020; García-Pedraza et al., 2021, 2022; Fernández-González et al., 2022, 2023, 2024; González-Hernández et al., 2023; Terol-Úbeda et al., 2025a, 2025b). In the present study, we show for the first time that peripheral 5-HT receptors modulate perivascular sensory CGRPergic outflow in female rats. Prejunctional 5-HT_{1F} receptors and pre and/or postjunctional 5-HT₇ receptors inhibited the perivascular CGRPergic neurotransmission. These findings extend previous evidence on serotonergic modulation of vascular function and support the existence of sex-dependent mechanisms in cardiovascular regulation.

There is strong evidence that BP regulation is influenced by biological effects of sex chromosomes, sex hormones and reproductive events (Gerds et al., 2022). During the fertile period, females have lower BP than age-matched males, mainly due to the reproductive hormone oestradiol, which downregulates vascular sympathetic nerve activity, attenuates calcium signaling in vascular, renal and cardiac cells, and controls the synthesis of potent vasoconstrictors, such as angiotensin II (Gerds et al., 2022). In accordance with this, and as previously demonstrated (Terol-Úbeda et al., 2025b), in our experimental model of 14-16-week-old female (cycling period) anaesthetised pithed rats (with no central influence on BP), MBP and HR were significantly lower (around 10 mmHg and 10 bpm, respectively) compared with male rats.

As previously established in pithed rats to induce vasodepressor responses, MBP was sustainedly elevated by a methoxamine infusion. Methoxamine enhances peripheral vascular resistance *via* α_1 -adrenoreceptor activation, while the increase in HR is mediated through cardiac α_1 -adrenoreceptor activation (González-Hernández et al., 2011; Cuesta et al., 2014). In this context, the methoxamine-induced increase in MBP was higher in female than in male rats. In line with these findings, previous evidence indicates sex-dependent differences in α - and β -adrenergic responsiveness across several vascular territories (Passmore et al., 2005; Alexandre et al., 2017; Sherwood et al., 2017), which may contribute to the enhanced pressor effect observed in females.

It is noteworthy that, in both male and female rats, the attenuation of the methoxamine-induced increase in MBP produced by the infusion of 5-CT (0.001 µg/kg/min) or AS-19 (20 µg/kg/min) likely reflects the activation of vasorelaxant 5-HT_{1/5/7} or 5-HT₇ receptors, respectively, leading to a long-lasting vasodilation. Previous studies from our group demonstrated 5-HT₇ receptor-mediated vasodilation in male pithed rats under comparable experimental conditions (Cuesta et al., 2014). Similarly, intravenous infusion of 5-CT decreased MBP in both male (García-Pedraza et al., 2021)

and female (Terol-Úbeda et al., 2025b) pithed rats without methoxamine. In addition, several pharmacological observations support the involvement of 5-HT₇ receptors in the vasodilator effect of 5-CT in female rats: (i) 5-CT-induced vasodilation was abolished by pretreatment with GR127935 plus SB258719 (5-HT_{1B/1D/1F} and 5-HT₇ receptor antagonist, respectively); (ii) selective activation of 5-HT_{1B/1D} and 5-HT_{1F} receptors did not modify the MBP values during methoxamine infusion; and (iii) blockade of 5-HT_{5A} receptors failed to affect 5-CT-induced vasodilation. Together, these findings support that the vasodilator effect of 5-CT in female rats is mainly mediated by 5-HT₇ receptors, as previously shown in males (Cuesta et al., 2014).

Under the presence of hexamethonium (to block autonomic outflow) and methoxamine, electrical stimulation of perivascular sensory CGRPergic nerves elicited frequency-dependent vasodepressor responses in both male and female rats, without significant variations in HR. Similarly, decreases in MBP induced by intravenous administration of exogenous α -CGRP in female rats were dose-dependent, as previously described in males (Cuesta et al., 2014). The inhibition of the electrically induced vasodepressor responses by the 5-HT_{1/5/7} receptor agonist 5-CT, observed in both male and female rats, demonstrates that the serotonergic system modulates perivascular sensory CGRPergic neurotransmission. In male rats, this 5-CT-mediated inhibition is consistent with previous findings showing that activation of prejunctional 5-HT_{1B/1F} and 5-HT₇ receptors reduces CGRP release from perivascular sensory nerves (González-Hernández et al., 2010, 2011; Cuesta et al., 2014). At the prejunctional level, 5-HT can modulate the release of autonomic and sensory neurotransmitters, including noradrenaline, acetylcholine and CGRP, facilitating hypertensive or hypotensive effects (Marichal-Cancino et al., 2020; Fernández-González et al., 2022, 2023, 2024; González-Hernández et al., 2023; Terol-Úbeda et al., 2025a, 2025b). In both male and female rats, 5-CT inhibits the vasoconstriction induced by sympathetic stimulation, involving 5-HT_{1D/1A} receptor subtypes in males whereas in females only 5-HT_{1D} receptors are implicated (Morán et al., 1994; García et al., 2005; García-Pedraza et al., 2013, 2021; Terol-Úbeda et al., 2025a, 2025b).

Given this sex-dependent serotonergic regulation of vascular function, it is plausible that hormonal influences contribute to differences in 5-HT-mediated modulation of perivascular sensory neurotransmission. Oestrogens regulate the gene expression of 5-HT receptors, promote 5-HT synthesis by increasing tryptophan hydroxylase activity, and reduce 5-HT degradation and reuptake through modulation of monoamine oxidases and the 5-HT reuptake transporter, respectively (Nappi et al., 2022). Moreover, oestrogen, 5-HT and CGRP receptors coexist within the trigeminovascular system (Aggarwal et al., 2012), supporting a functional link between serotonergic and CGRPergic pathways in migraine pathophysiology, a female-predominant disorder. Women experience more frequent, longer, and severe migraine attacks than men, and although serotonergic antimigraine drugs have demonstrated clinical efficacy in both sexes, most trials lack sufficient statistical power to assess potential sex-related differences in therapeutic response (Elgendy et al., 2019; de Vries et al., 2020). These considerations guided the present investigation to identify the 5-HT receptor (sub)types involved in the 5-CT-induced inhibition of perivascular sensory CGRPergic outflow in female rats.

The receptor (sub)types involved in the 5-CT-induced inhibition of perivascular CGRPergic outflow were first confirmed in male rats, and the role of 5-HT_{5A} receptors was explored, as their contribution had not been previously investigated. Activation of 5-HT_{1B}, 5-HT_{1F}, and 5-HT₇ receptors, using the selective agonists CP-93,129, LY344864, and AS-19, respectively, inhibited the vasodepressor responses induced by sensory CGRPergic stimulation, as previously described (González-Hernández et al., 2010, 2011; Cuesta et al., 2014). Although 5-HT_{5A} receptors were excluded in the present study, given that 5-CT maintained its inhibitory action in the presence of the 5-HT_{5A} receptor antagonist SB699551, these receptors have been implicated in autonomic cardiovascular regulation,

particularly in the inhibition of cardioaccelerator sympathetic outflow under specific experimental conditions in male rats (García-Pedraza et al., 2018, 2020). In female rats, the inhibitory effect of 5-CT on the vasodepressor responses induced by CGRPergic stimulation was reproduced by 5-HT_{1F} and 5-HT₇ receptor agonists (LY344864 and AS-19, respectively), whereas neither 5-HT_{1A} nor 5-HT_{1B/1D} receptor agonists (8-OH-DPAT and sumatriptan, respectively) modified the electrically induced vasodilations. As in males, 5-HT_{5A} receptors were unlikely to contribute to the 5-CT-mediated inhibition of the vasodepressor sensory CGRPergic drive, as this effect persisted after SB699551 administration. Consistent with this, previous studies reported no role for 5-HT_{5A} receptors in the serotonergic modulation of vascular sympathetic outflow in female rats (Terol-Úbeda et al., 2025b). The participation of 5-HT_{1F} and 5-HT₇ receptors in females was further confirmed using a mixture of GR127935 plus SB258719, which completely reversed the 5-CT-induced inhibition of perivascular sensory CGRPergic outflow. Altogether, these results highlight the sex-dependent serotonergic inhibition of perivascular sensory CGRPergic innervation, as in male rats 5-HT_{1F} and 5-HT₇, but also 5-HT_{1B}, receptors were involved as inhibitors of neurovascular CGRPergic drive (González-Hernández et al., 2010, 2011; Cuesta et al., 2014).

To clarify the nature of the 5-HT receptors involved in the inhibition of CGRPergic vascular responses in female rats, we determined whether LY344864 or AS-19 modified the vasodepressor responses to exogenous α -CGRP. Although other effects (as altered vascular responsiveness to CGRP) cannot be fully excluded, we conclude that 5-HT_{1F} receptor-mediated inhibition was prejunctional in nature, whereas 5-HT₇ receptors were pre and/or postjunctional in nature, since AS-19 also decreased the vasodepressor responses induced by exogenous α -CGRP. The latter differs from results in male rats, where 5-HT₇ receptors implicated in serotonergic inhibition of perivascular sensory CGRPergic outflow were purely prejunctional (Cuesta et al., 2014).

Although previous studies have reported sex-related differences in the serotonergic modulation of vascular sympathetic neurotransmission, affecting both the receptor (sub)types involved and their pre and/or postjunctional localization (Terol-Úbeda et al., 2025b), evidence of sexual dimorphism in peripheral 5-HT₁ and 5-HT₇ receptor function has remained limited (Spies et al., 2020). The present findings in female rats further illustrate the complexity of the serotonergic system, revealing a novel peripheral, sex-dependent mechanism in the modulation of perivascular CGRPergic responses. Sexual dimorphism influences not only the 5-HT receptors involved in the inhibition of the vasodepressor responses induced by sensory CGRPergic stimulation, but also the nature of these receptors.

Some limitations of the present study should be considered. Experiments were performed under pithed condition, thus excluding any influence of the CNS on cardiovascular regulation, and therefore the central component of migraine is not studied. The experimental design did not allow determination of the mechanisms underlying tachyphylaxis of the CGRPergic vasodepressor responses. Moreover, sensory nerve activity was not recorded directly; instead, electrically evoked CGRP release in the systemic vasculature was inferred from the magnitude of the vasodepressor responses (Cuesta et al., 2014). Additionally, this study did not assess ovarian hormones or the estrous cycle stage of female rats, since we only considered animals of reproductive age. Anyhow, our present results contribute to a better understanding of sex-related differences in basic cardiovascular pharmacology, a fundamental aspect for developing effective therapeutic strategies for both women and men with cardiovascular diseases (CVD). As several antimigraine therapies, including triptans and ditans, target serotonergic and CGRP-related pathways (Viticchi et al., 2022; Kalkman et al., 2023), and patients with migraine exhibit a higher incidence of CVD (Linstra et al., 2017 Rajendran et al., 2023), clarifying sex-specific serotonergic-CGRPergic interactions is essential

to improve therapeutic efficacy and minimize cardiovascular adverse effects. Given that CVD remain the leading cause of death among women worldwide (Vervoort et al., 2024), and migraine represents a major vascular risk factor in females, our findings reinforce the importance of conducting preclinical pharmacological studies in both male and female animal models to better predict sex-specific cardiovascular responses.

In conclusion, the present study demonstrates that 5-CT inhibits perivascular sensory CGRPergic neurotransmission in both male and female rats. Unlike males, where the 5-CT effect is mediated by prejunctional 5-HT_{1B/1F/7} receptors, in cycling-age female rats this inhibitory effect involves prejunctional 5-HT_{1F} and pre and/or postjunctional 5-HT₇ receptors. These findings reveal a sex-dependent serotonergic modulation of vascular CGRPergic function, highlighting its potential relevance in neurovascular pharmacology.

5 Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author upon reasonable request.

6 Ethics statement

All animal experiments were reviewed and approved by the Bioethics Committee of the University of Salamanca (Protocol ID1171). Procedures were conducted in compliance with the current Spanish (R.D. 53/2013) and European Union (Directive 2010/63/EU) regulations. This study adheres to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments; Percie du Sert et al., 2020).

7 Author contributions

A.C.T.-Ú.: Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft. A.M.: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing. J.Á.G.-P.: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review and editing. M.G.-D.: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review and editing. All authors read and approved the final manuscript.

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10 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

11 Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

12 Abbreviations

5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine
Δ MBP	Changes in mean blood pressure
BP	Blood pressure
Bpm	Beats/min
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CVD	Cardiovascular diseases
D-R curve	Dose-response curve
EtOH	Ethanol
HR	Heart rate
MBP	Mean blood pressure
NANC	Non-adrenergic non-cholinergic
S-R curve	Stimulus-response curve

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Figures

Figure 1. Study design showing the number of animals used in the two main sets and their subdivisions (n = 5 each). Vasodepressor responses were obtained either by electrical stimulation of the perivascular sensory outflow (set 1; groups 1-2: male rats; groups 3-4: female rats) or i.v. bolus administration of exogenous α -CGRP (set 2: female rats). D-R curve: dose-response curve; EtOH: ethanol; S-R curve: (electrical) stimulus-response curve.

Figure 2. Original experimental tracings showing mean blood pressure (MBP; mmHg), heart rate (HR; beats/min, bpm) and vasodepressor responses induced by (A) electrical stimulation of the perivascular CGRPergic outflow or (B) i.v. bolus administration of α -CGRP, during continuous i.v. infusion of saline (1 mL/h) in female pithed rats.

Figure 3. Effect of continuous i.v. perfusion of saline (1 mL/h; control), 5-CT (0.001 μ g/kg/min), CP-93,129 (20 μ g/kg/min), LY344864 (20 μ g/kg/min), 5-CT (0.001 μ g/kg/min) in the presence of SB699551 (1 mg/kg i.v.) or AS-19 (20 μ g/kg/min) (n = 5 each) on the vasodepressor responses induced by electrical stimulation of the CGRPergic outflow in male rats. Data were analysed by two-way ANOVA followed by Dunnett's *post hoc* test. All values are expressed as mean $\pm$ SEM. * P < 0.05 vs saline. Δ MBP: changes in mean blood pressure.

Figure 4. Effect of continuous i.v. perfusion of saline (1 mL/h; control), ethanol 5% (EtOH, 1 mL/h), 5-CT (0.001 μ g/kg/min) or AS-19 (20 μ g/kg/min) (n = 5 each) on the vasodepressor responses induced by electrical stimulation of the CGRPergic outflow in female rats. Data were analysed by two-way ANOVA followed by Dunnett's *post hoc* test. All values are expressed as mean $\pm$ SEM. * P < 0.05 vs saline. Δ MBP: changes in mean blood pressure.

Figure 5. Effect of continuous i.v. perfusion of saline (1 mL/h; control), 8-OH-DPAT, sumatriptan or LY344864 (20 μ g/kg/min and n = 5 each) on the vasodepressor responses induced by electrical stimulation of the CGRPergic outflow in female rats. Data were analysed by two-way ANOVA followed by Dunnett's *post hoc* test. All values are expressed as mean $\pm$ SEM. * P < 0.05 vs saline. Δ MBP: changes in mean blood pressure.

Figure 6. Effect of continuous i.v. perfusion of 5-CT (0.001 μ g/kg/min) alone or in the presence of SB699551 (1 mg/kg i.v.) or a cocktail of GR127935 (310 μ g/kg i.v.) + SB258719 (1 mg/kg i.v.) on the vasodepressor responses induced by electrical stimulation of the CGRPergic outflow in female rats. Data were analysed by two-way ANOVA followed by Dunnett's *post hoc* test. All values are expressed as mean $\pm$ SEM. * P < 0.05 vs saline; # P < 0.05 vs 5-CT alone. Δ MBP: changes in mean blood pressure.

Tables

Table 1. Changes in mean blood pressure (Δ MBP; mmHg) and heart rate (Δ HR; bpm) 15 min after starting saline or 5-HT agonist perfusion in pithed rats under methoxamine infusion (20 μ g/kg/min).

Wistar Rat	Treatment	Dose (μ g/kg/min)	Δ MBP (mmHg)	Δ HR (bpm)
Males	Saline	1 mL/h	2.3 $\pm$ 5.7	13.0 $\pm$ 1.5
	EtOH 5%	1 mL/h	-3.8 $\pm$ 0.6	6.8 $\pm$ 2.5
	5-CT	0.001	-38.2 $\pm$ 2.9 *	5.6 $\pm$ 4.6
	AS-19	20	-47.8 $\pm$ 14.1 *	11.3 $\pm$ 13.5
	CP-93,129	20	-14.1 $\pm$ 5.4	18.0 $\pm$ 3.1
	LY344864	20	-1.2 $\pm$ 2.8	7.7 $\pm$ 4.3
Females	Saline	1 mL/h	-0.4 $\pm$ 0.3	5.6 $\pm$ 3.2
	EtOH 5%	1 mL/h	-5.6 $\pm$ 3.9	7.7 $\pm$ 7.9
	5-CT	0.001	-36.7 $\pm$ 6.8 *	7.0 $\pm$ 3.5
	AS-19	20	-44.0 $\pm$ 5.6 *	15.3 $\pm$ 1.8
	8-OH-DPAT	20	-2.3 $\pm$ 3.9	7.4 $\pm$ 4.5
	Sumatriptan	20	-0.3 $\pm$ 2.0	3.6 $\pm$ 6.0
	LY344864	20	-7.2 $\pm$ 4.1	6.7 $\pm$ 7.5

All values are expressed as mean $\pm$ SEM (n = 5). Data were analysed by t test with Welch correction.

* P < 0.05 vs baseline. bpm: beats/min.

Table 2. Effect of continuous i.v. infusion of ethanol 5% (EtOH, 1 mL/h), LY344864 and AS-19 (20 μ g/kg/min each 5-HT agonist) on the vasodepressor responses induced by i.v. administration of α -CGRP (0.1, 0.3 and 1 μ g/kg) in female pithed rats.

Treatment	α -CGRP 0.1 μ g/kg	α -CGRP 0.3 μ g/kg	α -CGRP 1.0 μ g/kg
Control (saline)	-17.4 $\pm$ 0.7	-37.9 $\pm$ 0.8	-71.7 $\pm$ 3.8
EtOH 5%	-17.1 $\pm$ 0.3	-36.8 $\pm$ 0.8	-72.7 $\pm$ 0.5
LY344864	-19.9 $\pm$ 1.6	-41.2 $\pm$ 1.0	-78.7 $\pm$ 3.7
AS-19	-10.2 $\pm$ 1.2 *	-19.1 $\pm$ 2.3 *	-38.1 $\pm$ 5.4 *
Measurement	Δ MBP (mmHg)		

All values are expressed as mean $\pm$ SEM (n = 5). Data were analysed by two-way ANOVA followed by Dunnett's *post hoc* test. * P < 0.05 vs control. Δ MBP: changes in mean blood pressure.

Graphical abstract

Sex-dependent modulation of CGRPergic neurovascular activity by 5-CT in rats

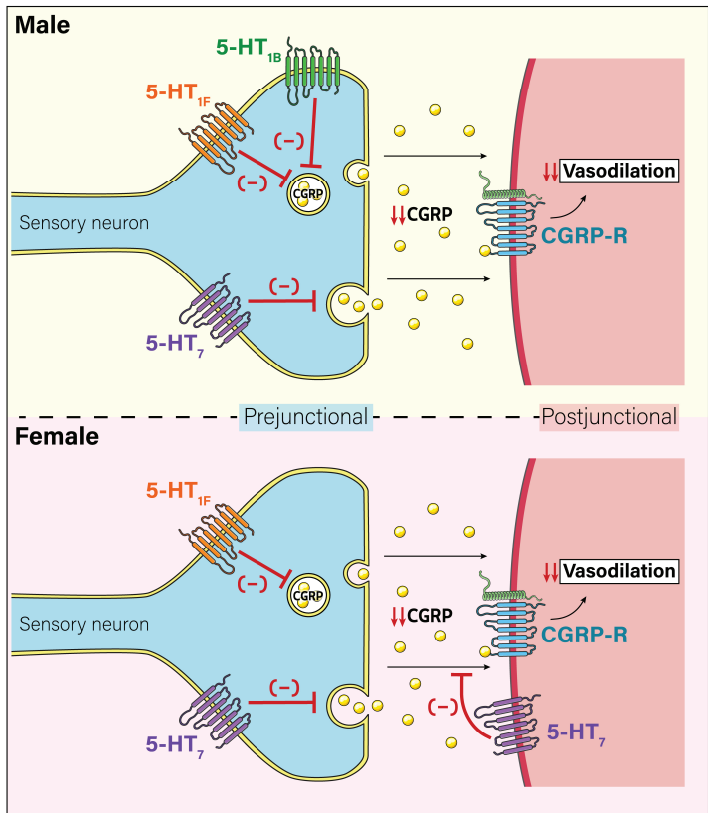
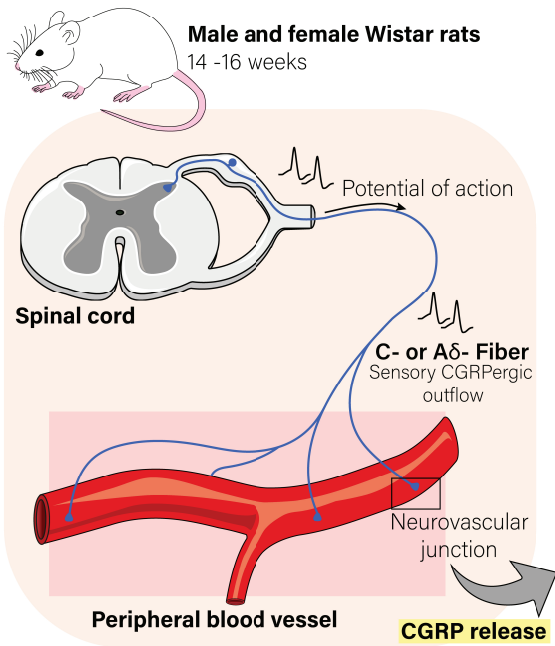


Figure 1

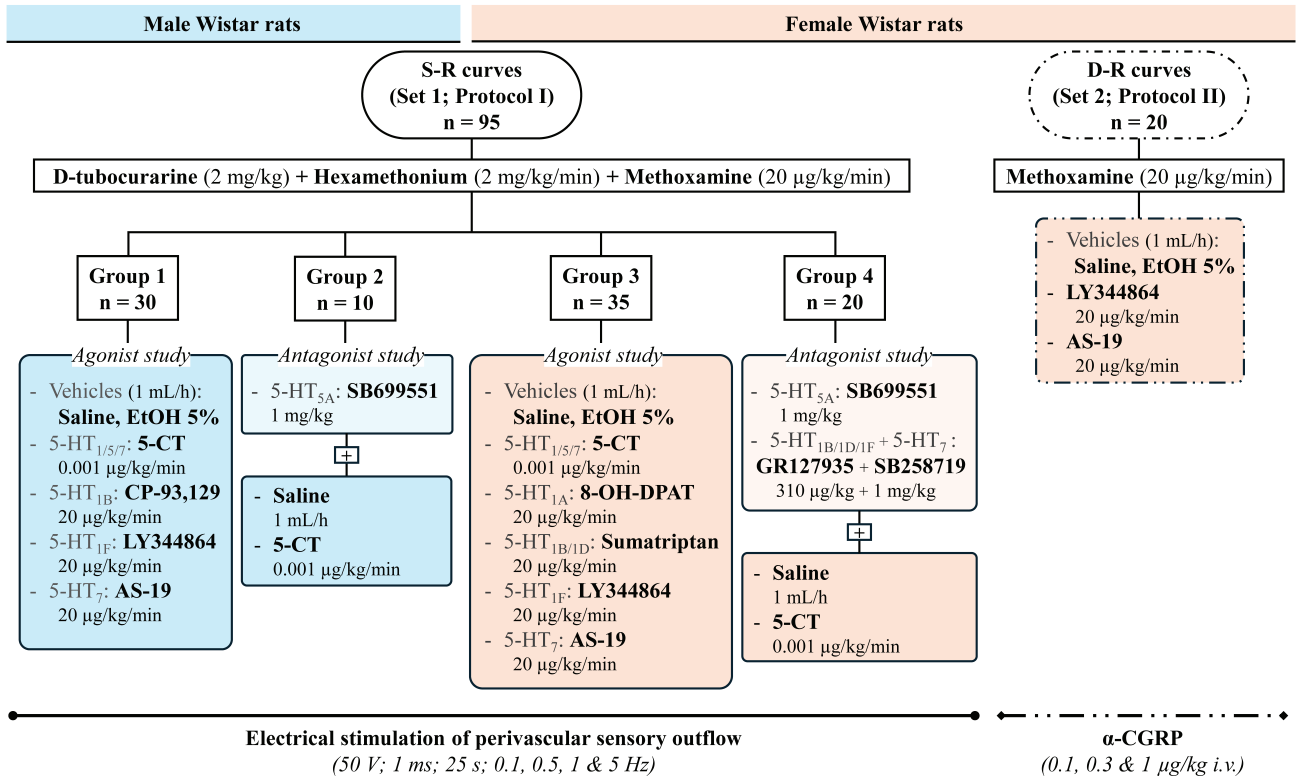


Figure 2

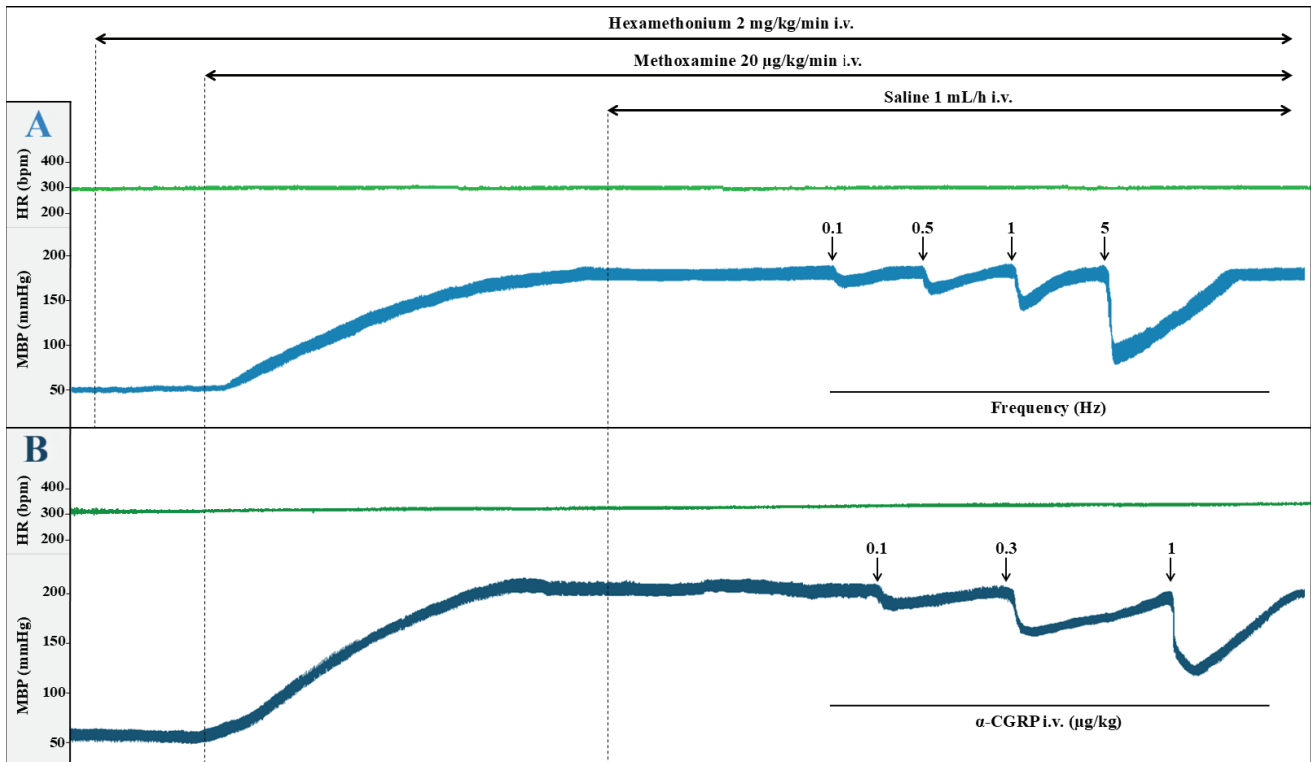


Figure 3

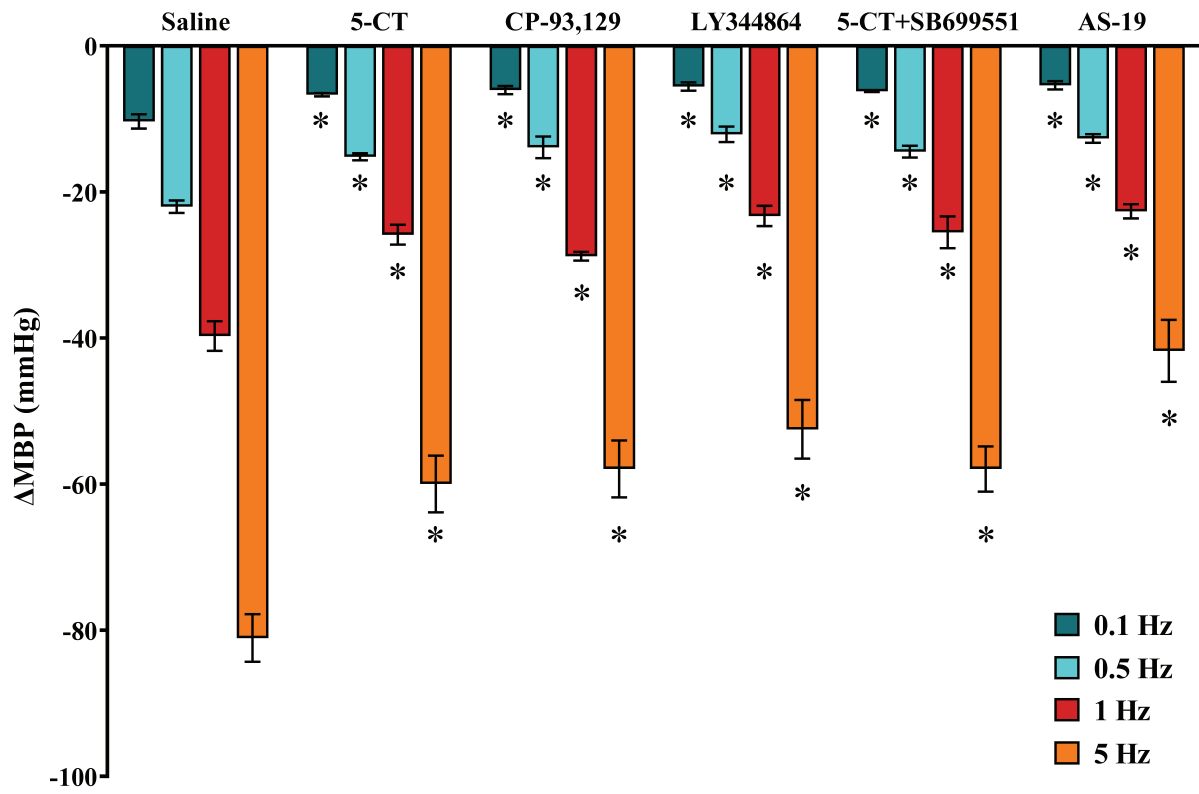


Figure 4

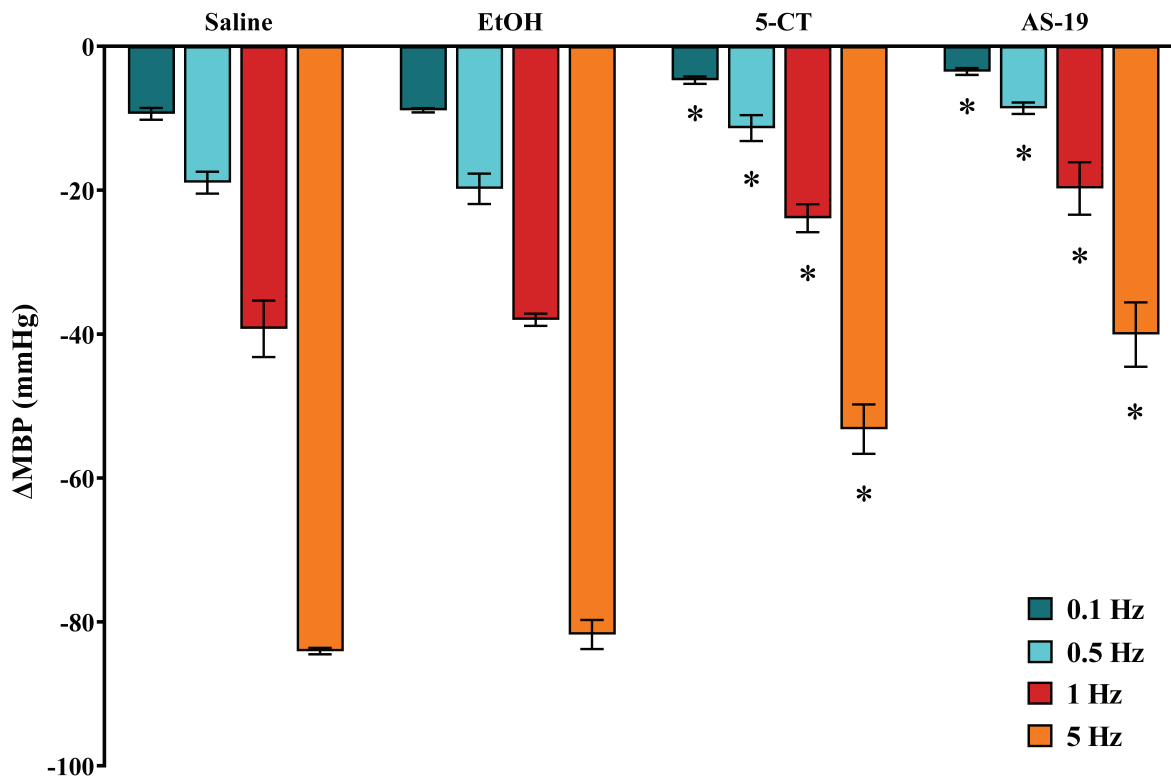


Figure 5

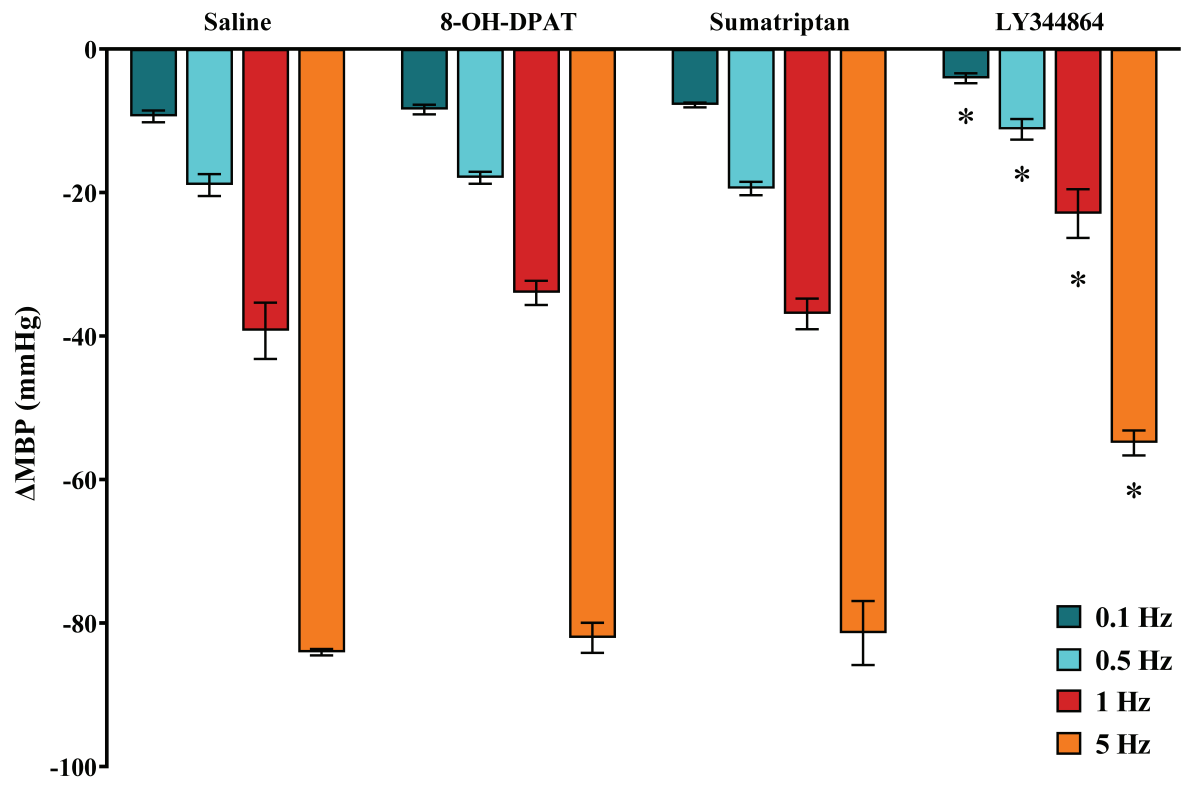
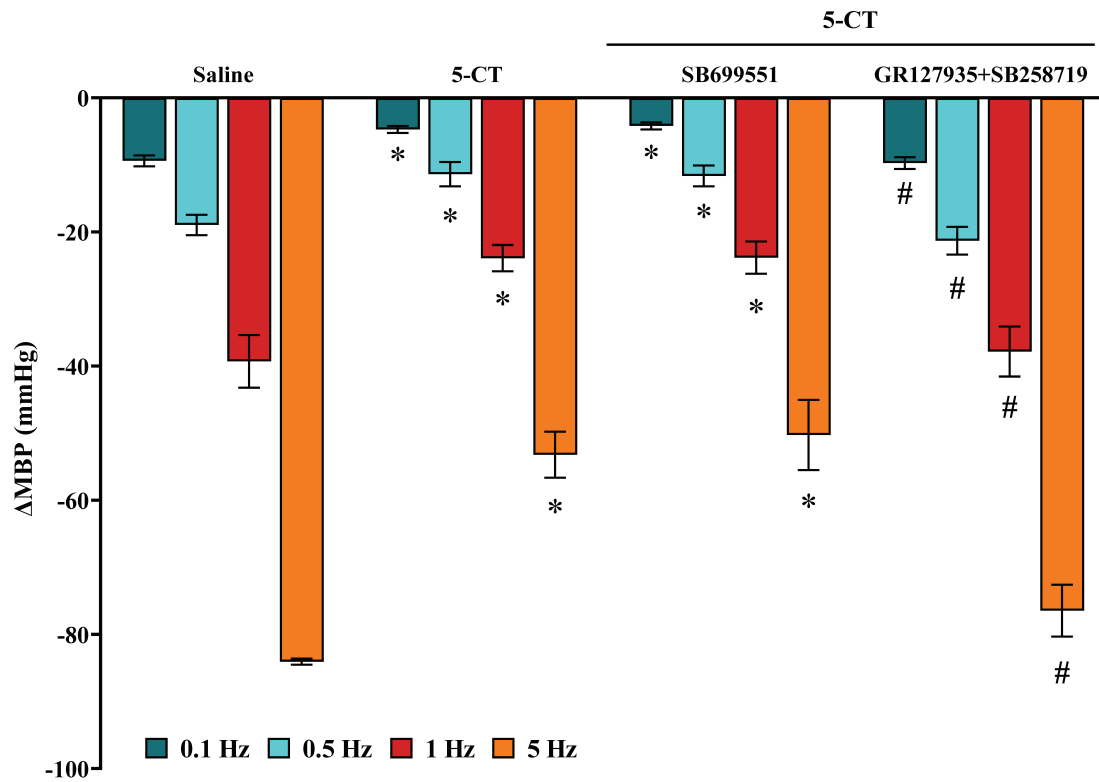


Figure 6



CONCLUSIONES

1. El sexo biológico influye en el control de la presión arterial basal, siendo significativamente menor en ratas hembra que en machos, sin observarse diferencias en la frecuencia cardíaca. Sin embargo, la magnitud de las respuestas vasoconstrictoras inducidas por estimulación simpática total, así como las respuestas vasodilatadoras obtenidas por estimulación de la inervación sensorial CGRPérgica, son similares en ratas de ambos sexos.

2. La 5-HT ejerce una acción inhibidora más potente sobre las respuestas vasopresoras inducidas por estimulación simpática en ratas hembra que en machos. Dicho efecto está mediado por la activación de receptores 5-HT_{1D} presinápticos; por el contrario, la activación de receptores 5-HT_{2A} presinápticos y 5-HT₃ pre y/o postsinápticos potencia la neurotransmisión simpática vascular en hembras.

3. En ratas hembra, la acción simpato-inhibidora vascular de serotonina mediada por la activación de receptores 5-HT_{1D} implica la apertura de canales K_{ATP} a través de la vía de EDH. En cambio, la potenciación de la neurotransmisión simpática vascular por activación de los receptores 5-HT_{2A} está mediada por la liberación de Ang II, mientras que el efecto simpato-potenciador asociado a los receptores 5-HT₃ no involucra vías indirectas.

4. La neurotransmisión sensorial CGRPérgica perivascular está influenciada por el sexo biológico. En ratas de ambos sexos, 5-CT, agonista 5-HT_{1/5/7}, inhibe dicha neurotransmisión CGRPérgica; sin embargo, mientras que en machos este efecto está mediado por la activación de receptores 5-HT_{1B/1F/7} presinápticos, en hembras implica la activación de receptores 5-HT_{1F} presinápticos y 5-HT₇ pre y/o postsinápticos.

En resumen, el sexo biológico influye en el tono vascular basal y en los mecanismos serotoninérgicos que regulan tanto la neurotransmisión simpática vascular como la inervación sensorial CGRPérgica perivascular, modificando el tipo y subtipo de receptores

de 5-HT implicados, su localización en la unión neuroefectora, y las vías indirectas involucradas. Estos hallazgos podrían abrir nuevas estrategias terapéuticas dependientes del sexo en el tratamiento de enfermedades cardiovasculares y neurovasculares.

CONCLUSIONS

1. Biological sex influences the control of basal blood pressure, which is significantly lower in female than in male rats, with no differences observed in heart rate. However, the magnitude of vasopressor responses induced by sympathetic stimulation and the vasodilator responses obtained by stimulation of sensory CGRPergic outflow are similar in both sexes.

2. 5-HT exerts a more potent inhibitory effect on vasopressor responses induced by sympathetic stimulation in female than in male rats. This effect is mediated by the activation of prejunctional 5-HT_{1D} receptors; conversely, activation of prejunctional 5-HT_{2A} and pre and/or postjunctional 5-HT₃ receptors enhances vascular sympathetic neurotransmission in females.

3. In female rats, the vascular sympatho-inhibitory effect of serotonin mediated by 5-HT_{1D} receptor activation involves the opening of K_{ATP} channels through the EDH pathway. In contrast, the potentiation of vascular sympathetic neurotransmission produced by 5-HT_{2A} receptor activation is mediated by Ang II release, whereas the 5-HT₃-induced sympatho-potentiation does not involve any indirect pathway.

4. Perivascular sensory CGRPergic neurotransmission is influenced by biological sex. In rats of both sexes, 5-CT, a 5-HT_{1/5/7} agonist, inhibits this CGRPergic drive; however, in males this effect is mediated by prejunctional 5-HT_{1B/1F/7} receptors, whereas in females it involves prejunctional 5-HT_{1F} and pre and/or postjunctional 5-HT₇ receptors.

In summary, biological sex influences basal vascular tone and the serotonergic mechanisms regulating both vascular sympathetic neurotransmission and perivascular sensory CGRPergic outflow, modifying the 5-HT receptor types and subtypes involved, their localisation at the neuroeffector junction, and the indirect pathways

implicated. These findings may contribute to the development of novel sex-dependent therapeutic strategies for cardiovascular and neurovascular diseases.

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ANEXOS**Anexo I**

Fernández-González, J. F., García-Pedraza, J. Á., Ordóñez, J. L., **Terol-Úbeda, A. C.**, Martín, M. L., Morán, A., & García-Domingo, M. (2023). Renal sympathetic hyperactivity in diabetes is modulated by 5-HT_{1D} receptor activation via NO pathway. *International Journal of Molecular Sciences*, 24(2), 1378. <https://doi.org/10.3390/ijms24021378>

Anexo II

Fernández-González, J. F., García-Pedraza, J. Á., **Terol-Úbeda, A. C.**, Martín, M. L., Morán, A., & García-Domingo, M. (2024). Chronic sarpogrelate treatment improves renal sympathetic hyperactivity in experimental diabetes. *Biomedicine & Pharmacotherapy*, 176, 116814. <https://doi.org/10.1016/j.biopha.2024.116814>

Anexo I

***Renal sympathetic hyperactivity in diabetes is modulated by
5-HT_{1D} receptor activation via NO pathway***

Fernández-González, J. F., García-Pedraza, J. Á., Ordóñez, J. L., **Terol-Úbeda, A. C.**,
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Article

Renal Sympathetic Hyperactivity in Diabetes Is Modulated by 5-HT_{1D} Receptor Activation via NO Pathway

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Abstract: Renal vasculature, which is highly innervated by sympathetic fibers, contributes to cardiovascular homeostasis. This renal sympathetic outflow is inhibited by 5-HT in normoglycaemic rats. Considering that diabetes induces cardiovascular complications, we aimed to determine whether diabetic state modifies noradrenergic input at renal level and its serotonergic modulation in rats. Alloxan diabetic rats were anaesthetized (pentobarbital; 60 mg/kg i.p.) and prepared for in situ autoperfusion of the left kidney to continuously measure systemic blood pressure (SBP), heart rate (HR), and renal perfusion pressure (RPP). Electrical stimulation of renal sympathetic outflow induces frequency-dependent increases (Δ) in RPP (23.9 ± 2.1 , 59.5 ± 1.9 , and 80.5 ± 3.5 mm Hg at 2, 4, and 6 Hz, respectively), which were higher than in normoglycaemic rats, without modifying HR or SBP. Intraarterial bolus of 5-HT and 5-CT (5-HT_{1/5/7} agonist) reduced electrically induced Δ RPP. Only L-694,247 (5-HT_{1D} agonist) reproduced 5-CT inhibition on sympathetic-induced vasoconstrictions, whereas it did not modify exogenous noradrenaline-induced Δ RPP. 5-CT inhibition was exclusively abolished by i.v. bolus of LY310762 (5-HT_{1D} antagonist). An inhibitor of guanylyl cyclase, ODQ (i.v.), completely reversed the L-694,247 inhibitory effect. In conclusion, diabetes induces an enhancement in sympathetic-induced vasopressor responses at the renal level. Prejunctional 5-HT_{1D} receptors, via the nitric oxide pathway, inhibit noradrenergic-induced vasoconstrictions in diabetic rats.

Keywords: 5-HT_{1D} receptor; diabetes; diabetic complications; renal vasculature; sympathetic neurotransmission; vasopressor responses



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1. Introduction

In the 21st century, human well-being is threatened not only by emerging illnesses but also by chronic diseases, which entail a worldwide social, economic, and health problem. Diabetes, which is rapidly increasing all over the world, is one of the most feared ones, not only for the presence of hyperglycaemia as a result of insulin resistance or secretion failure [1], but also for the development of complications that lead to dysfunction and collapse of different organs and systems, such as kidney, blood vessels, or nerves [2–4]. Within this framework, diabetic nephropathy has become one of the most widespread chronic microvascular diabetic complications, which is characterized by progressive renal function decline [5]. Although the kidney is arguably the most important target of microvascular alterations in diabetes, the development of autonomic diabetic neuropathy in patients may contribute to this damage and originate dysregulation of cardiovascular homeostasis [6,7].

In this sense, noradrenergic innervation plays a crucial role both in renal, and in the whole cardiovascular system, regulation. At the renal level, the sympathetic nervous system is the main actor in blood pressure regulation, since it is involved in tubular sodium reabsorption, renin release, and renal vascular resistance. It is widely described that impairment of sympathetic activity is related to increased vasopressor responses, endothelial

alterations, and augmented oxidative stress that come up with vascular damage [8,9]. Moreover, the increased sympathetic outflow may contribute to thrombogenesis as it disturbs platelet activation and aggregation [10]. All these interrelated dysfunctions have been described as diabetic complications [11–13].

The serotonergic system plays a pivotal function in cardiovascular homeostasis, both by directly regulating blood vessel contraction and dilation in different vascular beds such as mesentery, hindquarters, and kidneys [14–16], or by indirectly modulating the vegetative nervous system at the vascular and cardiac levels [17–20]. In this sense, 5-HT causes rat vascular sympatho-inhibition mainly by activation of 5-HT_{1D} receptors [18,19,21]. However, experimental diabetes, as well as pharmacological 5-HT modulation by the antagonism of 5-HT₂ receptor, modify the serotonergic effect on the sympathetic discharge of rat vasculature [22–25], maintaining the main vasodilatory effect of 5-HT. Furthermore, the inhibition of 5-HT reuptake during diabetes crucially modifies serotonergic regulation of the noradrenergic system, showing both vasodilatory and sympatho-excitatory action [26]. Serotonin is also able to play a neuroinhibitor role of sympathetic drive in different vascular beds (mesentery, renal); specifically, at renal level, our research group has shown that prejunctional 5-HT_{1D} receptor activation inhibits renal vasopressor noradrenergic outflow, involving nitric oxide (NO) production [18].

Considering that (a) diabetes is linked to long-term micro- and macrovascular problems that impair large blood vessels and the kidney (diabetic nephropathy), (b) the kidney is richly innervated by sympathetic fibers which are dysregulated during diabetes (diabetic neuropathy), (c) 5-HT_{1D} receptor activation, via the NO pathway, modulates noradrenergic input at renal level in normoglycaemic state, and that (d) presently, there is an active search for new therapeutical approaches to minimize renal damage in diabetes, it becomes urgent to explore the serotonergic system's role in these complications to find new possible pharmacological targets. Thus, our work intended to determine whether the serotonergic system modulates noradrenergic input in rat renal vascular beds during diabetes, establishing the receptor type/subtype(s), nature, and possible endogenous mediators involved.

2. Results

2.1. Systemic Haemodynamic Parameters in Diabetic Rats

The administration of alloxan induced a hyperglycaemic state after two days; glycaemia increased from 112 ± 3 mg/dL (day 0) to 548 ± 7 mg/dL (day 2) ($p < 0.05$ vs. day 0). The hyperglycaemic state was maintained until day 28: 554 ± 6 mg/dL ($p < 0.05$ vs. day 0). In relation to body weight, there was significant decrease at day 2: 288 ± 2 g vs. 305 ± 1 g at day 0 ($p < 0.05$ vs. day 0) that was restored at day 28 (304 ± 3 g; no statistical differences vs. day 0).

After anaesthesia and surgical procedure, basal systemic blood pressure (SBP), renal perfusion pressure (RPP), and heart rate (HR) were 97 ± 3 mm Hg, 113 ± 2 mm Hg, and 308 ± 6 beats/min, respectively. These values were not significantly modified after i.v. administration of saline, other vehicles (ethanol (EtOH) 5%, HCl 0.01 M, or PEN, consisting in a mixture of polyethylene glycol 33%, EtOH 33%, 0.2 M NaOH 34%), serotonergic receptor type/subtype antagonists, glibenclamide, ODQ or indomethacin. Intraarterial administration of vehicles (saline, EtOH 5% or HCl 0.01 M; 10 μ L) or serotonergic receptor type/subtype agonists did not modify per se any of the basal haemodynamic parameters. Only 5-HT at doses of 0.1 μ g/kg and 0.4 μ g/kg or α -methyl-5-HT (0.4 μ g/kg) provoked significant increases in RPP (up to 142 ± 8.9 , 175 ± 12.0 and 158 ± 17.2 mm Hg, respectively) that came back to basal values within 2 min.

2.2. Effects of Saline or 5-HT on the Electrically Induced Δ RPP in Diabetic Rats

The increases in RPP (Δ RPP) induced by electrical stimulation were frequency-dependent (23.9 ± 2.1 , 59.5 ± 1.9 and 80.5 ± 3.5 mm Hg at 2, 4 and 6 Hz, respectively; stimulation response (S-R) curve E0). These vasopressor responses were due to selective stimulation

since no effects were observed in HR or SBP. These values were not altered after i.a. administration of saline (Table 1), EtOH 5% or HCl 0.01 M.

Table 1. Effect of saline and 5-HT on the increases in renal perfusion pressure induced by renal sympathetic stimulation in diabetic rats.

Compounds		Stimulation Frequencies (Hz)		
		2	4	6
Saline (10 μ L)		24.0 ± 1.6	58.1 ± 2.7	81.0 ± 4.2
5-HT (μ g/kg, i.a.)	0.0125	19.5 ± 4.2 *	40.6 ± 7.9 *	61.0 ± 9.2 *
	0.1	16.2 ± 2.8 *	32.7 ± 3.8 *	53.7 ± 6.3 *
	0.4	11.8 ± 6.0 *	26.3 ± 9.7 *	51.2 ± 9.9 *
		ΔRPP (mm Hg)		

Effect of i.a. bolus injections of saline (10 μ L, $n = 5$) or 5-HT (0.0125, 0.1 and 0.4 μ g/kg; $n = 5$ for each treatment) on the increases in renal perfusion pressure (ΔRPP) evoked by renal sympathetic stimulation (2, 4 and 6 Hz) in the in situ-autoperfused kidney of diabetic rats. * $p < 0.05$ vs. saline.

5-HT at the doses of 0.0125, 0.1 and 0.4 μ g/kg, administered intraarterially, significantly inhibited the ΔRPP in a dose- and frequency-dependent way (Table 1).

2.3. Effect of Serotonergic Receptor Type Agonists on the Increases in RPP Induced by Electrical Stimulation of Renal Periaxillary Nerves in Diabetic Rats

The i.a. administration of 0.4 μ g/kg of α -methyl-5-HT (α -m-5-HT), 1-phenylbiguanide (1-PBG), cisapride and AS-19 (5-HT₂, 5-HT₃, 5-HT₄ and 5-HT₇ receptor agonists, respectively) did not evoke any effect in the electrically induced ΔRPP (Figure 1). The only drug that reproduced serotonin inhibitory action was 5-CT, a 5-HT_{1/5/7} receptor agonist (Figure 1). The 5-CT inhibitory effect (0.1, 0.4 and 1.0 μ g/kg) was produced in a dose- and frequency-dependent way (Figure 2), as occurred with 5-HT.

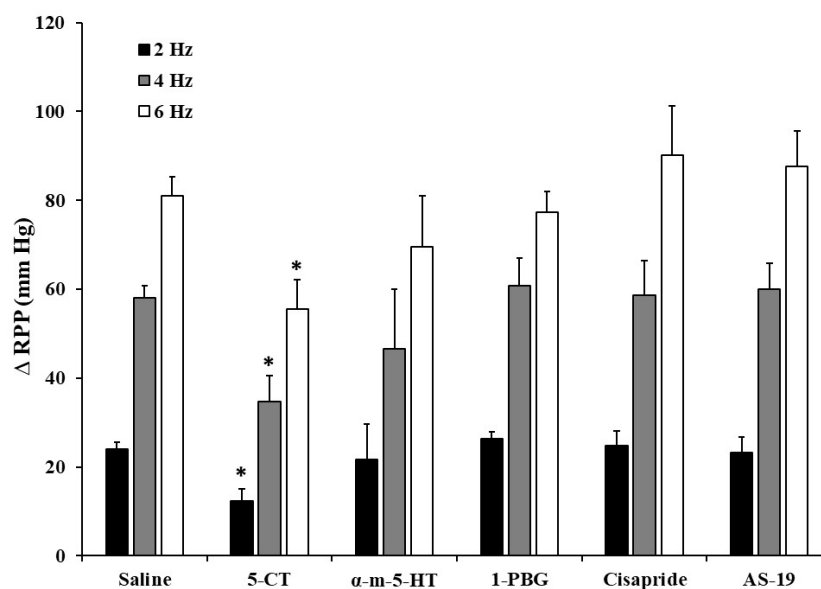


Figure 1. Effect of i.a. administration of serotonergic receptor agonists on the electrically induced increases in renal perfusion pressure in diabetic rats. Effect of i.a. bolus of saline (10 μ L) or the serotonergic receptor agonists 5-CT (5-HT_{1/5/7}), α -methyl-5-HT (5-HT₂), 1-PBG (5-HT₃), cisapride (5-HT₄), or AS-19 (5-HT₇) (0.4 μ g/kg each agonist) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves in diabetic rats. * $p < 0.05$ vs. saline. α -m-5-HT, α -methyl-5-HT; 1-PBG, 1-phenylbiguanide.

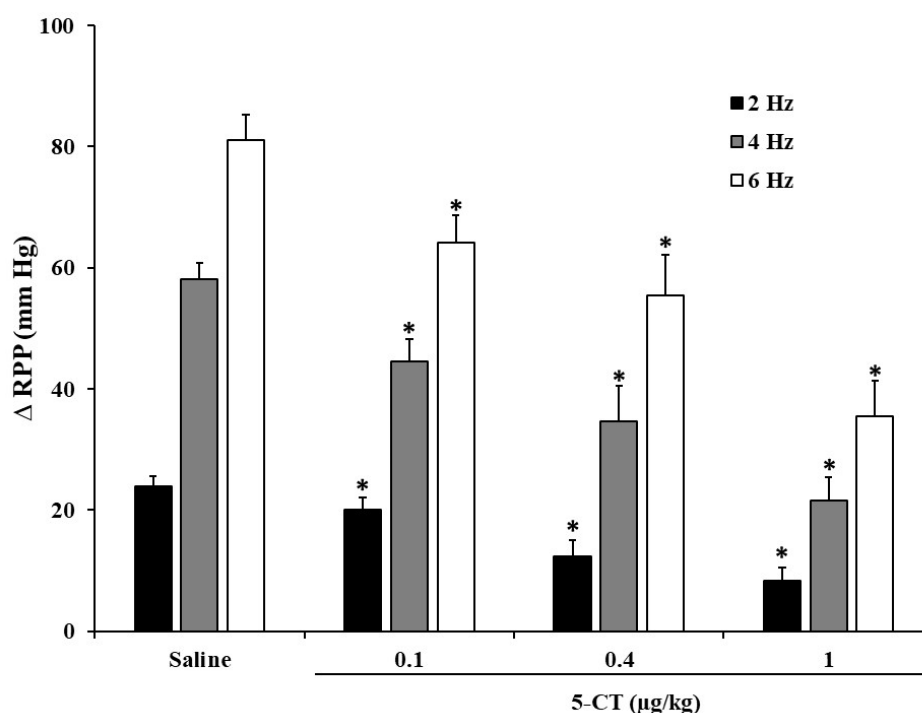


Figure 2. Effect of i.a. bolus administration of increasing doses of 5-CT on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves in diabetic rats. Effect of i.a. bolus of saline (10 μ L), and the serotonergic 5-HT_{1/5/7} receptor type agonists, 5-CT (0.1, 0.4 and 1.0 μ g/kg), on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves in diabetic rats. * $p < 0.05$ vs. saline.

2.4. Changes on the Electrically Induced Δ RPP by the i.a. Administration of 5-HT₁ Receptor Subtype Agonists in Diabetic Rats

Intraarterial administration of neither 8-OH-DPAT, CGS-12066B, nor BRL-54443 (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1F}, respectively, at a dose of 0.4 μ g/kg each) induced any change in the electrically induced renal vasopressor responses in diabetic rats (Figure 3). In contrast, only the i.a. bolus administration of L-694,247 (0.4 μ g/kg), a selective 5-HT_{1D} receptor agonist, was able to reproduce the 5-HT and 5-CT inhibition of the Δ RPP produced by renal periaarterial nerve stimulation at all stimulation frequencies (Figure 3).

2.5. Influence of Intravenous Administration of SB 699551 or LY310762 on the Saline or 5-CT Effect on the Renal Vasopressor Responses Induced by Electrical Stimulation in Diabetic Rats

The i.v. administration of neither the selective 5-HT_{5A} receptor antagonist (SB 699551) nor the selective 5-HT_{1D} antagonist (LY310762) (1 mg/kg each one) (nor their vehicle, saline 1 mL/kg i.v.) modifies per se the renal pressor responses induced by sympathetic electrical stimulation in diabetic rats.

In any case, i.a. administration of 0.4 μ g/kg of 5-CT in the presence of SB 699551 induced an inhibition of the vasopressor response (Figure 4), which means that 5-HT_{5A} is devoid of any action on the inhibitory effect produced by 5-CT.

The i.v. administration of the 5-HT_{1D} antagonist, LY310762, was able to block the inhibitory effect provoked by 5-CT on the sympathetic vasopressor responses in the kidney of diabetic rats (Figure 4).

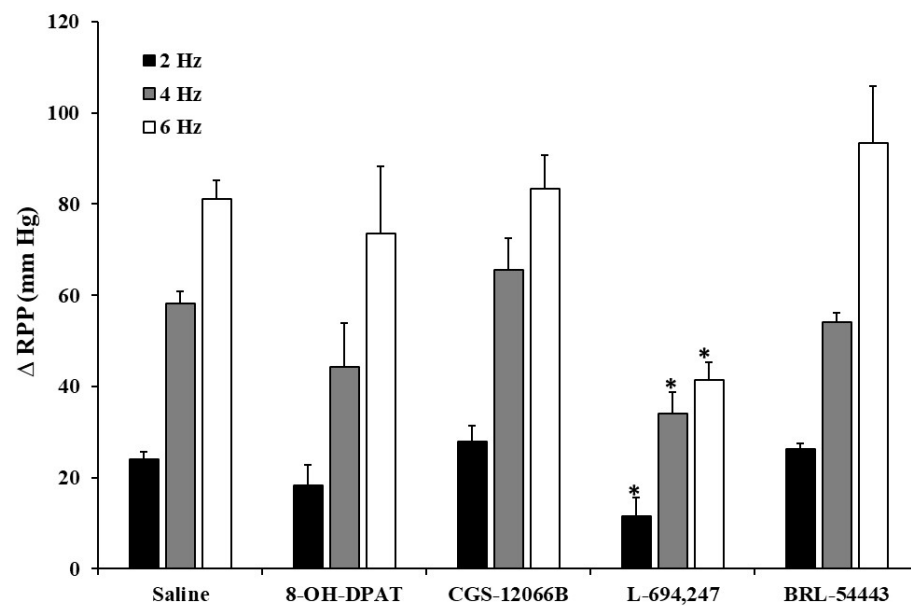


Figure 3. Effect of i.a. bolus administration of 5-HT₁ receptor subtype agonists on the renal vasopressor responses in diabetic rats. Effect of i.a. bolus of saline (10 μ L), and the serotonergic receptor subtype agonists 8-OH-DPAT (5-HT_{1A}), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}), and BRL-54443 (5-HT_{1F}) (0.4 μ g/kg each agonist), on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves in diabetic rats. * $p < 0.05$ vs. saline.

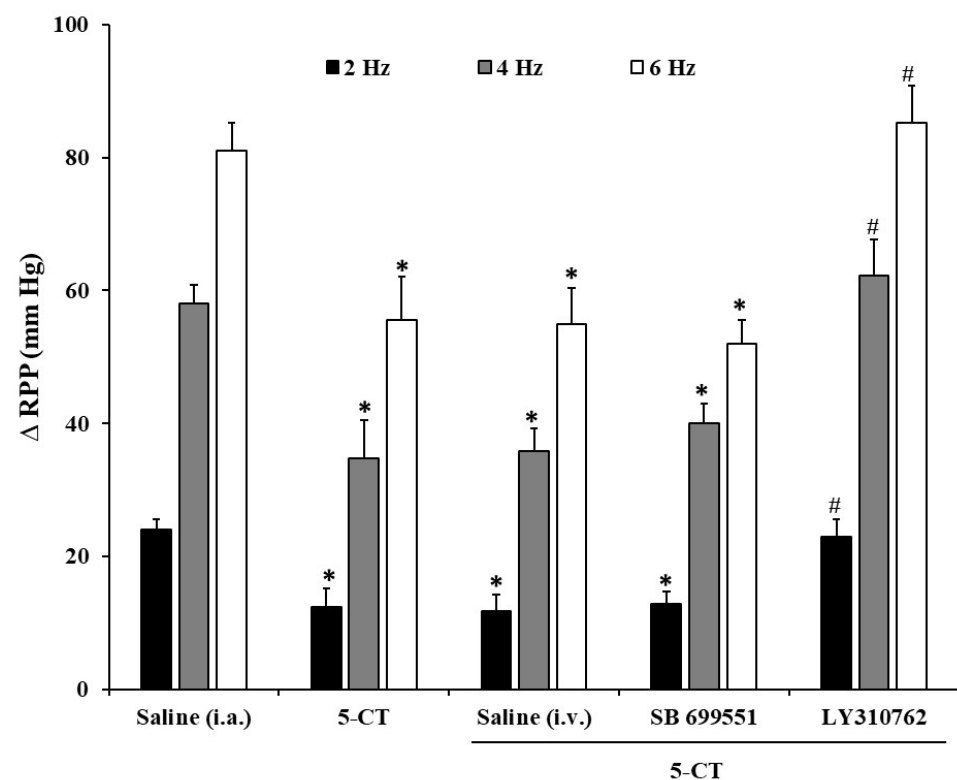


Figure 4. Effect of i.v. pretreatment of serotonergic receptor subtype antagonists on the 5-CT inhibitory effect of the renal sympathetic-induced vasopressor responses in diabetic rats. Effect of i.a. bolus of saline (10 μ L) or 5-CT (0.4 μ g/kg) in the absence or the presence of i.v. pretreatment with saline (1 mL/kg), SB 699551, or LY310762 (1 mg/kg each) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves in diabetic rats. * $p < 0.05$ vs. saline i.a.; # $p < 0.05$ vs. 5-CT.

2.6. Role of Saline or L-694,247 (5-HT_{1D} Receptor Agonist) on the Renal Vasopressor Responses Induced by i.a. Administration of Exogenous Noradrenaline (NA) in Diabetic Rats

The dose-response (D-R) curve E'0 induced by the i.a. administration of NA was 37.1 ± 3.7 , 73.8 ± 10.3 , and 109.6 ± 13.2 mm Hg at NA i.a. bolus of 0.05, 0.1, and 0.4 µg/kg doses, respectively. The increase in RPP (D-R curve E'0) caused by the i.a. administration of exogenous NA (control group) remained stable after i.a. bolus of saline (Figure 5). Interestingly, i.a. bolus of L-694,247 (0.4 µg/kg) failed to inhibit the pressor responses to i.a. administration of exogenous NA (Figure 5).

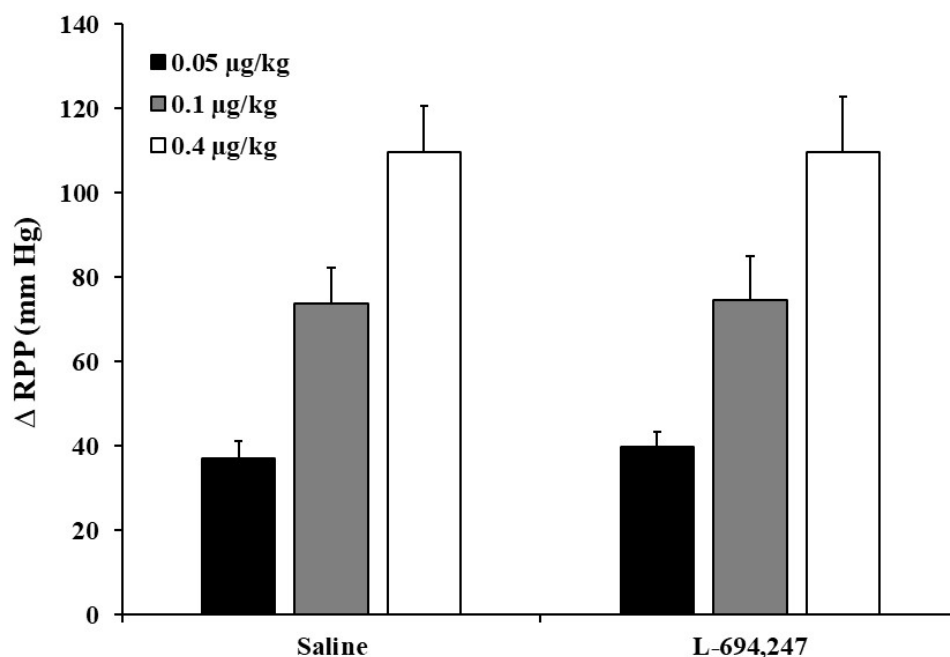


Figure 5. Effect of L-694,247 on the noradrenaline-induced increases in renal perfusion pressure in diabetic rats. Effect of i.a. bolus injections of saline (10 µL) or L-694,247 (0.4 µg/kg) on the increases in renal perfusion pressure (ΔRPP) evoked by i.a. administration of exogenous noradrenaline (0.05, 0.1 and 0.4 µg/kg) in the in situ-autoperfused kidney of diabetic rats. No statistical significance.

2.7. Influence of Intravenous Administration of Vehicle, ODQ, Glibenclamide, or Indomethacin on the Saline- or L-694,247-Effect on the Renal Vasopressor Responses Induced by Electrical Stimulation in Diabetic Rats

The i.v. bolus of neither the vehicles (saline, PEN; 1 mL/kg each), indomethacin (2 mg/kg), glibenclamide (20 mg/kg), nor ODQ (10 µg/kg) alters per se the ΔRPP induced by electrical stimulation at 2, 4, and 6 Hz.

L-694,247 inhibition (0.4 µg/kg, i.a.) of electrically induced renal vasoconstrictions was exclusively abolished by i.v. administration of 10 µg/kg of the guanylyl cyclase inhibitor, ODQ (Figure 6). In contrast, both the i.v. administration of glibenclamide (a specific inhibitor of ATP-sensitive K⁺ channels) and indomethacin (a non-selective cyclooxygenase (COX) inhibitor) failed to reverse the L-694,247 inhibitory action on the renal vasopressor responses of diabetic rats.

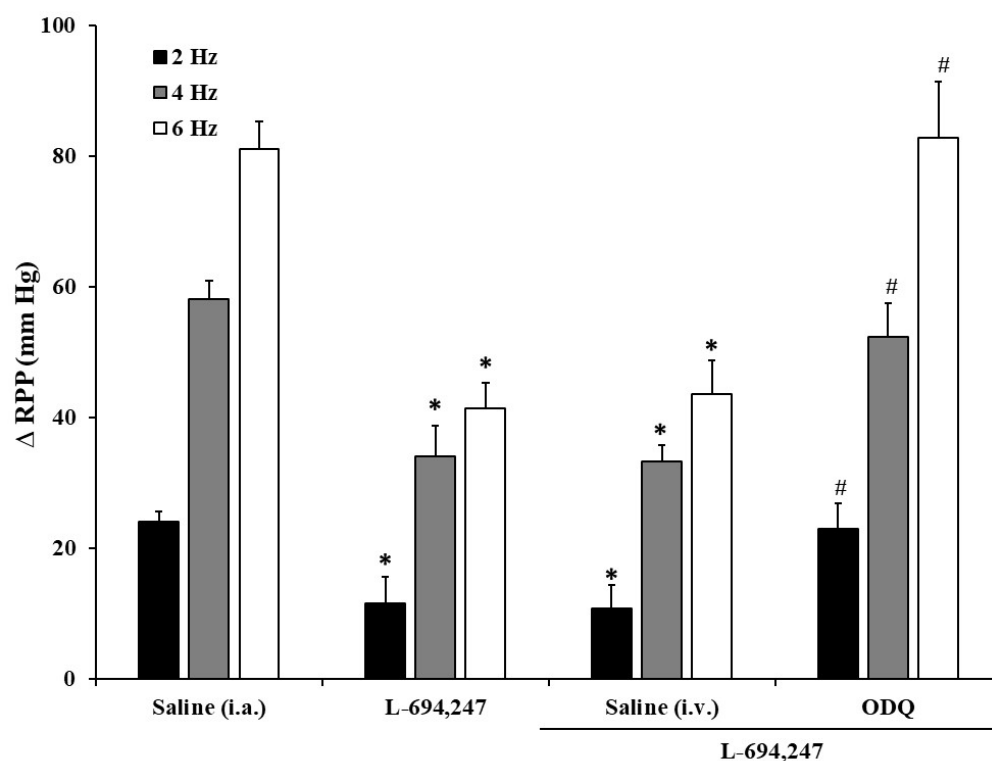


Figure 6. Effect of i.v. administration of ODQ on the 5-HT_{1D} agonist inhibitory action of the renal vasopressor responses in diabetic rats. Effect of i.a. bolus of saline (10 μ L) or L-694,247 (0.4 μ g/kg) in the absence or the presence of i.v. pretreatment with saline (1 mL/kg) or ODQ (10 μ g/kg) on the vasopressor responses elicited by electrical stimulation of renal sympathetic nerves in diabetic rats. * $p < 0.05$ vs. saline i.a.; # $p < 0.05$ vs. L-694,247.

3. Discussion

Our work brings to light that diabetes in rats augments renal vasopressor responses induced by sympathetic input, exposing that serotonergic inhibition of noradrenergic outflow, in the in situ-autoperfused rat kidney, is mediated by 5-HT_{1D} receptor subtype activation that involves the NO pathway (Figure 7).

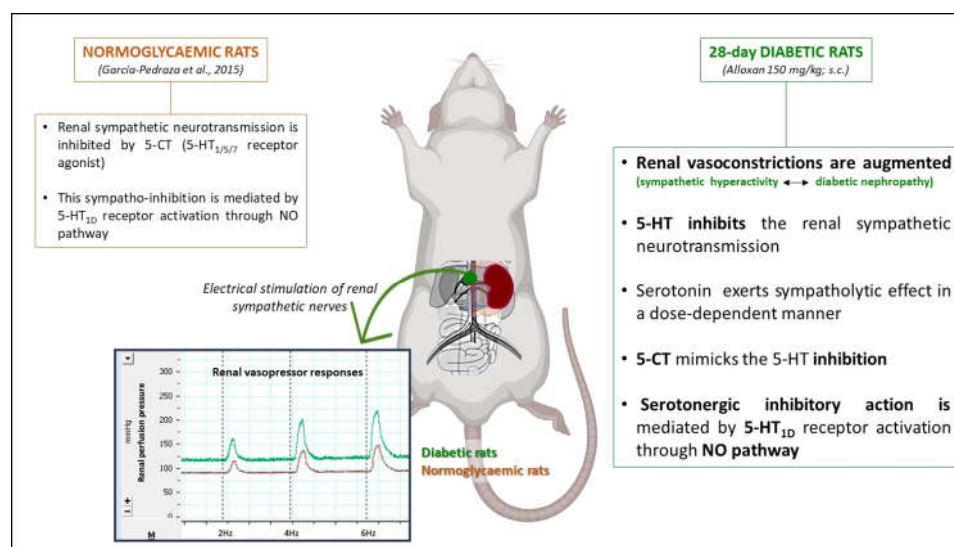


Figure 7. Scheme summarizing the main outcomes in 5-HT modulation of the renal sympathetic neurotransmission in normoglycaemic and diabetic animals [18].

To study these actions, we used a chemically induced diabetic model in rats [27]. Alloxan administration induces a pattern similar to human type 1 diabetes due to its selective damage to the pancreatic β -cells in the islets of Langerhans that produce insulin. This drug caused increased glycaemia after day 2 post-injection, with a consequent loss of weight. Hyperglycaemia stayed at the same rate during the 28-day period, whereas weight slightly increased, reaching values similar to those prior to alloxan administration [17,25,26].

After anaesthesia, our technique of in situ autoperfusion of rat kidney is a specific in vivo experimental model, first tuned up by Fink and Brody (1978) [28] and modified by us [16,18], which continuously measures SBP, HR, and renal blood flow, assessing quick changes in the latter due to i.a. drug administration or to sympathetic periarterial renal nerve stimulation. Basal SBP in diabetic animals (current data) is similar to normoglycaemic animals [18]; however, RPP is significantly higher, while HR is diminished. These differences may be explained by the renal damage originated by the hyperglycaemia, which is usually accompanied by renal microvascular alterations, glycosuria [29], and heart compensatory mechanisms that lead to alterations in HR. Although the development of hypertension during diabetes is well-known, in our 28-day diabetic model, in anaesthetized rats, there is no change in SBP compared to normoglycaemic animals [18], probably due to the duration of diabetic state, since we have already described increased SBP in a 56-day diabetic model using the same experimental technique [30]. None of the i.v. or i.a. administered drugs modified these basal parameters, with the exception of 5-HT at high doses and α -m-5-HT (5-HT₂ receptor agonist), which augmented RPP, coming back to basal values within 2 min. This fact has been previously shown by us [18] and may be explained by the vasoconstrictor role of this 5-HT receptor type at renal level [16,30–32].

The renal vascular bed is a master modulator of blood pressure homeostasis. Thus, diabetic nephropathy originates renal dysfunction that may induce increased renal vascular resistance and, subsequently, chronic kidney damage, that runs in hypertension and vascular altered homeostasis. In this sense, the adrenergic system plays a crucial role, since sympathetic nerves extensively innervate the kidney [33,34]. Renal nerves follow the renal arteries and innervate not only the vasculature but also the juxtaglomerular apparatus and the basement membrane of epithelial cells within the nephron. Thus, electrical stimulation of renal periarterial nerves induces frequency-dependent increases in RPP due to NA release [18]. The Δ RPP are augmented in diabetic animals in comparison with normoglycaemic individuals [18], which suggests the development of diabetic neuropathy, with a marked sympathetic hyperactivity that, in the long term, exacerbates cardiovascular risk in diabetic patients [35,36]. In addition, the fact that increases in renal vasopressor responses after i.a. exogenous NA are higher in diabetic than in normoglycaemic animals supports both the development of diabetic peripheral neuropathy and microvascular complications in the kidney [37,38]. Our group has already shown cardiovascular sympathetic overactivity as a marker of cardiovascular impairment in diabetes [22,25,26] or hypertension [39].

The serotonergic system acts as modulator, both potentiating and inhibiting noradrenergic or vagal outflow at cardiac and vascular levels in different experimental models [21–24,40,41]. Furthermore, our group has described the serotonergic inhibitory action of noradrenergic input at mesenteric and renal levels [18,19,39]. Moreover, in diabetic animals, 5-HT is also able to reduce vasopressor responses to sympathetic stimulation in the kidney (current data). In any case, it is important to remark that serotonin doses utilized are higher than in normoglycaemic animals [18], probably due to diabetic damage both at neurohumoral and microvascular levels [2–4], which would make it necessary to increase serotonin doses to be able to reduce vasopressor responses induced by sympathetic hyperactivity [8,9]. These results show the importance of the peripheral effects of this biogenic amine, and in general, of the whole serotonergic system, and open new frontiers in the search for novel treatments in pathologies, such as renovascular and neuropathic diabetic complications.

To further analyse the 5-HT receptor type involved in this serotonergic inhibitory effect on renal noradrenergic input at vascular level, we administered selective agonists (i.a. bolus). Our results allowed us to conclude that only the selective 5-HT_{1/5/7}, 5-CT, was able to reproduce the serotonin inhibitory effect in a dose-dependent manner as serotonin did, as occurred in the normoglycaemic model [18], whereas neither α -methyl-5-HT, 1-PBG, cisapride, nor AS-19 (5-HT₂, 5-HT₃, 5-HT₄ and 5-HT₇ receptor agonists, respectively) modified the electrically induced renal vasopressor responses. These data differ from the serotonergic sympatholytic effect at vascular level in a pharmacological-altered model, where both 5-HT₁ and 5-HT₇ receptor types were involved [24] in the inhibitory action. As currently, no selective 5-HT_{5A} receptor agonist is described, to study this receptor type effect, we utilised a 5-HT_{5A} selective serotonergic antagonist (SB 699551) prior to 5-CT administration [25]. As SB 699551 did not reverse 5-CT inhibitory action, we could devoid 5-HT_{5A} receptor type of any sympatholytic effect in the kidney vasculature of diabetic animals and confirm that the sole serotonergic receptor type involved in the sympatho-inhibitory effect on vasopressor responses is the 5-HT₁ receptor type, which is in agreement with previous results by our group at vascular level in normoglycaemic and diabetic rats [21,22], and at renal or mesenteric levels in normoglycaemic rats [18,19], but differs from those obtained in the mesentery of hypertensive rats, where 5-HT₄ was the responsible receptor type of the serotonergic inhibitory effect [39].

In the search for more specific therapeutic approaches in diabetic complications, we determined the 5-HT₁ receptor subtype/s involved in this inhibitory effect, and established that only the receptor 5-HT_{1D} is implicated in serotonergic inhibitory effect on electrically induced renal vasoconstrictions, since L-694,247 (5-HT_{1D} agonist) [42] reproduced 5-HT and 5-CT inhibitory action, but this selective 5-HT_{1D} agonist was not able to reduce vasoconstrictor responses induced by i.a. administration of exogenous NA; thus, we propose a prejunctional locus for 5-HT_{1D} receptors. To confirm these results, we i.v. administered LY310762 (a 5-HT_{1D} receptor antagonist) [43] prior to i.a. bolus of 5-CT, which completely blocked its sympatholytic effect. Hence, we disclose that prejunctional 5-HT_{1D} receptor activation is the main factor responsible for the serotonergic sympatholytic effect at renal level in diabetic rats. The 5-HT_{1D} receptor, at prejunctional locus, has already been described as sympatholytic at the renal level [18], and at cardiac and vascular levels, in pithed rats as well [21,40] in normoglycaemic animals. In any case, it is worthwhile to mention that short-term diabetic state modifies the serotonergic profile involved in the sympatho-modulation, unmasking peripheral prejunctional 5-HT_{1A} and 5-HT_{5A} at vascular and cardiac level, respectively, in pithed rats [22,41].

Endothelial dysfunction contributes to the pathogenesis of microvascular diabetic complications. Thus, considering that (a) endothelial relaxation depends on several agents such as NO [44], prostacyclin [45], and/or endothelium-derived hyperpolarizing factor (EDHF) [46], (b) endothelial-dependent vasorelaxation is altered in diabetes [47], and (c) the NO synthesis was involved in 5-HT_{1D} renal sympatho-inhibitory effect in normoglycaemic rats [18], we determined the possible role of these vasodilator agents in the serotonergic inhibitory action in diabetic rats using ODQ (a guanylyl cyclase inhibitor), indomethacin (a COX_{1/2} inhibitor), or glibenclamide (an ATP-sensitive K⁺ channels blocker) [48–50]. As was observed in normoglycaemic animals, only the NO pathway is involved in the 5-HT_{1D} renal sympatholytic action in diabetes, which is also in agreement with previous results by us in the diabetic pithed rat model [22,51]. Although there is evidence that, during diabetes, microvascular endothelial dysfunction is primarily characterized by impaired endothelial repair due to enhanced oxidative stress and a decreased release of NO [52], our present results show that at renal level, the NO pathway maintains its relevant role in mediating serotonergic modulation of noradrenergic-induced vasopressor responses.

This work has some constraints: taking into account our experience, experiments were only performed in males; thus, there is no sex bias. Although haemodynamic parameters (SBP and HR) are in the normal range for anaesthetized rats, our technique requires invasive surgery, and finally, we should mention that renal sympathetic nerve activity is not directly

measured, but indirectly estimated through the induced changes in renal perfusion pressure. In any case, it is noteworthy that this work, in basic pharmacology, has highlighted that serotonergic modulation of NA release at renal level, via 5-HT_{1D} receptor activation and, consequently, NO pathway in diabetic individuals, may be a therapeutic approach to reduce sympathetic hyperactivity closely tied to renal microvascular complications. Currently, renal sympathetic denervation has become, despite its side effects (renal artery injury and deterioration of kidney function, among others), one of the treatment options in pathologies with increased sympathetic nerve activity, such as cardiovascular complications of diabetes or hypertension [53,54]. Our results set down the basis to use serotonergic inhibition of noradrenergic outflow as a feasible alternative to complete renal sympathetic ablation in cardiovascular and renal complications derived from diabetes.

4. Materials and Methods

4.1. Drugs Employed

The compounds and suppliers used in the experiments described throughout the manuscript were: sodium pentobarbital (Dolethal®; Vetoquinol, Madrid, Spain); heparin sodium from Rovi (Madrid, Spain); atropine sulphate (Scharlau, Barcelona, Spain); indomethacin (Acofarma, Barcelona, Spain); alloxan monohydrate, 1-PBG, BRL-54443 maleate salt, glibenclamide, NA bitartrate, and 5-HT hydrochloride (Merck Life Sciences S.L.U., Madrid, Spain); 5-CT, 8-OH-DPAT, α -methyl-5-HT, AS-19, cisapride, CGS-12066B, L-694,247, LY310762 hydrochloride, ODQ, and SB 699551 (Tocris Bioscience, Bristol, UK).

All the compounds were dissolved in saline at the time of experimentation, except AS-19 (dissolved in EtOH 5%), cisapride (0.01 M HCl), and both indomethacin and glibenclamide (dissolved in a mixture of PEN). None of the vehicles alter either RPP or SBP.

4.2. General Methods

A total of 135 male Wistar rats (275–325 g) became diabetic by a single subcutaneous injection of alloxan (150 mg/kg) and were housed for 28 days in a 12/12 h light–dark cycle under specific temperature (22 ± 2 °C) and humidity (50%) conditions, with free availability of food and tap water.

Before alloxan administration and until day 28, animals were weighed and non-fasting blood glucose levels were periodically measured with a glucometer (Accu-chek® Aviva, Roche Diagnostics; Barcelona, Spain). Rats with blood glucose levels <250 mg/dL (non-diabetic) were discarded.

After 28 days of the diabetes induction, animals were anaesthetized (sodium pentobarbital, 60 mg/kg, i.p.) and prepared for the in situ autoperfusion of left kidney as previously described by our group [16,18]: catheters were placed in the trachea, the right and left carotid arteries (to measure SBP and HR, and RPP, respectively), and jugular and femoral veins (for i.v. administration).

The in situ perfusion of the left kidney was performed according to the method of Fink and Brody (1978) [28] modified by Morán et al. (1997) [31]. The renal vascular bed was perfused using an extracorporeal circuit and a constant flow Gilson peristaltic pump from the left carotid artery to the left renal artery [16,18,28,30,55], which was exposed by midline laparotomy and deflection of the intestines to the right side of the animal. A loose tie was placed around the aorta 1 cm below the left renal artery and 1 cm above the iliac bifurcation. To prevent blood clots, heparin sodium was administered (5 mg/kg, i.v.) and intravenous infusion of saline (0.9% NaCl) was initiated at a rate of 2 mL/h and continued throughout the experiment. Atropine (1 mg/kg) was administered intravenously before the saline infusion in order to block potential cholinergic effects.

Both the distal portion of the external circuit coming from the left carotid and the right carotid were connected to two different pressure transducers connected to an e-corder 410 amplifier (Model ED410, Cibertec, Spain) for measurement of the RPP and SBP and HR, respectively.

At the beginning of each experiment, the flow was adjusted to make the RPP equal to the SBP. Flow was constant throughout the experiments; thus, variations in the RPP reflected variations in renal vascular resistance. The flow rate through the renal vascular beds ranged from 2 to 2.9 mL/min [18].

The diabetic animals were initially divided into two main clusters (see Figure 8), so that the effects produced by different 5-HT agents could be investigated on the vasopressor responses induced by: (i) electrical stimulation of sympathetic renal nerves (S-R group, $n = 125$); or (ii) i.a. bolus injections of exogenous NA (D-R group, $n = 10$). In the vasopressor S-R curves and D-R curves elicited by electrical stimulation and exogenous NA, respectively (see Figure 8), each response was elicited under unaltered values of resting blood pressure. The electrical stimuli (12.5 ± 2.5 V; 1 ms; 2, 4 and 6 Hz), as well as dosing with NA (0.05, 0.1 and 0.4 $\mu\text{g/kg}$), were given using sequential schedule at 3–5 min intervals. At each frequency, stimulation was continued until the response was maximal (5–10 s), and basal perfusion pressure was restored immediately after interruption of the stimulation.

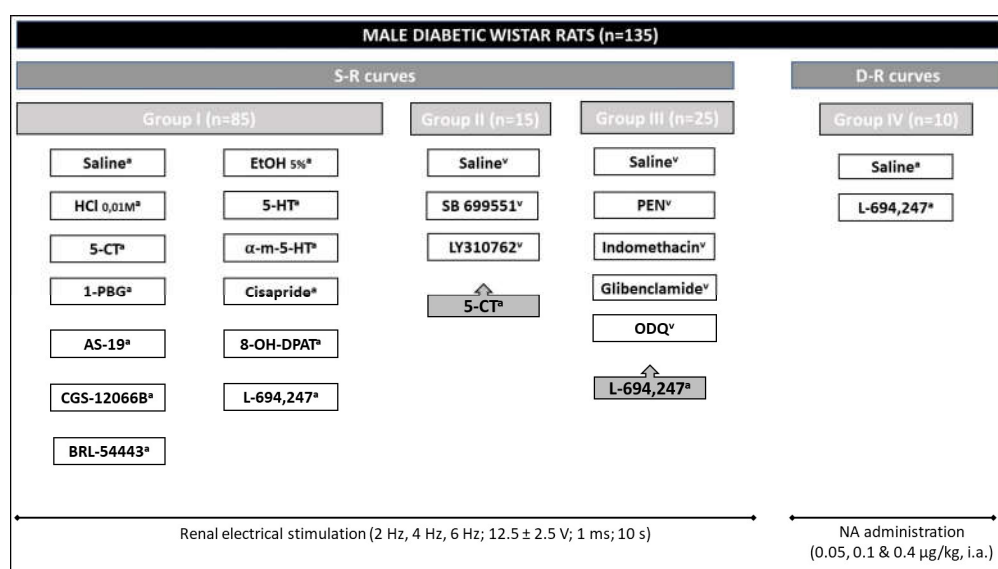


Figure 8. Scheme of experimental protocols, showing the number of animals used. Experimental protocols showing the two main sets of diabetic animals and the different groups used in these experiments, in which renal vasopressor responses are obtained by renal nerve sympathetic stimulation (S-R curves) or i.a. bolus of noradrenaline (NA) (D-R curves). ^a i.a. administration; ^v i.v. administration; α -m-5-HT: α -methyl-5-HT; S-R: (electrical) stimulus-response; D-R: dose-response (to exogenous i.a. NA); PEN: Polyethylene glycol/Ethanol/NaOH 33:33:34.

4.3. Experimental Protocols

After the animals had been in a stable haemodynamic condition for at least 15 min, baseline values of SBP, HR, and RPP were measured.

4.3.1. Electrical Stimulation of the Periarterial (Vasopressor) Renal Nerves

The first main group (S-R curves; Figure 8) was designed to study the influence of 5-HT agonists and antagonists on renal sympathetic neurotransmission in diabetic rats. In addition, the implication of possible indirect pathways on 5-HT effect was determined.

Increases in RPP were obtained by the electrical stimulation of the periarterial renal nerves, by placing a small bipolar electrode close to the origin of the left renal artery connected to a Cibertec Stimulator CS-9 using square wave pulses at increasing stimulation frequencies (2, 4 and 6 Hz). In that way, the control S-R curve (E0) was completed in 15 min. Afterwards, the rats were divided into three different groups. The first one (Group I; $n = 85$; Figure 8) received intraarterial bolus injections of a maximum volume of 10 μL using a micro-syringe via the distal cannula: (a) saline, (b) ethanol 5%, (c) HCl 0.01 M, (d) 5-HT (0.0125, 0.1 and 0.4 $\mu\text{g/kg}$), (e) 5-CT (5-HT_{1/5/7} agonist; 0.1, 0.4 and 1.0 $\mu\text{g/kg}$)

and the following agonists at a dose of 0.4 µg/kg: α-methyl-5-HT (5-HT₂), 1-PBG (5-HT₃), cisapride (5-HT₄), AS-19 (5-HT₇), 8-OH-DPAT (5-HT_{1A}), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}), and BRL-54443, (5-HT_{1F}). After 5 min of the corresponding i.a. administration, a new S-R curve (E1) was obtained as described above for the S-R curve E0.

The second cluster (Group II; n = 15; Figure 8) was carried out to confirm the 5-HT receptors involved in the serotonergic modulation of the renal sympathetic nerve activity. These animals were administered i.v. vehicle (saline, 1 mL/kg) or 1 mg/kg of the following antagonists, SB 699551 (5-HT_{5A}) or LY310762 (5-HT_{1D}). The corresponding curves (E0_{saline}, E0_{SB 699551} and E0_{LY310762}) were completed after 10 min, respectively. Then, the animals received an i.a. administration of 5-CT (0.4 µg/kg). After 5 min of i.a. administration, a new S-R curve (E1) was obtained.

The third group (Group III; n = 25; Figure 8) was performed to determine the indirect pathways involved in the serotonergic effect on renal sympathetic nerve activity. These animals received, intravenously, as follows: (a) saline (1 mL/kg), (b) PEN (1 mL/kg), (c) a non-selective COX inhibitor, indomethacin (2 mg/kg), (d) an ATP-sensitive K⁺ channels blocker, glibenclamide (20 mg/kg), or (e) a selective inhibitor of soluble guanylyl-cyclase, ODQ (10 µg/kg), 10 min before its corresponding S-R curve. After that, the animals received an i.a. bolus of L-694,247 (0.4 µg/kg) to obtain a new S-R curve (E1).

4.3.2. Administration of Exogenous NA

In another set of animals (Group IV; Figure 8; n = 10) prepared as described above, the bipolar electrode was omitted and D-R curves by intraarterial administration of exogenous NA (0.05, 0.1 and 0.4 µg/kg) were constructed before (E'0) and 5 min after (E'1) administration of i.a. saline (10 µL) or L-694,247 (0.4 µg/kg).

4.4. Data Presentation and Statistical Procedures

All the data in the text, tables, and figures, are presented as mean ± SEM of five experiments. Changes in renal vascular resistance are stated as increases in RPP (mm Hg), in comparison with the corresponding baseline value. Statistical significance was carried out with one-way ANOVA followed by Student–Newman–Keul's post hoc test. Statistical significance was accepted at $p < 0.05$.

5. Conclusions

This study discloses that twenty-eight-day experimental diabetes originates an enhancement of the renal sympathetic activity and that the serotonergic system modulates this sympathetic drive: activation of prejunctional 5-HT_{1D}, through NO pathway, reduces noradrenergic-evoked vasopressor responses in the kidney. Thus, this study enlarges the knowledge of complex regulation mechanisms occurring in renal diabetic complications (including both neuropathy and microvascular alterations), and unmask new therapeutic approaches based on 5-HT_{1D} receptor activation to diminish renal sympathetic outflow and, therefore, tackle diabetic renal disturbances.

Author Contributions: A.M.: Design of in vivo experiments, Analysis of data, and Writing—Reviewing and Editing. J.Á.G.-P.: In vivo experiments, Analysis of data, and Writing—Reviewing and Editing. A.C.T.-Ú.: In vivo experiments. J.L.O.: In vivo experiments. J.F.F.-G.: In vivo experiments, Analysis of data, and Writing—Reviewing and Editing. M.L.M.: Design of experiments. M.G.-D.: Design of experiments, Analysis of data, and Writing—Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The main data are included in this manuscript. All data are available from the corresponding author on reasonable request.

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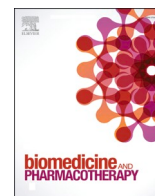
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Anexo II

Chronic sarpogrelate treatment improves renal sympathetic hyperactivity in experimental diabetes

Fernández-González, J. F., García-Pedraza, J. Á., **Terol-Úbeda, A. C.**, Martín, M. L., Morán, A., & García-Domingo, M.

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Chronic sarpogrelate treatment improves renal sympathetic hyperactivity in experimental diabetes

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Sympatholytic effect

ABSTRACT

Diabetes and derived complications, especially diabetic nephropathy and neuropathy annually cause great morbimortality worldwide. 5-hydroxytryptamine (5-HT) acts as a modulator of renal sympathetic input and vascular tone. In this line, 5-HT₂ receptor blockade has been linked with reduced incidence and progression of diabetic microvascular alterations. In this work, we aimed to determine, in diabetic rats, whether 5-HT₂ blockade ameliorates renal function and to characterize the serotonergic modulatory action on renal sympathetic neurotransmission. Diabetes was induced in male Wistar rats by alloxan administration (150 mg/kg, s.c.), and sarpogrelate (30 mg/kg-day, p.o.; 5-HT₂ antagonist) was administered for 14 days (DM-S). Normoglycemic and diabetic (DM) animals were maintained as aged-matched controls. At 28th day, DM-S animals were anesthetized and prepared for the *in situ* autoperfusion of the kidney. Renal vasoconstrictor responses were induced electrically or by i.a. noradrenaline (NA) administration. The role of 5-HT and selective 5-HT agonist/antagonist were studied on these renal vasopressor responses. Sarpogrelate treatment decreased renal sympathetic-induced vasopressor responses, reduced renal hypertrophy and kidney damage markers increased in DM. Intraarterial 5-HT inhibited the sympathetic-induced renal vasoconstrictions, effect reproduced by 5-CT, AS-19, L-694,247 and LY 344864 (5-HT_{1/5/7}, 5-HT₇, 5-HT_{1D} and 5-HT_{1F} receptor agonists, respectively). Blocking 5-HT_{1D/1F/7} receptors completely abolished the 5-CT sympatho-inhibition. NA vasoconstrictions were not altered by any of the 5-HT agonists tested. Thus, in experimental diabetes, chronic sarpogrelate treatment reduces renal damage markers, kidney hypertrophy and renal sympathetic hyperactivity and modifies serotonergic modulation of renal sympathetic neurotransmission, causing a sympatho-inhibition by prejunctional 5-HT_{1D/1F} and 5-HT₇ activation.

1. Introduction

Diabetes mellitus has emerged as a major global health challenge, affecting millions of people worldwide; moreover, it is expected to be the most common chronic disease in a few decades [1]. One of the most significant associated complications is the diabetic nephropathy, a progressive kidney condition that conduces to end-stage renal disease [2], to which various mechanisms (including inflammation, oxidative stress, altered signaling pathways) and the existence of other comorbidities [3,4] contribute. One of these concomitant pathologies is the diabetic neuropathy, characterized by a sympathetic imbalance, that is partly responsible of renal damage development [5].

Serotonergic system, due to its great variety of receptors [6] stands out not only by its central function in anxiety or depression (among others), but also due to its peripheral regulatory role in vascular function, platelet aggregation, and tissue remodeling. Recent studies have suggested a potential link between 5-hydroxytryptamine (5-HT) and the pathogenesis of diabetic nephropathy, outlining new possible therapeutic strategies consisting of targeting different 5-HT receptor type/subtypes [7,8]. In fact, in renal vasculature, 5-HT exerts both direct actions [9–11] and indirect effects by modulating sympathetic renal innervation in normoglycemic and diabetic animals [7,12]. 5-HT₂ receptors, widely expressed in renal tissues, are of particular interest since they mediate deleterious effects such as vasoconstriction [10,13],

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inflammation, and fibrosis, which are key processes in the development and progression of diabetic nephropathy [8,14].

The prevalence of nephropathy as a diabetic complication continues to increase day by day. Hence, finding innovative therapeutic strategies to reduce final renal failure is a must in the XXI century. Sarpogrelate, a selective 5-HT₂ receptor antagonist [15], has emerged as a potential therapeutic drug for various vascular complications associated with diabetes [16]; thus, blocking 5-HT₂ receptor may also become a new possible target to modify/improve pathogenic mechanisms underlying diabetic nephropathy [17,18], closely related to other long-term diabetic complications, such microvascular alterations/damage or diabetic neuropathy.

In this line, our work analyzed the effect of chronic blockade of 5-HT₂ receptors on kidney dysfunction and renal adrenergic damage in diabetes, as well as determined the serotonergic profile involved in the modulation of renal sympathetic input in sarpogrelate-treated diabetic animals.

2. Material and methods

2.1. Ethical approval of the study protocol

The experimental protocol was approved by the Research Ethical Committee of University of Salamanca (ID number 0001003) according with regulation provided by the European Union of use of animals for scientific purposes and Spanish guidelines (Directive 2010/63/EU; R.D. 53/2013).

2.2. Drugs utilized

Each drug and its respectively supplier used in the experiment were: sarpogrelate hydrochloride from VulcanChem (Los Angeles, CA, US); heparin sodium from Rovi (Madrid, Spain); sodium pentobarbital (Doletal®; Vetoquinol, Madrid, Spain); atropine sulphate (Scharlau, Barcelona, Spain); 1-phenylbiguanide (1-PBG), 5-hydroxytryptamine (5-HT) hydrochloride, alloxan monohydrate and noradrenaline (NA) bitartrate (Merck life Sciences, Madrid, Spain); 5-

carboxamidotriptamine (5-CT), 8-OH-DPAT, AS-19, CGS 12066B dimaleate, cisapride, GR 127935 hydrochloride, L-694,247, LY 344864 hydrochloride, SB 258719 hydrochloride and SB 699551 (Tocris Bioscience, Bristol, UK).

All these compounds were dissolved in saline at the experimentation time, except AS-19 (dissolved in ethanol, 5 %) and cisapride (0.01 M HCl). None of the vehicles exhibit properties to alter renal perfusion pressure (renal-PP), heart rate (HR) or systemic blood pressure (SBP).

2.3. General methods

Diabetes mellitus was induced in animals (male Wistar rats; 300 ± 25 g) by the administration of alloxan (150 mg/kg, s.c.) [7,19,20]. Diabetic animals were maintained for 28 days in the animal facilities of the University of Salamanca, with food and drink *ad libitum*, controlled temperature and humidity conditions (22 °C and 50 %, respectively) in 12/12 h light-dark cycle.

Body weight and non-fasting glycemia were periodically controlled using a glucometer (*Accu-chek*®, Aviva, Roche Diagnostics; Barcelona, Spain). Glycemia over 250 mg/dL was set as diabetic-glycemia values. Animals with glycemia under this value were discarded.

From day 14, animals received sarpogrelate (30 mg/kg-day, p.o.; dissolved in drinking water) [19,21] to achieve the sarpogrelate-treated diabetic group (DM-S).

In parallel, a normoglycemic group of animals (NG) and a non-treated diabetic group of animals (DM) were maintained for 28 days as controls to perform complementary studies (Fig. 1)

2.4. Experimental protocols: renal sympathetic neurotransmission study in sarpogrelate-treated diabetic rats

After 28 days of diabetes induction, DM-S animals were anesthetized (pentobarbital, 60 mg/kg, i.p.) and prepared for the *in situ* autoperfusion of the left kidney as previously described by our group [7,12]. Jugular and femoral veins were cannulated for drugs i.v. administration. Both carotid arteries were cannulated, the right for the hemodynamic control of the animal and the left carotid artery to establish renal autoperfusion

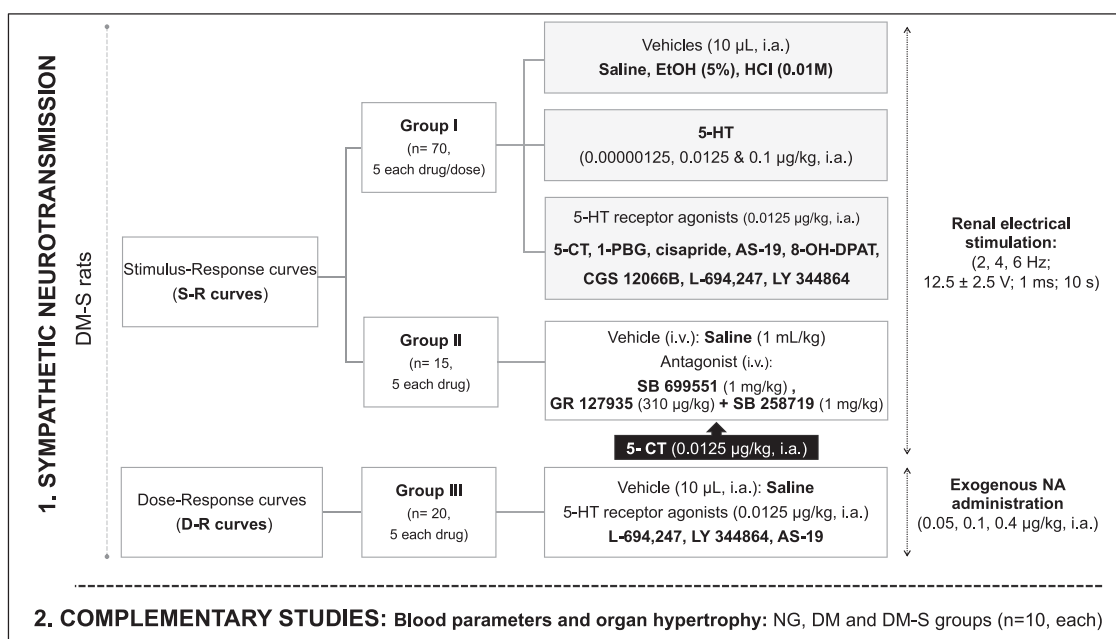


Fig. 1. Experimental design, including protocols and number of animals used. (1) The sympathetic neurotransmission study in sarpogrelate-treated diabetic group (DM-S) was divided into two main clusters, inducing the vasopressor responses by either (i) renal periaarterial nerves electrical stimulation (S-R curve) or (ii) i.a. bolus noradrenaline (NA) administration (D-R curves). (2) The complementary studies were carried out in normoglycemic (NG), diabetic (DM) and DM-S rats, analyzing organ hypertrophy and blood renal parameters.

using an extracorporeal circuit to the cannulated left renal artery through a Gilson peristaltic pump [7,12]. Both carotid arteries were connected to different pressure transducers, ensembled to an e-corder 410 amplifier (Model ED410, Cibertec, Spain) to record the renal-PP, SBP and HR.

Once the circuit was established, heparin sodium (5 mg/kg, i.v.) was administered as a preventive blood clotting agent, and saline i.v. infusion was initiated at 2 mL/h rate (to maintain volemia in the animals). Atropine (1 mg/kg) was intravenously administered as a muscarinic blocker.

At the beginning of the experiment, flow was adjusted to equate renal-PP and SBP. Flow was kept constant until the end of the procedure, at a flow rate (from 2 to 2.9 mL/min) established through the peristaltic pump from left carotid artery to the renal vascular bed [7]. After 15 min of surgery, the hemodynamic condition was stable. At this time the baseline values of SBP, HR and renal-PP were recorded. Afterwards, the sympathetic renal nerves were electrically stimulated or the i.a. NA administration was performed (as indicated below).

At this point, the DM-S group (n=105; Fig. 1) was divided into two different clusters to investigate the effect of 5-HT-receptors agonists/antagonist on the vasopressor responses induced by (a) the electrical stimulation of periarterial renal nerves (stimulus-response (S-R) curves) or (b) the i.a. NA administration (dose-response (D-R) curves). The renal electrical stimuli (2, 4, 6 Hz; 12.5 ± 2.5 V; 1 ms; 10 s) and the NA administration (0.05, 0.1 and 0.4 $\mu\text{g/kg}$, i.a.) were fixed in sequential 3–5 min intervals. The responses obtained showed increases in renal-PP (without alteration of SBP) and renal-PP came back to basal values immediately after the stimulation ended or the NA administration.

2.4.1. Periarterial renal nerves electrical stimulation

Increases in renal-PP (Δ renal-PP) were performed by electrical stimulation of the renal artery at increasing frequencies (by placing an electrode in the left renal artery coupled to a Cibertec Stimulator C9, using the conditions previously described) at 5 min intervals between each frequency. The control S-R curve (E0) was achieved in 15 min.

The first DM-S group (S-R curve, Fig. 1) was performed to study the effect of 5-HT receptor selective agonists on the renal sympathetic vasoconstrictor responses induced by electrical stimulation. This group (group I, total n=70; n=5 for each agonist and dose) received the intraarterial bolus injection in a maximum volume of 10 μL using a micro-syringe of: (i) saline, ethanol 5 % or HCl 0.01 M (vehicles), (ii) 5-HT (0.0000125, 0.0125 and 0.1 $\mu\text{g/kg}$), (iii) the selective 5-HT receptor agonists, at 0.0125 $\mu\text{g/kg}$: 5-CT (5-HT_{1/5/7}), 1- PBG (5-HT₃), cisapride (5-HT₄), AS-19 (5-HT₇), 8-OH-DPAT (5-HT_{1A}), CGS 12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}), LY 344864 (5-HT_{1F}). After 5 min of the i. a. administration, a new S-R curve (E1) was executed as described for the E0 (control curve).

In the second one (group II, n=15), the effect of the selective 5-HT_{1/5/7} agonist (5-CT, 0.0125 $\mu\text{g/kg}$) was studied in presence of the different 5-HT receptor antagonists: SB 699551 (5-HT_{5A}; 1 mg/kg; i.v.) a mixture of GR 127935 (5-HT_{1B/1D/1F}; 310 $\mu\text{g/kg}$; i.v.) plus SB 258719 (5-HT₇, 1 mg/kg; i.v.) or their vehicle (saline, 1 mL/kg, i.v.). This set of experiments was used to confirm the receptor type/subtype(s) involved in the modulation of the renal sympathetic nerve vascular activity. Firstly, after 10 min of each antagonist (or their vehicle) administration the E0_{saline} (control), E0_{SB 699551} or E0_{GR 127935+SB 258719} were completed as previously described. Then, in the presence of each antagonist, the animals received 0.0125 $\mu\text{g/kg}$ of 5-CT (i.a) and a new S-R curve (E1) was performed.

2.4.2. Vasopressor responses by exogenous NA

In the third group of DM-S animals (group III, n= 20), the Δ renal-PP were induced by increasing doses of exogenous NA administered i.a. (as previously reported). The D-R curve was performed before (E0') and after 5 min of i.a. administration of saline (10 μL), L-694,247, LY 344864 or AS-19 (0.0125 $\mu\text{g/kg}$, each) to obtain a new D-R curve (E1').

2.5. Blood parameters and organs hypertrophy determination

Complementary studies were performed in three groups of animals: NG, DM and DM-S (n=10 each). After the anesthetic administration (pentobarbital, 60 mg/kg, i.p.), blood samples were collected from the carotid artery, and centrifuged at 3000 rpm for 10 min. Serum samples were kept at -80 °C until analysis.

To determine renal function, total proteins, blood urea nitrogen (BUN) and creatinine were measured in each group. To do so, serum samples were placed in multiparametric SpotChem EZ® analytic strips and analyzed using the biochemistry autoanalyzer SpotChem EZ® (ARKRAY, Japan). Creatinine clearance was calculated from every animal blood creatinine and weight value by using ACLARA, a web-based, openly available estimated creatinine clearance (eClCr) calculator (<http://idal.uv.es/acalara>) [22].

The right kidneys and left ventricles were collected and prepared as previously stated [21]. Then, left ventricle hypertrophy (LVH) index was calculated using left ventricle weight/tibia length ratio (mg/mm), whereas the renal hypertrophy (RH) index was calculated considering the kidney weight/tibia length ratio (mg/mm).

2.6. Statistical evaluation and data presentation

All data in the text, figures, and tables are presented as mean $\pm$ S.E. M. of five experiments, unless otherwise stated. Changes in the renal vascular resistance from the baseline values, both induced by electrical stimulation or i.a. NA administration are presented as Δ renal-PP (mm Hg). Comparison of the results from the experimental groups and their corresponding control group was carried out with one-way ANOVA followed by the Student-Newman-Keul's post hoc test. Statistical significance was accepted at $p < 0.05$.

Note that: Δ renal-PP after saline i.a. administration did not differ from those obtained in the E0 curve (nothing), hence to simplify statistical analysis is only carried out vs saline.

3. Results

3.1. Metabolic and hemodynamic parameters in DM-S rats

In DM-S, the glycemic values augmented from 112 ± 3 mg/dL (day 0; prior alloxan administration) to 537 ± 7 mg/dL (day 2; post-alloxan administration), ($p < 0.05$ vs day 0). This hyperglycemic state was maintained until the day 28 (548 ± 10 mg/dL), and it did not differ from the DM control group (554 ± 6 mg/dL).

However, body weight significantly decreased two days after alloxan administration (279 ± 2 g vs 300 ± 2 g prior administration; $p < 0.05$ vs day 0), and this body weight was maintained until day 28.

After anesthesia, SBP, HR and renal-PP were 91 ± 3 mm Hg, 295 ± 6 beats per min (bpm) and 109 ± 4 mm Hg, respectively in the DM-S animals. These basal values were not modified by the i.a. administration of selective 5-HT receptor agonists, vehicles (saline, ethanol 0.5 % and HCl 0.01 M) or the i.v. 5-HT antagonist administrations. In contrast, only i.a. administration of higher doses of 5-HT (0.0125 and 0.1 $\mu\text{g/kg}$) evoked a transient but significant Δ renal-PP (13.6 ± 3.5 and 16.2 ± 3.0 , respectively; $p < 0.05$ vs basal values).

3.2. Renal sympathetic-induced vasopressor responses in diabetic rats treated with sarpogrelate

The electrical stimulation of renal periarterial nerves induced endogenous NA release, and consequently immediate and frequency-dependent Δ renal-PP, that were 23.9 ± 2.1 , 59.5 ± 1.9 and 80.5 ± 3.5 for 2, 4 and 6 Hz, respectively (S-R curve; Fig. 2). Similarly, exogenous NA i.a. administration in ascending doses induced dose-dependent renal vasopressor responses (Δ Renal-PP), that were 26.5 ± 3.5 , 42.2 ± 3.9 and 72.5 ± 8.5 mm Hg for 0.05, 0.1 and 0.4 $\mu\text{g/kg}$ i.a. NA

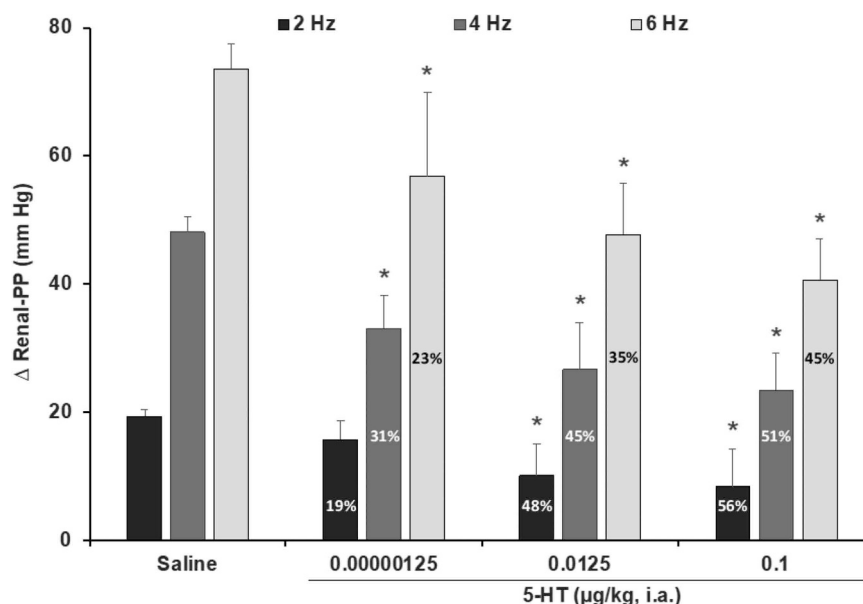


Fig. 2. Effect of intraarterial bolus of 10 μL of saline (control; n=5) and increasing doses of 5-HT (0.00000125–0.1 μg/kg; n=5 each dose) on the increases in renal perfusion pressure (Δ Renal-PP) evoked by the selective stimulation of renal sympathetic nerves in the in situ autoperfused kidney of sargogrelate-treated diabetic rats. Percentual values shown inside the bars indicates the percentages of inhibition vs saline. *p < 0.05 vs saline using one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

doses, respectively (D-R curve, control). These vasoconstrictor responses (electrical or exogenous NA) were selectively induced in the renal vascular bed, so neither SBP nor HR were altered (data not shown).

3.3. Effect of vehicles, 5-HT or selective 5-HT receptor type agonists on the renal vasopressor responses induced by electrical stimulation in DM-S rats

The Δ renal-PP due to electrical stimulation at increasing frequencies (2, 4 and 6 Hz; Fig. 2) were not modified by the intraarterial administration of 10 μL of saline (Fig. 2), ethanol 5 % or HCl 0.01 M (data not shown). In contrast, i.a. 5-HT administration (0.00000125, 0.0125 and 0.1 μg/kg) induced a dose and frequency-dependent inhibition of the sympathetic-induced Δ renal-PP (Fig. 2) in DM-S rats.

As shown in Fig. 3, the electrically evoked Δ renal-PP were not

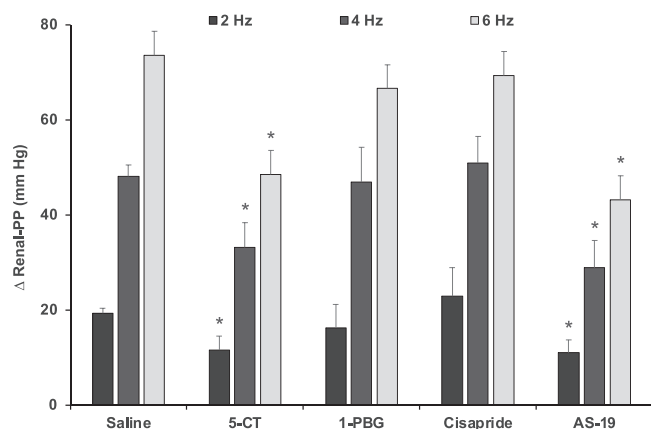


Fig. 3. Effect of the i.a. bolus of saline (10 μL; n=5) and 0.0125 μg/kg of either 5-CT (5-HT_{1/5/7} receptor agonist), or 1-PBG (5-HT₃ receptor agonist), or cisapride (5-HT₄ receptor agonist) or AS-19 (5-HT₇ receptor agonist) (n=5 each agonist) on the vasoconstrictor responses (Δ Renal-PP) elicited by electrical stimulation of renal sympathetic nerves in the in situ autoperfused left kidney of sargogrelate-treated diabetic rats. *p < 0.05 vs saline, using one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

altered by the intraarterial administration of the 5-HT₃ or 5-HT₄ receptor agonists (1-PBG or cisapride, respectively at 0.0125 μg/kg). In contrast, i.a. 5-CT (5-HT_{1/5/7} agonist; 0.0125 μg/kg) mimicked the 5-HT inhibitory effect on the vasopressor responses (Fig. 3). To the same extent, AS-19 (5-HT₇ selective agonist; 0.0125 μg/kg, i.a.) significantly reduced the electrical-induced renal vasoconstrictor responses (Fig. 3).

3.4. Effect of the different 5-HT₁ receptor subtype agonists on the increases in renal perfusion pressure evoked by electrical stimulation in DM-S rats

To further analyze the 5-HT receptor subtype/s involved in the renal serotonergic sympathoinhibitory action, different 5-HT₁ subtype agonists were intraarterially administered. 8-OH-DPAT (5-HT_{1A}) and CGS 12066B (5-HT_{1B}) failed to modify the sympathetic-induced Δ renal-PP (Fig. 4). On the other hand, both L-694,247 (5-HT_{1D}) and LY 344864 (5-HT_{1F}) significantly inhibited the electrically induced renal vasoconstrictions (Fig. 4).

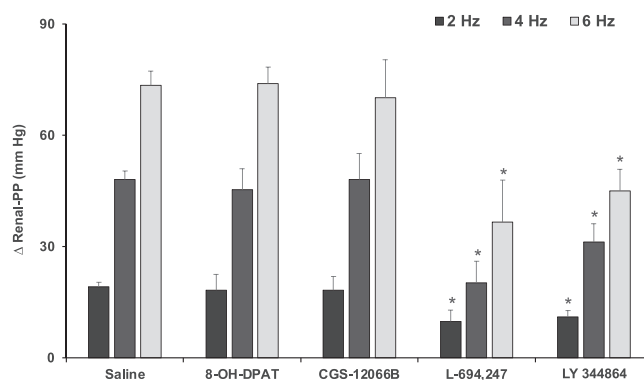


Fig. 4. Effect of i.a. bolus of saline (10 μL; n=5), 8-OH-DPAT (5-HT_{1A}), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) or LY 344864 (5-HT_{1F}) (0.0125 μg/kg and n=5 for each agonist) on the vasopressor responses (Δ Renal-PP) elicited by electrical stimulation of the periaarterial renal nerves in sargogrelate-treated diabetic rats. *p < 0.05 vs saline, with one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

3.5. Impact of saline or different 5-HT receptor type/subtype antagonist on the 5-CT renal sympatho-inhibitory effect in DM-S rats

The i.v. administration of the 5-HT_{5A} selective antagonist (SB 69951, 1 mg/kg), or a mixture of GR 127935 (5-HT_{1B/1D/1F} selective antagonist, 310 µg/kg) plus SB 258719 (5-HT₇ selective antagonist, 1 mg/kg) did not induce *per se* any effect in the renal vasopressor responses induce by electrical stimulation of periarterial renal nerves (data not shown). Furthermore, antagonist's vehicle (saline, 1 mL/kg, i.v.) did not modify the 5-CT-induced renal sympathetic inhibition (Fig. 5). The i.v. administration of 1 mg/kg of SB 699551 (5-HT_{5A} antagonist) was not able to abolish the renal sympatho-inhibitory effect of 5-CT (0.0125 µg/kg, i.a.). Whereas, i.v. administration of a cocktail of GR 127935 (5-HT_{1B/1D/1F} antagonist, 310 µg/kg) and SB 258719 (5-HT₇ antagonist, 1 mg/kg) completely blocked the inhibitory effect of 5-CT on the Δ renal-PP (Fig. 5).

3.6. Effect of selective 5-HT agonists on the Δ renal-PP induced by the intraarterial administration of exogenous NA

The i.a. administration of exogenous NA evoked (Δ renal-PP) in a dose dependent manner, as described in 3.2. Neither saline (10 µL) nor any of the 5-HT selective receptor agonists tested: L-694,247 (5-HT_{1D}), LY 344864 (5-HT_{1F}) and AS-19 (5-HT₇) (at a dose 0.0125 µg/kg each agonist) of modified these vasopressor responses (Table 1).

3.7. Organ hypertrophy and blood parameters

The LVH index did not show differences among NG, DM or DM-S rats (data not shown); however, RH index (mg/mm) of the NG, DM and DM-S animals were 29.3 ± 1.1, 40.4 ± 1.4* and 35.1 ± 1.1*[#], respectively; (*p < 0.05 vs NG; #p < 0.05 vs DM; n=10, each group). As described in Table 2, the diabetic state (DM group) decreased the total proteins concentration (p < 0.05) in the serum samples compared with the NG group. Although sarpogrelate-treatment (DM-S group) seems to increase total protein concentration, the values obtained were not significantly different from either DM or NG groups. In relation to other renal function markers, BUN and creatinine were significantly increased in DM animals compared with NG group (p < 0.05) (Table 2). The chronic blockade of 5-HT₂ was able to reduce BUN in diabetic animals (p < 0.05 vs diabetic), although DM-S BUN values did not reach NG group BUN values (p < 0.05 vs normoglycemic). Besides, the DM-S rats showed restored normoglycemic creatinine values (p < 0.05 vs diabetic).

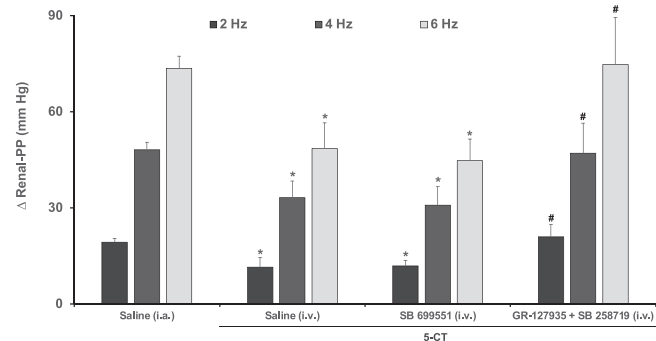


Fig. 5. Effect of the i.a. administration of 5-CT (0.0125 µg/kg) in the presence of saline (1 mL/kg, i.v.), SB 699551 (1 mg/kg, i.v.) or a cocktail of GR 127935 (310 µg/kg, i.v.) + SB 258719 (1 mg/kg, i.v.) on the vasopressor responses (Δ Renal-PP) elicited by electrical stimulation of renal sympathetic nerves in sarpogrelate-treated diabetic rats. n=5 for each treatment. *p < 0.05 vs i.a. saline; #p < 0.05 vs 5-CT, using one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

Table 1
Effect of i.a. bolus of saline (10 µL), L-694,247, LY 344864 or AS-19 (0.0125 µg/kg, each agonist) on the vasopressor responses elicited by the i.a. administration of noradrenaline (0.05, 0.1 and 0.4 µg/kg) in diabetic rats treated with sarpogrelate.

i.a. NA doses (µg/kg)			
Drugs	0.05	0.1	0.4
Saline	29.9 ± 2.3	45 ± 3.2	78.9 ± 5.5
L-694,247	29.9 ± 6.9	40.5 ± 7.0	77.5 ± 9.7
LY 344864	26.2 ± 4.0	40.1 ± 7.2	70.6 ± 8.9
AS-19	29.8 ± 4.3	41.5 ± 5.7	71.3 ± 8.1
Δ Renal-PP (mm Hg)			

Note that responses induced by the i.a. administration of exogenous NA administration were not modified by any of the selective 5-HT receptor type agonists (p > 0.05 vs saline)

Table 2
Renal parameters comparison among the different study groups from serum blood samples (normoglycemic, diabetic and diabetic treated with sarpogrelate).

	NG	DM	DM-S
Total proteins (g/dL)	4.87 ± 0.13	4.34 ± 0.16*	4.64 ± 0.47
Blood Urea Nitrogen (mg/dL)	22.44 ± 0.91	35.58 ± 1.45*	28.38 ± 4.48* [#]
Creatinine (mg/dL)	0.64 ± 0.02	0.81 ± 0.05*	0.64 ± 0.06 [#]
†eClCr (mL/min)	0.94 ± 0.03	0.66 ± 0.06*	0.81 ± 0.04* [#]

†Estimated creatinine clearance (eClCr) was calculated by online ACLARA calculator from Intelligent Data Analysis Laboratory (IDAL) from University of Valencia, from a single value of blood creatinine (mg/dL) and body weight (g). NG: normoglycaemic group; DM: non-treated (control) diabetic group; DM-S: sarpogrelate-treated diabetic group. Values shown as means ± S.E.M. of n=10. *p < 0.05 vs normoglycemic; #p < 0.05 vs diabetic.

4. Discussion

4.1. General: metabolic and haemodynamic parameters

Due to the great prevalence of diabetes nowadays, chronic derived complications have become a worldwide public health. The most common associated pathologies are microvascular alterations, neuropathy (characterized by sympathetic hyperactivity) and renal function alterations [3,4]. The combination of these three may lead to final renal failure and death [23]. Hence, understanding this intricate interplay, and finding new pharmacological strategies to containing it, has emerged as a must nowadays and in the coming years. In this line, modulating the serotonergic system (due to its great variety of receptors and peripheral actions) seems a promising procedure for the development of novel therapeutic interventions.

In this spirit, we studied the effect of the 5-HT₂ chronic blockade on the renal sympathetic neurotransmission and its modulation by the serotonergic system in experimental diabetic animals. Moreover, we determined the changes that this treatment may induce in renal function during diabetes. To do so, we utilized sarpogrelate as a 5-HT₂ antagonist because it has already been postulated as a pharmacological tool in diabetic microvascular complications and in renal function damage induced by diabetes [17,18,24,25].

Alloxan is a commonly used diabetogenic compound in experimental rodent models [19,20,26]. Chronic hyperglycemia appears on the second day post-subcutaneous administration (due to β-cell destruction) and is maintained during the 28 days in which the animals remain penned in the animal facilities [7,20]. The chronic hyperglycemia leads to a variety of complications, including renal damage and neuropathy [19,27].

14-day oral sarpogrelate treatment, at a dose of 30 mg/kg.day [19, 21] does not modify glycemia in diabetic animals as stated by us and other authors [19,28].

The *in vivo* experimental model (*in situ* autoperfused rat kidney) utilized in our experiments allows to characterize the direct action of a drug in the kidney having into account all physiopathological compensatory responses in the entire organism [10]. In this work, we continuously recorded SBP, HR and renal blood pressure, assessing changes in the renal blood flow (without modifying SBP and HR) induced either by selective stimulation of sympathetic periarterial renal nerves or i.a. administration of exogenous NA in sarpogrelate-treated diabetic rats, as previously described in other experimental models [9, 19].

The renal system is stated as the leading long-term regulator of blood pressure; thus, renal alterations together with hypertension (one of common diabetes comorbidities) are directly involved in the renal damage [29] and final kidney failure. Our experimental technique allows us to establish the effect of chronic blockade of 5-HT₂ receptors on hemodynamic parameters (HR, SBP and RPP). On this basis, elevated renal-PP and SBP was already reported before in a long-term diabetic animal model using the same experimental technique [30], which indicates the acute development of diabetic hypertension. Recently, our group has demonstrated that renal-PP is also augmented in a short-term model of diabetes, whereas there are no changes in SBP compared to normoglycemic animals [7,9,12], probably due to the timing of diabetes. Although sarpogrelate treatment has been demonstrated to reduce high blood pressure in awake diabetic animals [21,31], our current data, in anaesthetized animals, show that 14-day oral sarpogrelate treatment does not alter SBP or the augmented perfusion pressure in the kidney during diabetes. Similarly, the significant decrease observed in HR in diabetic rats [7] *versus* normoglycemic is not modified by the chronic blockade of 5-HT₂ receptors.

It is well established that diabetes induces changes in the sympathetic nervous system [32,33]. The hyperactivation of adrenergic input in diabetes is showed in different manners: higher adrenergic responses [7] and increases in systemic and/or renal/adrenal catecholamines concentration [34,35], among others. Moreover, adrenergic innervation is extensive in the kidney [36]. Thus, renal sympathetic hyperactivity, previously described in several diabetic animal models, [7,37] may contribute to the development of hypertension (one of common diabetes comorbidities), and progressively, generate a cross-talk that contributes to renal damage [29]. Therefore, controlling the renal sympathetic (hyper)activity may be a target in diabetes in order to reduce the associated complications [37,38]. In this sense, we demonstrate that vasoconstrictor responses induced by either electrical stimulation of the renal sympathetic nerves (that induces endogenous NA release) or exogenous NA administration are significantly lower in the DM-S rats than in the DM ones [7]. These results in the renal bed are in agreement with those recently reported by us in total vascular noradrenergic innervation [19], where the vasopressor responses were attenuated in sarpogrelate-treated in comparison to non-treated diabetic rats. This fact proves that sarpogrelate treatment regulates the sympathetic hyperactivity originated by diabetes, and consequently, decreases the magnitude of the increased vasopressor responses due to diabetic state [19, current data]. Hence, chronic treatment with a 5-HT₂ blocker becomes a potential target in the regulation of sympathetic hyperactivity, which highly contributes to cardiovascular and renal complications during diabetes.

4.2. Inhibitory profile of renal sympathetic neurotransmission of different 5-HT agonists in DM-S animals

The serotonergic system is established as one of the modulators of the blood pressure control. Anyhow, none of the administered 5-HT agonists (i.a.) or antagonists (i.v.) altered basal parameters (SBP, HR or renal-PP). Only the higher doses of 5-HT (0.0125 and 0.1 µg/kg, i.a.) increased renal-PP that came back to basal values within 2 min. It is important to remark that this fact has been previously shown by us both in normoglycemic and diabetic rats [7,12] and it may be due to the

vasoconstrictor role of serotonin at renal level mainly mediated by 5-HT₂ receptor activation [10,13,30]. In fact, chronic sarpogrelate treatment halves this acute vasoconstrictor effect of 5-HT (0.1 µg/kg, i. a.; current results) compared to diabetic rats [7].

In relation to serotonergic modulatory role of sympathetic activity, the inhibitory effect of 5-HT on the sympathetic neurotransmission has been reported before in different experimental model and vascular territories [9,19,25,39,40].

As 5-HT₂ receptors are (1) functional and widely expressed in renal tissues and (2) mediate deleterious effects such as vasoconstriction [10, 13], inflammation, and fibrosis, which are key processes in the development and progression of diabetic nephropathy [8,14], our hypothesis remained that chronic blockade of 5-HT₂ receptor might unmask or potentiate other 5-HT receptor activation to control sympathetic hyperactivity during diabetes. Our study confirms that 5-HT inhibitory effect is revealed at lower doses in diabetes (sarpogrelate-treated or not) than in normoglycemic animals. As stated, the 0.0125 µg/kg dose of 5-HT inhibited the sympathetic responses in the DM and in DM-S animals [7; current data], but not in the normoglycemic animals [12]. Of particular interest is that the 5-HT₂ blockade induces, in DM-S group, a higher percentage of 5-HT inhibition than in DM animals, which, in fact, would indicate that, in diabetic animals chronically treated with sarpogrelate, lower doses of 5-HT may induce a greater inhibitory action.

In DM-S rats, the inhibitory serotonergic profile is mainly mediated by the 5-HT_{1/7} receptor type, since the selective receptor agonists 1-PBG (5-HT₃) and cisapride (5-HT₄) did not modify the vasopressor responses in the renal bed of DM-S rats. Only the non-selective 5-HT_{1/5/7} agonist, 5-CT, and the selective 5-HT₇ agonist, AS-19, reproduced 5-HT renal sympatho-inhibitory action, and 5-HT_{5A} receptor was devoid of this inhibitory action since the selective 5-HT_{5A} blockade (SB 699551) did not abolish 5-CT action on renal noradrenergic input. In pithed rats, 5-HT₁ receptor type has longly been described as sympathoinhibitory at vascular level in normoglycemic and diabetic state [20,41]. Furthermore, in this experimental model (normoglycemic pithed rat) treated with sarpogrelate, as occurs in our current data in the kidney, 5-HT₇ is unmasked as sympatholytic [25]. Anyhow, in diabetic pithed rats, the 5-HT_{5A} receptor was involved in the serotonergic sympathoinhibitory action at cardiac level [39].

To further analyse the 5-HT receptor subtype involved in the renal sympatholytic effect of 5-HT, we utilized selective agonists. Neither 8-OH-DPAT nor CGS-12066B (5-HT_{1A} and 5-HT_{1B} receptor agonists, respectively) were able to reproduce 5-CT inhibitory action; however, L-694,247 (5-HT_{1D}) and LY 344864 (5-HT_{1F}) mimicked serotonin inhibitory effect.

To confirm the 5-HT_{1D/1F} and 5-HT₇ involvement in the serotonergic sympatholytic action, we used a mixture of GR 127935 (5-HT_{1B/1D/1F}) plus SB 258719 (5-HT₇), that completely abolished 5-CT inhibition of the vasoconstrictor responses (see results, 3.5 section).

All these data evidence that chronic blockade of 5-HT₂ receptors in experimental diabetes show up the 5-HT_{1D}, 5-HT_{1F} and 5-HT₇ receptors as responsible of the renal vasculature sympathoinhibition by the serotonergic system, which is partly in agreement with results in DM animals [7], where 5-HT_{1D} receptors were the sole responsible of the sympatho-inhibitory action. Thus, sarpogrelate treatment in diabetic rats unmasks the 5-HT_{1F} and 5-HT₇ receptors involved in the inhibition of the renal sympathetic vascular tone. The 5-HT_{1D} receptor has been entailed in the renal noradrenergic-neurotransmission inhibition also in normoglycemic animals [9]. Moreover, the inhibitory role of this receptor subtype has already been described in the sympathetic vascular and cardiac innervation [41,42] and, also, in the cardiac vagal input [43, 44] in different experimental models. Also, the 5-HT₇ activation has been reported as a sympathoinhibitor in sarpogrelate-treated normoglycemic [25] and diabetic [19] pithed animals. In addition, this inhibitory effect by 5-HT₇ could be explained by the activation of G_s protein, and consequently, the K⁺ channel activation followed of the membrane hyperpolarization. Of particular interest is the participation

of 5-HT_{1F} in the sympatho-inhibitory effect, that has never been described before at renal level; however, our results tally with those obtained at cardiac level in sarpogrelate-treated normoglycemic pithed animals [45]. The unmasking of 5-HT_{1F} receptor as sympatho-modulator at renal level in DM-S rats may become of great interest, since there are some studies that showed up how 5-HT_{1F} receptor activation play a significant role in improving renal damage [46, 47]. Both 5-HT_{1D/1F} receptors acts as neurotransmitter inhibitor by the inhibition of G_{i/o} protein and adenylyl cyclase inhibition [6,48].

The implication of these three receptor subtypes (5-HT_{1D/1F} and 5-HT₇) could, at least in part, explain the inhibitory effect of 5-HT in DM-S rats at lower doses than in DM rats. Furthermore, sarpogrelate treatment enhances the endothelial function in the diabetic rats thought reducing oxidative stress or inflammatory factors [18,21].

The administration of increasing doses of NA induced dose-dependent vasoconstrictor responses on the renal vasculature. The intraarterial administration of the L-694,247, LY 344864 and AS-19 (5-HT_{1D}, 5-HT_{1F} and 5-HT₇ receptor agonist, respectively at a dose of 0.0125 µg/kg each) did not modify the NA-induced the Δ renal-PP. This leads us to confirm the prejunctional nature of this inhibition. On this basis, the prejunctional locus of serotonergic receptors as inhibitors of noradrenergic input was previously demonstrated at renal, cardiac and vascular levels in normoglycemic [9,12,41,42] and diabetic animals [7, 20,39].

4.3. Renal parameters and organs hypertrophy

Diabetic renal disease clinical manifestations appear to be heterogeneous. Anyhow, kidney hypertrophy, total proteins, creatinine levels (and/or creatinine clearance) and blood urea nitrogen are common markers to evaluate the functionality of the kidney [8,49]. In this sense, we tried to determine whether our experimental model of diabetes originates alteration in these parameters; the present results confirm increased levels of serum creatinine and BUN, and decreased levels of serum total proteins and creatinine clearance (estimated by ACLARA, a web-based, openly available estimated creatinine clearance (eClCr) calculator [22]) which confirm that 28-day alloxan-induced hyperglycemia induces renal disturbances, as previously described [50,51]. 14-day sarpogrelate-treatment in diabetic animal is able to reduce the elevation of both serum BUN and creatinine values in diabetic animals; and also, 5-HT₂ receptor blockade significantly reversed eClCr, although it did not come back to control values (NG group). Anyhow, we may affirm that changes induced in the renal function by diabetes tend to improve with 5-HT₂ blockade.

This fact is also consistent with the decrease observed of RH rate in the diabetic rats treated with sarpogrelate from the diabetic control ($p < 0.05$) [21, current data]. Although some studies, using other models of sarpogrelate treatment, did not find significant effect of the 5-HT receptor blockade on the renal parameters or organ hypertrophy in diabetic rats [24], our results agree with those from other authors who reported that blocking 5-HT₂ receptor ameliorates altered renal parameters in chronic kidney disease in rats [52] or mice [49].

4.4. Final considerations

The present work has some curbs to be taken into account: (1) our experimental model *in vivo* allows to determine renal sympathetic activity as an indirect assessment by measuring Δ renal-PP (which is not a direct measurement of NA release), (2) we selectively measure renal-PP/sympathetic innervation in the left kidney, and not in the right kidney, but it is important to remark that perform the *in situ* autoperfusion of the right kidney might lead to the blockage of the mesenteric arterial irrigation (by surgical necessities), which could induce a loss in the hemodynamic control and several systemic changes and (3) our results are based in males; thus, we may further analyze these results in female rats to avoid sex or gender bias; anyhow, it must be considered that in a

pithed rat model, the sympathetic input at vascular level shows no differences between male and female rats [53].

Despite all these constraints, current data bring to light new possibilities in the treatment of diabetic complications. We have demonstrated that chronic sarpogrelate treatment attenuates the augmented diabetic renal vascular responses due to sympathetic hyperactivity [7]. Moreover, sarpogrelate chronic therapy reduce renal hypertrophy and ameliorates markers of renal dysfunction (BUN or creatinine), which can trigger damage in the kidney during diabetes. Besides, 5-HT₂ blockade shows up the inhibitory effect of prejunctional 5-HT_{1D/1F} and 5-HT₇ on the renal sympathetic outflow. Thus, modulating serotonergic system may contribute to ameliorate renal alterations (reduce hypertrophy and improve markers) and reduce the microvascular disturbances due to noradrenergic hyperactivation during diabetes.

5. Conclusions

Chronic 5-HT₂ receptor blockade (by sarpogrelate) reduces renal hypertrophy, ameliorates renal dysfunction markers (BUN and creatinine), decreases the renal sympathetic hyperactivity and shows up a renal sympatholytic effect by prejunctional 5-HT_{1D}, 5-HT_{1F} and 5-HT₇ receptor activation in experimental diabetes. So, these results open new ways to explore in the current treatment of diabetic complications, postulating the 5-HT₂ antagonism as a pharmacological tool to mitigate kidney dysfunction in diabetes.

CRedit authorship contribution statement

Anais-Clara Terol-Úbeda: Methodology, Investigation, Data curation. **José-Ángel García-Pedraza:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Juan-Francisco Fernández-González:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Asunción Morán:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **María-Luisa Martín:** Data curation, Conceptualization. **Mónica García-Domingo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Authors' statement

Anais Clara Terol-Úbeda: Performed *in vivo* experiments, performed study of blood parameters. Asunción Morán: Designed the *in vivo* experiments, Analyzed the data, and Writing-Reviewing and Editing. José Ángel García-Pedraza: Performed *in vivo* experiments, designed blood parameters and organ hypertrophy study, Analyzed the data, and Writing-Reviewing and Editing. Juan Francisco Fernández-González: Performed *in vivo* experiments, study blood parameters and organ hypertrophy, Analyzed the data, and Writing-Reviewing and Editing. María Luisa Martín: Designed the *in vivo* experiments. Mónica García-Domingo: Designed the *in vivo* experiments and blood parameter study, Analyzed the data, and Writing-Reviewing and Editing. All authors read and approved the final manuscript.

Declaration of Competing Interest

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

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