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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-HT_3 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 $\mu g \cdot kg^{-1}$). 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-HT_3 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-HT_4, 5-HT_{5A} or 5-HT_7) modified these vasoconstrictions. Only 1-PBG (5-HT_3) 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-HT_3 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.

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

KEYWORDS
5-HT, 5-HT_{1D} receptor, 5-HT_{2A} receptor, 5-HT_3 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-HT_2 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 system is mediated by 5-HT_{1D} and 5-HT_{1A} receptors (García-Pedraza et al., 2013; Morán et al., 1998), but **5-HT_3** 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

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-HT_3 receptors potentiate vascular noradrenergic outflow 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-HT_3 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.

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 ± 2°C).

2.2 | Surgical procedure and general methods

The animals were anaesthetised with pentobarbital (60 mg·kg^{-1}; i.p.) and a tracheal cannula was placed for artificial respiration (1-ml air-·100 g^{-1}, 50 strokes·min^{-1}). A pithing 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 UI·kg^{-1}) and atropine (1 mg·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).

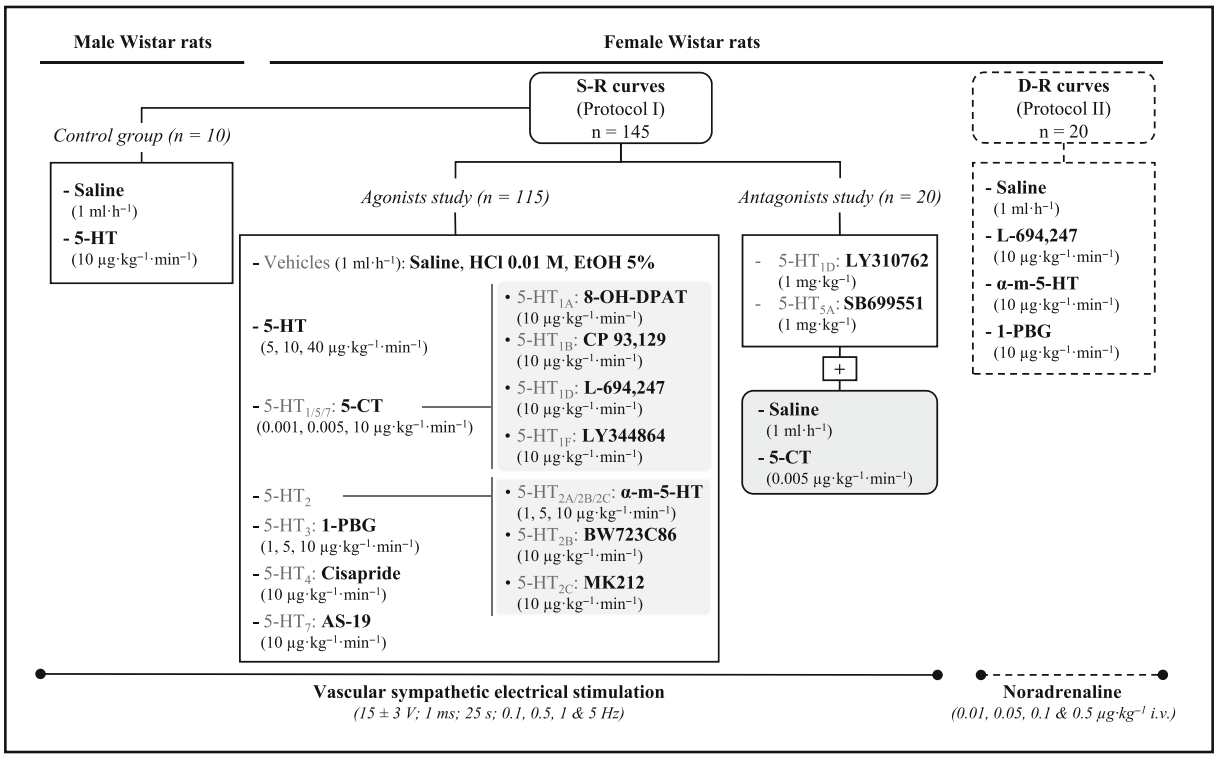

FIGURE 1 Scheme of experimental protocols, showing the number of animals used. Experimental protocols showing the two main sets and the different groups used in these experiments, in which vasopressor responses are obtained by electrical sympathetic stimulation (S–R curves) or i.v. bolus of noradrenaline (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 ml·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 mg·kg^{-1}, i.v.) to prevent electrically-induced muscular spasms. The entire sympathetic outflow from the spinal cord was stimulated as previously stated: 15 ± 3 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 ml·h^{-1}, control group) or 5-HT (10 µg·kg^{-1}·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 ml·h^{-1}), (b) 5-HT (endogenous ligand; 5, 10 and 40 µg·kg^{-1}·min^{-1}) or selective 5-HT receptor (sub)type agonists: (c) 5-CT (5-HT$_{1/5/7}$; 0.001, 0.005 and 10 µg·kg^{-1}·min^{-1}), (d) 8-OH-DPAT (5-HT$_{1A}$; 10 µg·kg^{-1}·min^{-1}), (e) CP 93,129 (**5-HT$_{1B}$**; 10 µg·kg^{-1}·min^{-1}), (f) L-694,247 (5-HT$_{1D}$; 10 µg·kg^{-1}·min^{-1}), (g) LY344864 (**5-HT$_{1F}$**; 10 µg·kg^{-1}·min^{-1}), (h) α-methyl-5-HT (5-HT$_2$; 1, 5 and 10 µg·kg^{-1}·min^{-1}), (i) BW723C86 (**5-HT$_{2B}$**; 10 µg·kg^{-1}·min^{-1}), (j) MK212 (**5-HT$_{2C}$**; 10 µg·kg^{-1}·min^{-1}), (k) 1-PBG (5-HT$_3$; 1, 5 and 10 µg·kg^{-1}·min^{-1}), (l) cisapride (**5-HT$_4$**; 10 µg·kg^{-1}·min^{-1}) or (m) AS-19 (**5-HT$_7$**; 10 µg·kg^{-1}·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 mg·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

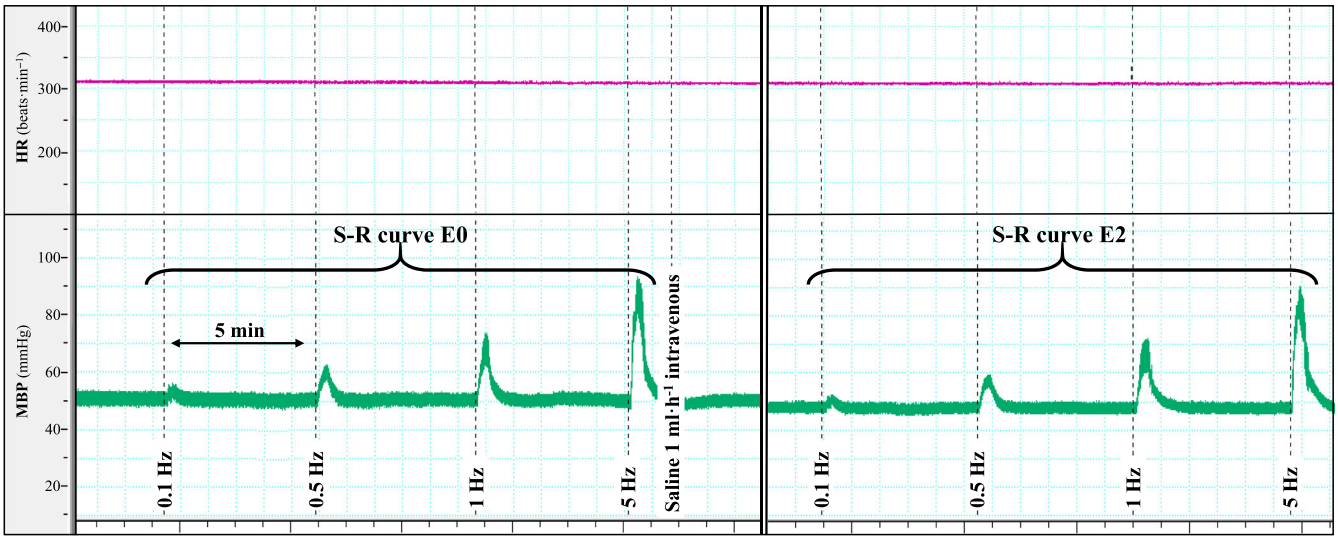

FIGURE 2 Original experimental tracing illustrating the vasopressor responses induced by electrical sympathetic stimulation before and during perfusion of saline (1 ml·h^{-1}) in female pithed rats. Heart rate (HR; beats·min^{-1}) and mean blood pressure (MBP; mmHg) are shown in the figure. S–R curves 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 ml·h^{-1}; n = 5 each) or 5-CT (0.005 μg·kg^{-1}·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 μg·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 ml·h^{-1}; n = 5), L-694,247, α-methyl-5-HT or 1-PBG (10 μg·kg^{-1}·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 ± 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 frequences 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 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**), (±)-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 ± 2 mmHg, respectively). Basal HR was similar in female and male animals (313 ± 4 and 306 ± 16 beats·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 μg·kg^{-1}·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 μg·kg^{-1}·min^{-1} of 5-CT was reversed by pretreatment with LY310762 (46 ± 3 mmHg).

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

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

Note: All values are expressed as means ± 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 ($10\ \mu g \cdot kg^{-1} \cdot min^{-1}$) 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$) 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 $5\text{-HT}_{1/5/7}$ receptor agonist, reduced the sympathetic-evoked vasopressor responses (Figures 3 and 4). Increasing doses of 5-CT (0.001, 0.005 and 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$) decreased the vasopressor responses in a frequency- and dose-dependent way (Figure 4). In contrast, 5-HT_2 and 5-HT_3 receptor agonists, α-methyl-5-HT and 1-PBG ($10\ \mu g \cdot kg^{-1} \cdot min^{-1}$ 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-HT_4 and 5-HT_7 receptor agonists, respectively), at a dose of $10\ \mu g \cdot kg^{-1} \cdot min^{-1}$ each, modified the sympathetic-induced vasoconstrictions (Figure 3).

3.4 | Role of 5-HT_1 or 5-HT_2 receptor subtype agonists on sympathetic vasopressor responses in female rats

The inhibitory effect of 5-CT was mimicked by i.v. infusion of $10\ \mu g \cdot kg^{-1} \cdot min^{-1}$ 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-HT_2 receptor subtype involvement in the enhancing effect of electrical-induced vasopressor responses, only $10\ \mu g \cdot kg^{-1} \cdot min^{-1}$ of α-methyl-5-HT, a non-selective 5-HT_2 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 $10\ \mu g \cdot kg^{-1} \cdot min^{-1}$ 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 ($1\ mg \cdot kg^{-1}$), or the selective 5-HT_{5A} antagonist, SB699551($1\ mg \cdot kg^{-1}$), did not modify per se the vasopressor

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

		Frequency of stimulation (Hz)			
	i.v. infusions (μg·kg^{-1}·min^{-1})	0.1	0.5	1	5
Male	Saline	2.4 ± 0.3	8.4 ± 0.8	18.5 ± 1.8	43.0 ± 3.8
	5-HT (10)	1.0 ± 0.1*	4.8 ± 0.4*	11.5 ± 1.2*	35.6 ± 3.5
Female	Saline	2.1 ± 0.1	9.8 ± 0.3	21.1 ± 0.7	46.3 ± 1.1
	5-HT (5)	1.8 ± 0.3	8.1 ± 1.6	16.4 ± 2.4	44.1 ± 2.7
	5-HT (10)	1.2 ± 0.2*	4.9 ± 1.4*	10.5 ± 2.2*	27.8 ± 2.7*
	5-HT (40)	0.5 ± 0.1*	2.6 ± 0.4*	4.1 ± 0.9*	11.3 ± 1.6*
		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.

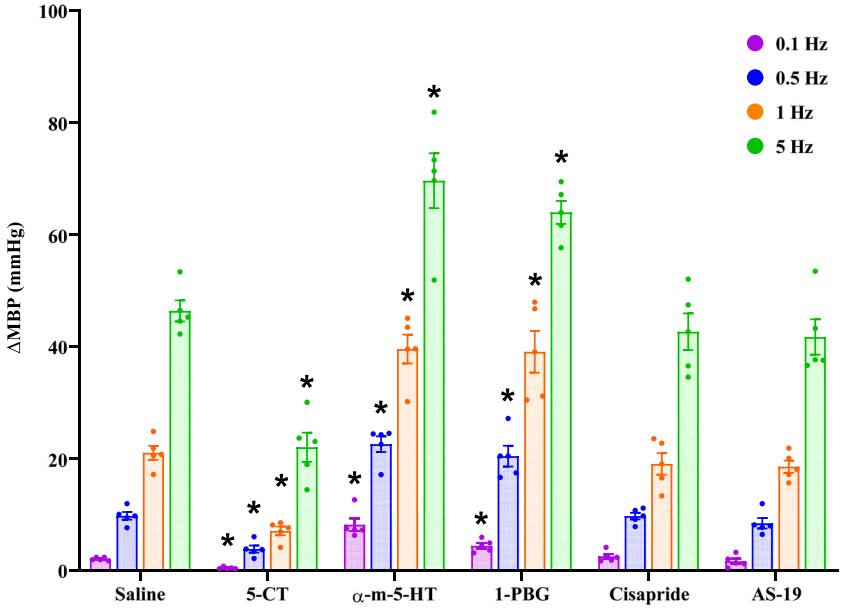

FIGURE 3 Effect of 5-HT receptor type agonists on the sympathetic vasopressor responses in female pithed rats. Increases in mean blood pressure (ΔMBP; mmHg) induced by electrical sympathetic stimulation during i.v. continuous perfusions (n = 5 each) of saline (control; 1 ml·h^{-1}) or 10 μg·kg^{-1}·min^{-1} of the following 5-HT receptor type agonists: 5-CT (5-HT$_{1/5/7}$), α-methyl-5-HT (α-m-5-HT; 5-HT$_2$), 1-PBG (5-HT$_3$), cisapride (5-HT$_4$) or AS-19 (5-HT$_7$) in female pithed rats. Data shown are individual values with means ± SEM. *P < 0.05, significantly different from saline; two-way ANOVA followed by Dunnett's post hoc test.

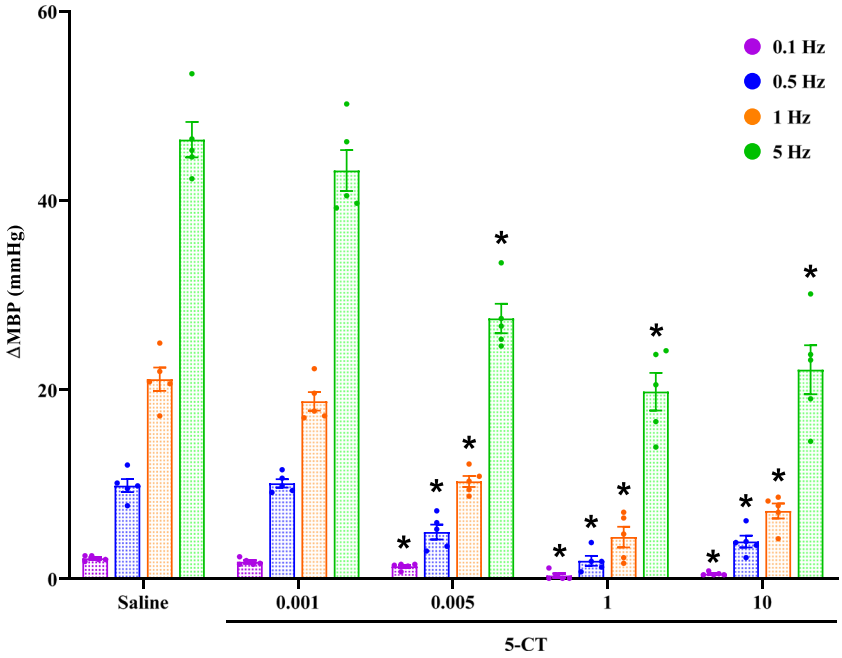

FIGURE 4 Effect of 5-CT on the sympathetic vasopressor responses in female pithed rats. Effect of the i.v. perfusion of saline (control; 1 ml·h^{-1}) or 5-CT (0.001, 0.005, 1 and 10 μg·kg^{-1}·min^{-1}; n = 5 for each dose) on the increases in mean blood pressure (ΔMBP; mmHg) induced by electrical sympathetic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

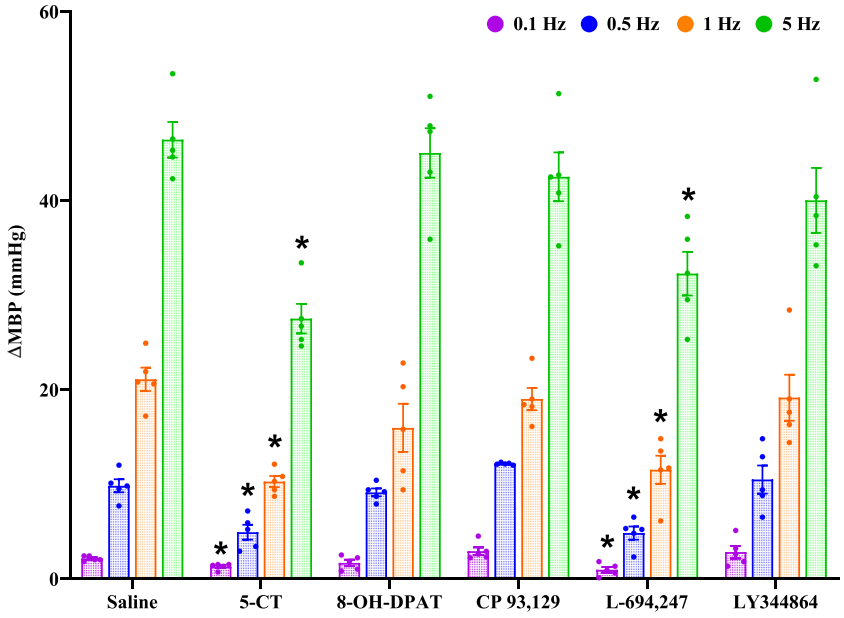

FIGURE 5 Effect of 5-HT$_1$ receptor (sub)type agonists on the sympathetic vasopressor responses in female pithed rats. Effect of i.v. continuous perfusion (n = 5 each) of saline (control; 1 ml·h^{-1}), 0.005 μg·kg^{-1}·min^{-1} of 5-CT and 10 μg·kg^{-1}·min^{-1} of 8-OH-DPAT, CP 93,129, L-694,247 or 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 ± SEM. * $P < 0.05$, significantly different from saline; two-way ANOVA followed by Dunnett's post hoc test.

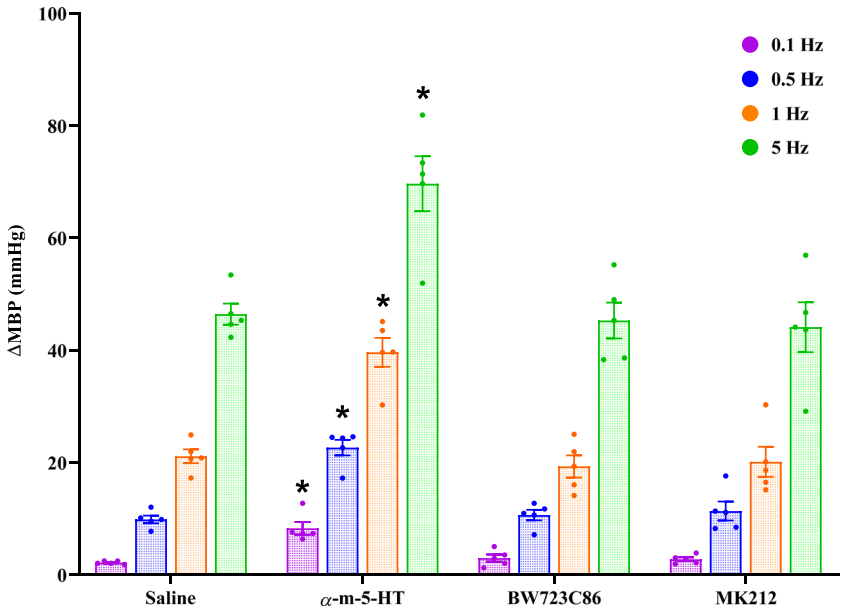

FIGURE 6 Effect of 5-HT$_2$ receptor subtype agonists on the sympathetic vasopressor responses in female pithed rats. Effect of i.v. continuous perfusion of saline (control; 1 ml·h^{-1}), α-m-5-HT (α-methyl-5-HT), BW723C86 or MK212 (10 μg·kg^{-1}·min^{-1} each; n = 5 for each treatment) on the increases in mean blood pressure (ΔMBP; mmHg) induced by electrical sympathetic stimulation in female pithed rats. Data shown are individual values with means ± 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 μg·kg^{-1}·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 μg·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 μg·kg^{-1}·min^{-1} each) did not modify the vasoconstrictor responses to exogenous noradrenaline (Table 3), whereas the i.v. perfusion of 1-PBG (10 μg·kg^{-1}·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

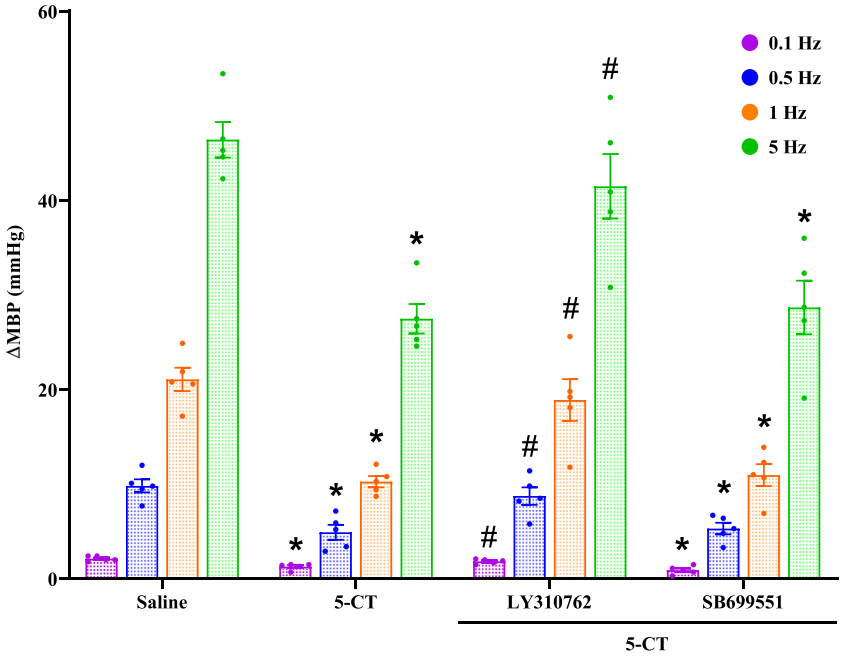

FIGURE 7 Effect of 5-HT_{1D} (LY310762) or 5-HT_{5A} (SB699551) receptor subtypes blockade on 5-CT inhibitory action on electrical-induced vasoconstrictions in female pithed rats. Effect of i.v. bolus injections of LY310762 or SB699551 (1 mg·kg^{-1} each) in the inhibitory effect of 5-CT (0.005 μg·kg^{-1}·min^{-1}) 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-HT_2 or 5-HT_3 receptor agonists on the vasoconstrictions induced by exogenous noradrenaline i.v. administration in female animals.

	Noradrenaline (μg·kg^{-1}; i.v. bolus)			
i.v. infusions (μg·kg^{-1}·min^{-1})	**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-HT_3 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; Gerdts 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-HT_3 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-HT_2 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-HT_3 (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 17β-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-HT_7, 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-HT_7 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-HT_2 and 5-HT_3 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-HT_3 receptor activation potentiated the noradrenergic-induced vasoconstrictions, whereas the 5-HT_2 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-HT_3 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-HT_3 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; Gerdts 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 physiophathology 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 prejunctional 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-HT_3 receptor activation increases sympathetic-induced vasconstrictions. 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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