Life Sciences 293 (2022) 120335

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Oral fluoxetine treatment changes serotonergic sympatho-regulation in experimental type 1 diabetes

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ARTICLE INFO

Keywords:
5-HT
Diabetes
Fluoxetine
Sympathetic neurotransmission
Vascular tone

ABSTRACT

Aims: This study investigated whether fluoxetine treatment changes the 5-HT regulation on vascular sympathetic neurotransmission in type 1 diabetes.

Main methods: Four-week diabetes was obtained by a single alloxan s.c. administration in male Wistar rats, administering fluoxetine for 14 days (10 mg/kg/day; p.o.). Systolic blood pressure, heart rate, glycaemia, body weight (BW) evolution, creatinine, and blood urea nitrogen (BUN) were monitored. Afterward, rats were pithed to perform the vascular sympathetic stimulation. 5-$HT_{1A/1D/2A}$ receptors expression was analysed by Western blot in thoracic aorta. Both i.v. norepinephrine and the electrical stimulation of the spinal sympathetic drive evoked vasoconstrictor responses.

Key findings: Fluoxetine treatment significantly reduced the BW gain, hyperglycaemia, creatinine, and BUN in diabetic rats. The electrical-produced vasopressor responses were greater in untreated than in fluoxetine-treated diabetic rats. 5-HT decreased the sympathetic-produced vasopressor responses. While 5-CT, 8-OH-DPAT and L-694,247 (5-$HT_{1/7}$, 5-HT_{1A} and 5-HT_{1D} agonists, respectively) reproduced 5-HT-evoked inhibition, the 5-HT_2 activation by α-methyl-5-HT augmented the vasoconstrictions. The 5-CT sympatho-inhibition was reversed by 5-HT_{1A} plus 5-HT_{1D} antagonists (WAY-100,635 and LY310762, respectively), whereas ritanserin (5-HT_{2A} antagonist) blocked the α-methyl-5-HT potentiating effect. Norepinephrine-generated vasoconstrictions were increased or diminished by α-methyl-5-HT or 5-CT, respectively. 5-$HT_{1A/1D/2A}$ receptors were expressed at vascular level, being 5-HT_{1A} expression increased by fluoxetine in diabetic rats.

Significance: Our findings suggest that fluoxetine improves metabolic and renal profiles, changes the vasopressor responses, and 5-HT receptors modulating sympathetic activity in diabetic rats: 5-$HT_{1A/1D}$ are involved in the sympatho-inhibition, while 5-HT_{2A} is implicated in the sympatho-potentiation, being both effects pre and/or postjunctional in nature.

1. Introduction

Diabetes mellitus and depressive disorder is one of the comorbidities that is associated with the greatest deterioration in the health of patients [1]. There is an interconnection between both pathologies, where the altered neurohumoral pathways of one can trigger the appearance of the other [2,3]. The longer duration together with the onset at early ages makes type 1 diabetes (T1D) more prone to suffer from these psychiatric disorders, enhancing the T1D-derived cardiovascular complications [3], which are the main causes of mortality in T1D [4,5]. Among their etiopathogenic factors, an increase in the noradrenergic neurotransmission causes vascular impairment, arterial hypertension, renal disease, and heart failure [6,7], besides being also common in depressive disorders [8].

It has been demonstrated that vascular sympathetic neurotransmission is modulated by the serotonergic system [9–11]. We have

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https://doi.org/10.1016/j.lfs.2022.120335
Received 21 December 2021; Received in revised form 14 January 2022; Accepted 14 January 2022
Available online 18 January 2022

previously established that 5-HT causes rat vascular sympatho-inhibition mainly by activation of 5-HT_{1D} receptors [9–11]. However, experimental T1D, as well as pharmacological 5-HT modulation by the antagonism of 5-HT_2 receptor, modified the serotonergic effect on sympathetic discharge of rat vasculature [12–14], maintaining the main vascular sympatholytic effect of 5-HT.

Otherwise, increasing 5-HT concentrations using fluoxetine as a selective serotonin reuptake inhibitors (SSRIs) is the keystone of antidepressant treatment [15]. Apart from their central neuronal effect, SSRIs affect the cardiovascular homeostasis, displaying positive and undesirable consequences [16–18]. In this line, we have recently shown that fluoxetine treatment in normoglycaemic rats caused a reduction in the vascular release of norepinephrine (NE), as well as a modification of the 5-HT receptors that modulate the noradrenergic innervation [19]. Under diabetes situation, SSRI treatment seems to improve the metabolic alteration, although there are conflicting data [20,21].

Considering that a) the sympathetic modulation is a promising target in T1D-related cardiovascular alterations, b) the serotonergic regulation is a key player in vascular sympathetic neurotransmission, and c) SSRI treatment has shown possible benefits in diabetes as well as inhibitory effects on vascular noradrenergic drive, this research aimed to investigate if 14-day administration of fluoxetine (SSRI) modifies the impact of 5-HT modulation on vascular sympathetic outflow and exerts beneficial cardiometabolic effects in type 1 diabetic rats.

2. Material and methods

2.1. Bioethical statement

We utilized male rats (Wistar strain), weighing from 260 to 290 g (n = 172). All protocols were authorised by Academic Bioethics Committee (ID number 0000357) and comply with current European and Spanish legislation (Directive 2010/63/EU and Spanish R.D. 53/2013).

2.2. Compounds

The drugs (and its corresponding suppliers) utilized were: fluoxetine hydrochloride (Normon; Madrid, Spain); heparin sodium (Roche; Madrid, Spain); atropine sulphate (Scharlau; Barcelona, Spain); sodium pentobarbital (Dolethal® from Vetoquinol; Madrid, Spain); d-tubocurarine hydrochloride, alloxan monohydrate, 5-HT creatinine-sulfate monohydrate, NE bitartrate, 1-phenylbiguanide (1-PBG), BW723C86, and CGS-12066B (Sigma-Aldrich; St. Louis, MO, US); 5-carboxamidotryptamine maleate (5-CT), 8-OH-DPAT, L-694,247, α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT), ritanserin, MK212 hydrochloride, WAY-100,635, and LY310762 (Tocris Bioscience; Bristol, UK).

All drugs were dissolved in saline, except ritanserin (in 0.04 M lactic acid). None of the vehicles significantly affected baseline blood pressure or heart rate (HR).

2.3. Common methods

T1D was induced in 165 animals by subcutaneous administration of alloxan (150 mg/kg). The rats were split up into two sets: non-treated (control) and fluoxetine-treated (fluox-t) diabetic rats (n = 25 and 140, respectively). All the rats were kept diabetic for 28 days. Systolic blood pressure (SBP), HR, body weight, and glycaemia were periodically measured (day 0, 2, 7, 14, 21 and 28). Animals that did not get non-fasting blood glucose concentrations >250 mg/dL at all-time points were withdrawn from the experiment. Blood glucose levels, SBP and HR were determined in awake animals, as previously described [12–14,19]. The remaining 7 rats were used as non-diabetic (normoglycaemic) control to measure blood parameters (see below).

In fluox-t diabetic group, fluoxetine was orally administered (10 mg/kg/day in drinking water), from day 14 to day 28 [19,22]. At this point, 10 rats (5 from non-treated and 5 from fluox-t diabetic group) were used for analysing the expression of 5-$HT_{1A/1D/2A}$ receptor subtypes in samples of thoracic aorta artery by Western blot (see "Western blot study" section). The other 155 rats (n = 155) were assigned for pharmacological trials in pithed animals (Fig. 1). Of these animals, 14 rats (7 from each diabetic group, non-treated and fluox-t diabetics) plus 7 normoglycaemic rats (non-diabetic control) were destined to examine renal markers from whole blood samples, analysing by SPOTCHEM® EZ SP-4430 (A. Menarini Diagnostics; Barcelona, Spain).

After 4-week diabetes period, animals were intraperitoneally anesthetized (sodium pentobarbital; 60 mg/kg) and pithed. From that moment till the end of the experiments mean blood pressure (MBP) and HR were continuously measured. Stimulation of complete adrenergic drive was performed as previously described [13,14]. Then, the effect of i.v. perfusion of 0.9% NaCl or the different 5-HT receptor agonists was studied on the increases in MBP produced by: noradrenergic electrical stimulation (n = 135; 20 rats from cluster 1, and 115 rats from cluster 2); or i.v. administration of NE (n = 20 rats from cluster 2).

No alteration in basal HR or MBP were remarked after the vasopressor stimulus-response (S-R) by preganglionic noradrenergic stimulation or after the dose-response (D-R) by exogenous NE [13,14].

2.4. Study protocols

After achieving stable haemodynamic values (~10 min), clusters 1 and 2 went through the next protocols (Fig. 1).

2.4.1. Procedure 1 - electrical stimulation of the vascular sympathetic innervation

The control S-R curve (E0) was obtained by electrical activation (27.5 ± 2.5 V; 25 s; 0.1, 0.5, 1, and 5 Hz) of the noradrenergic vasopressor outflow [13,14]. Later, rats were distributed into several groups:

The first group (cluster 1; control diabetic group; n = 20) received a continuous i.v. perfusion of nothing (control for all the agonists), 5-HT (10 μg/kg/min), selective 5-$HT_{1/7}$ (5-CT; 5 μg/kg/min), and 5-HT_{1A} (8-OH-DPAT; 10 μg/kg/min) receptor agonists. Then, two new E1 and E2 S-R curves were performed similarly to E0 [12–14,19].

In the first fluox-t diabetic group (cluster 2; n = 65), each rat received a continuous i.v. infusion of nothing (control for all the agonists), saline, 5-HT (5, 10 and 80 μg/kg/min), or 5 μg/kg/min of the selective: 5-$HT_{1/7}$ (5-CT), 5-HT_{1A} (8-OH-DPAT), 5-HT_{1B} (CGS—12066B), 5-HT_{1D} (L-694,247), 5-HT_2 (α-methyl-5-HT), 5-HT_{2B} (BW723C86), 5-HT_{2C} (MK212), and 5-HT_3 (1-PBG) receptor type/subtype agonists (Fig. 1). The S-R curves (E1 and E2) were constructed as previously indicated for control group.

The second fluox-t group (n = 25) was performed to stablish the action of the i.v. bolus administration of antagonists: WAY-100,635 (100 μg/kg), LY310762 (1 mg/kg), ritanserin (1 mg/kg), 5-$HT_{1A,}$ 5-HT_{1D}, or 5-HT_{2A} receptor antagonists, respectively, 0.04 M lactic acid (vehicle of ritanserin), or WAY-100,635 + LY310762 on the electrical-produced vasoconstrictions (Fig. 1). The $E0_{Antagonist}$ curve was performed 10 min after the administration. When it was finished, animals received a saline infusion, and E1 and E2 curves were conducted.

The third fluox-t group (n = 25) permitted us to stablish the function of the different receptor (sub)types implicated in the serotonergic modulatory action. These rats were subdivided into several subsets: (a) role of 5-HT_{1A} receptor: i.v. bolus administration of WAY-100,635 + i.v. infusion of 8-OH-DPAT; (b) involvement of 5-HT_{1D} receptor: i.v. bolus of LY310762 + i.v. perfusion of L-694,247; (c) implication of 5-$HT_{1A/1D}$ receptor: i.v. combination of LY310762 plus WAY-100,635 and, then, an i.v. infusion of 5-CT (5 μg/kg/min); (d) 5-HT_{2A} repercussion: animals received i.v. ritanserin + i.v. infusion of α-methyl-5-HT (5 μg/kg/min) or 5-HT (10 μg/kg/min). Then, S-R E1 and E2 curves were obtained as previously indicated (Fig. 1).

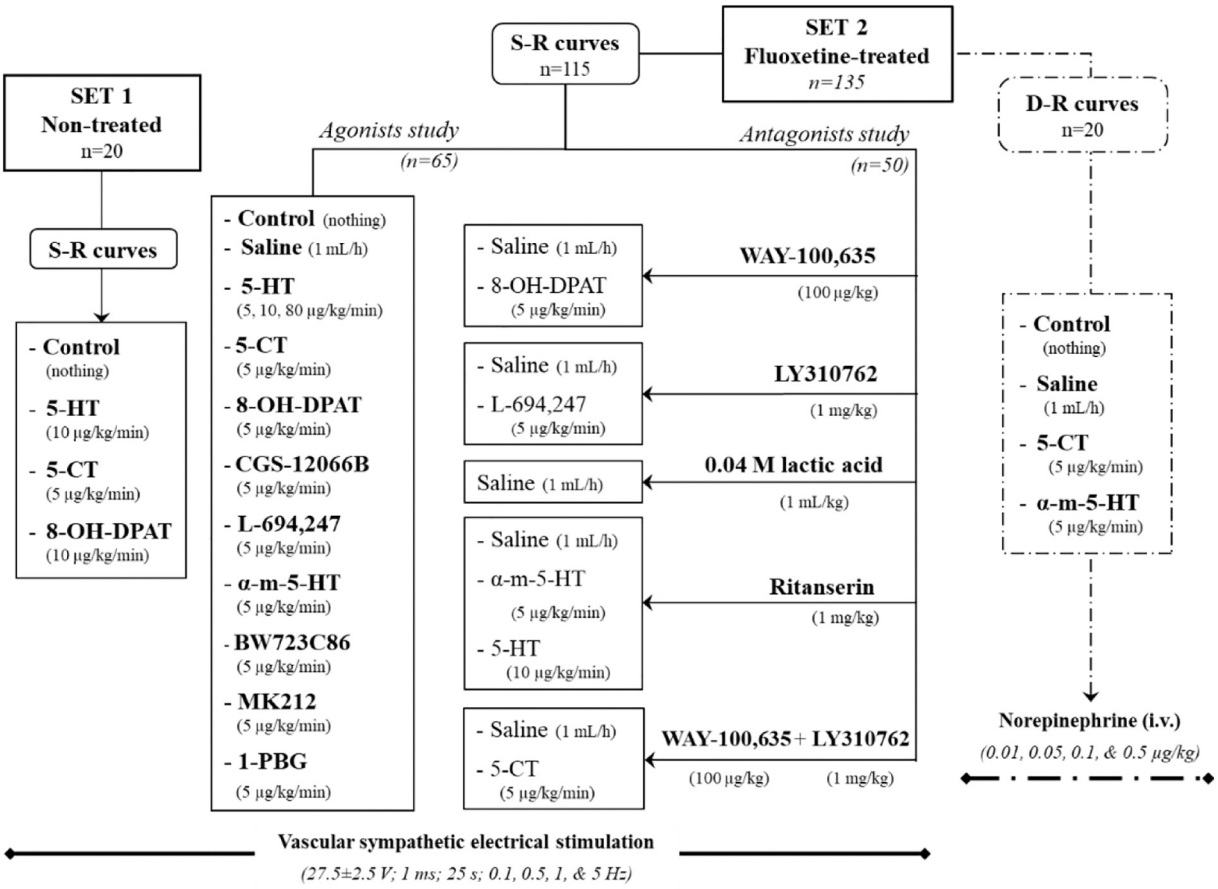

Fig. 1. Experimental groups showing the number of animals used. Number of pithed rats used in the two main sets (set 1: non-treated rats; set 2: fluoxetine-treated rats) as well as their divisions into further treatments ($n = 5$ each, with no exception). The vasopressor responses were obtained by either electrical sympathetic stimulation (set 1 and set 2, stimulus-response curves [S-R curves]; $n = 135$) or i.v. bolus injections of exogenous noradrenaline (set 2, dose-response curves [D-R curves]; $n = 20$). Agonists were administered as i.v. continuous infusion and antagonists were administered as i.v. bolus injections. α-m-5-HT, α-methyl-5-HT.

2.4.2. Procedure 2 - exogenous norepinephrine (i.v. bolus)

To explore the post and/or prejunctional nature, increasing doses of NE were administered in a group of fluox-t diabetic rats (cluster 2; $n = 20$; Fig. 1) to induce pressor responses. D-R curves by NE, were conducted before (E0) and during (E1 and E2) the continuous perfusion of nothing (control), saline, α-methyl-5-HT, or 5-CT (5 µg/kg/min each), as described in previous works [12–14,19].

The doses for all compounds have been chosen in the basis of preceding results [12–14,19,22]. Infusion of saline or 5-HT agonists was rated at 1 mL/h. The time lapse between the distinct stimulation frequencies or NE doses was ~5–10 min, always awaiting until MBP had returned to baseline values after vasopressor responses. E1 and E2 (or E1 and E2) curves were always similar; therefore, solely E2 (or E2) curves are presented in the figures.

2.5. Western blot study

Thoracic aortas were carefully isolated and cleaned of fat and connective tissue. Then, the aortas were disintegrated, lysed and samples were prepared as previously described [12]. Gels were prepared adding 2,2,2-trichloroethanol (Sigma-Aldrich) to visualize the proteins and to use the total amount of protein as a loading control (Stain Free). Gels were blotted onto polyvinylidene fluoride membranes (Bio-Rad), which were blocked with 5% BSA in tris-buffered saline (TBS) for 1 h at room temperature before incubation with the primary antibodies: 5-HT_{1A}, 5-HT_{1D}, and 5-HT_{2A} receptor subtypes (from *antibodies-online GmbH*) overnight at 4 °C. Blots were then washed in TBS–Tween, and then incubated with a fluorescent secondary antibody (StarBright B700, goat anti-rabbit, Bio-Rad). Bands were visualized with the ChemiDoc™ MP Imaging System (Bio-Rad). The analysis of the Stain Free and the bands was carried out with the ImageLab program (Bio-Rad). For this, the volume of the band of interest was quantified, normalizing it regarding the volume of its total protein. The target protein band intensity values are adequately corrected (to protein load), which allowed a precise comparison of target proteins among the samples.

2.6. Statistical evaluation

All results are represented as mean ± SEM of, at least, five experiments ($n = 5$). Electrically-induced or exogenous NE MBP variations were represented as increases in mmHg from the basal value. Statistic differences were analysed with one-way ANOVA followed by the *post hoc* Bonferroni's test, with the exception of the Western blot analysis ($n = 4$ for each group) where Wilcoxon test was utilized. Statistical significance was accepted at $p < 0.05$. In *in vivo* experiments (pithed rats), the statistical evaluation was completed *versus* control curves (E0 or E0).

3. Results

3.1. Cardiometabolic parameters

Table 1 shows the mean values of non-fasting blood glucose concentrations (mg/dL), HR (beats/min), SBP (mmHg) and body weight (g) at day 0 (prior to alloxan administration) and at day 28 after diabetes induction in rats. Alloxan caused a marked increase in blood glucose concentration from day 2 post-administration (data not shown),

Table 1
Monitored variables in control and 14-day fluoxetine-treated diabetic rats.

Day	Diabetic rats		
	0 (n)	Non-treated	Fluoxetine-treated
		28 (n)	28 (n)
Body weight (g)	275 ± 15 (165)	334 ± 7* (25)	284 ± 4# (140)
Glycaemia (mg/dL)	95 ± 4 (165)	488 ± 12* (25)	267 ± 10*# (140)
SBP (mmHg)	124 ± 4 (12)	142 ± 2* (6)	137 ± 4* (6)
Heart rate (beats/min)	330 ± 15 (12)	330 ± 6 (6)	310 ± 13 (6)

Body weight (g), non-fasting glycaemia (mg/dL), systolic blood pressure (SBP; mmHg), and heart rate (beats/min) in rats at day 0 (prior diabetes induction) and 28 days after inducing type 1 diabetes in fluoxetine-treated rats (14 days; 10 mg/kg/day; p.o.) or non-treated group.
* $p < 0.05$ *vs* the corresponding value at day 0.
# $p < 0.05$ *vs* the corresponding value in non-treated group at day 28.

maintaining this hyperglycaemia during the 28 days in the control diabetic group (Table 1). However, the fluoxetine treatment (during 14 days) significantly diminished the glycaemia in diabetic rats. Additionally, the fluoxetine treatment prevented the body weight gain of diabetic animals at 28 days post-induction compared to the non-treated diabetic group. The animals reached a hypertensive state (without modifying HR) at day 28 post-diabetes induction, which was not significantly changed in fluoxetine-treated diabetic rats.

Both blood urea nitrogen (BUN) and plasma creatinine concentrations were increased in control diabetic rats when compared with normoglycaemic animals (Table 2). However, fluoxetine treatment significantly reduced these renal blood parameters in diabetic rats (Table 2).

Basal MBP and HR, after anaesthesia and pithing, in fluox-t diabetic rats (n = 135) were 52 ± 1 mmHg and 277 ± 5 beats/min, respectively; and 53 ± 2 mmHg and 284 ± 8 beats/min, respectively, in control diabetic rats ($n = 20$). Neither i.v. perfusions/bolus of vehicles nor 5-HT agonists or antagonists in fluox-t diabetic rats significantly shifted these values, with the exception of: MBP and HR were increased by the perfusion of either 80 μg/kg/min of 5-HT and α-methyl-5-HT (Δ MBP: 30 ± 4 and 28 ± 4 mmHg, respectively; Δ HR: 40 ± 14 and 132 ± 15 beats/min, respectively); only 5-CT diminished MBP (Δ MBP: −17 ± 3 mmHg); and only 1-PBG perfusion enhanced HR (Δ HR: 40 ± 13 beats/min). Administration of WAY-100,635 + LY310762 (data not shown) prior to 5-CT reversed this vasodilator effect. On the other hand, ritanserin administration prior to perfusion of 10 μg/kg/min of 5-HT reduced MBP (Δ MBP: −13 ± 3 mmHg).

3.2. Vascular action of noradrenergic outflow stimulation or exogenous NE

Electrical stimulation of noradrenergic outflow (0.1–5 Hz) or i.v. administration of NE (0.01–0.5 μg/kg) induced instant increases in MBP, which were frequency- or dose-dependent (sympathetic stimulation or NE, respectively) (Figs. 2, 3 and Table 3). As previously stated [12–14,19], dose-dependent and transitory augmenting in HR was provoked by NE injections (not shown) in fluox-t diabetic rats; whereas the electrical-provoked vasopressor actions are caused by total noradrenergic stimulation, and slight fluctuations in HR were noticed [12–14,19].

The electrically induced gains (from basal values) in MBP (0.1, 0.5, 1, and 5 Hz) were 9.6 ± 1.3, 40.7 ± 3.6, 58.4 ± 3.2, and 103.5 ± 2.5 mmHg in control diabetic group; anyhow, these increases were significantly diminished in fluox-t diabetic rats: 3.7 ± 0.2, 23.4 ± 0.8, 41.8 ± 1.1, and 77.9 ± 1.4 mmHg (Fig. 2 (E0) and Fig. 3 (E0)). The vasopressor responses to exogenous NE in fluox-t hyperglycaemic animals are shown in Table 3 (E0 curve). Increases in MBP were reproducible, since they were basically unaltered after i.v. saline.

Table 2
Renal blood parameters in normoglycaemic, non-treated diabetic, and 14-day fluoxetine-treated diabetic rats.

Blood variables (mg/dL)	Control	Non-treated diabetic	Fluox-t diabetic
Blood urea nitrogen (BUN)	22.4 ± 0.9	36.3 ± 1.4*	22.7 ± 1.6#
Creatinine	0.62 ± 0.02	0.81 ± 0.05*	0.50 ± 0.10#

Renal blood variables (blood urea nitrogen, BUN; and creatinine; mg/dL) in normoglycaemic (control), non-treated diabetic, and fluoxetine-treated (Fluox-t; 10 mg/kg/day fluoxetine treatment for 14 days) diabetic rats ($n = 7$ each group).
* $p < 0.05$ *vs* control (normoglycaemic) group.
# $p < 0.05$ *vs* non-treated diabetic group.

3.3. Impact on the electrically evoked vasoconstrictions of 5-HT type/subtype receptor agonists

In control diabetic rats, perfusion of 5-HT reduced the noradrenergic-evoked vasopressor responses at all frequencies used, with the exception of 5 Hz (not shown). Besides, 5-CT and 8-OH-DPAT substantially diminished the sympathetic-produced vasoconstrictions (not shown), as previously demonstrated [13].

In fluox-t diabetic rats, 5-HT perfusions (5–80 μg/kg/min) originated a substantial reduction of the noradrenergic-induced vasoconstrictions in a dose- and frequency- dependent manner (Fig. 2A). While perfusion of α-methyl-5-HT considerably augmented the electrically induced vasoconstrictions at all frequencies tested (Fig. 2C),

5-CT significantly diminished the sympathetic-mediated vasopressor responses (Fig. 2B). On the other hand, 1-PBG did not alter the electrically induced vasoconstrictions (not shown).

3.4. Effect of 5-HT$_1$ or 5-HT$_2$ subtype agonists on noradrenergic vasopressor responses in fluox-t diabetic rats

The inhibitory effect of 5-CT was reproduced by i.v. perfusion of the 5-HT$_{1D}$ and 5-HT$_{1A}$ receptor agonists, L-694,247 and 8-OH-DPAT, respectively (Fig. 2B); whereas the 5-HT$_{1B}$ receptor agonist, CGS—12066B, did not reproduced the 5-HT inhibition (Fig. 2B).

Solely α-methyl-5-HT, a 5-HT$_2$ receptor agonist, substantially augmented the electrically induced noradrenergic vasoconstrictions (Fig. 2C); while the 5-HT$_{2C}$ and 5-HT$_{2B}$ receptor agonists, MK212 and BW723C86, respectively, did not modify the E0 (control) S-R curve (Fig. 2C).

3.5. Impact on the serotonergic regulation of adrenergic outflow by 5-HT receptor antagonists in fluox-t diabetic rats

The effect of several serotonergic antagonists was studied in the 5-HT modulation of noradrenergic-induced vasopressor effect in fluox-t hyperglycaemic group. WAY-100,635 (5-HT$_{1A}$ antagonist), LY310762 (5-HT$_{1D}$ antagonist), the cocktail of WAY-100,635 + LY310762, ritanserin (5-HT$_{2A}$ antagonist), or its vehicle (0.04 M lactic acid) had no effect *per se* on these vasopressor responses (data not shown; $p > 0.05$).

Pretreatment with WAY-100,635 or LY310762 abolished the inhibitory action of 8-OH-DPAT or L-694,247, respectively, in fluox-t diabetic rats (Fig. 3A). In this line, ritanserin pretreatment blocked the enhancing sympathetic effect produced by α-methyl-5-HT (Fig. 3B). The inhibitory action produced by the i.v. perfusion of (a) 5-CT was annulled by the i.v. mixture of WAY-100,635 + LY310762 (Fig. 3A), (b) 5-HT was enhanced by prior i.v. administration of ritanserin (Fig. 3B).

3.6. NE-induced vasoconstrictions in fluox-t diabetic rat: Influence of α-methyl-5-HT and 5-CT

The increments in MBP evoked by i.v. administration of NE remained stable after the perfusion of 0.9% NaCl (data not shown). As Table 3

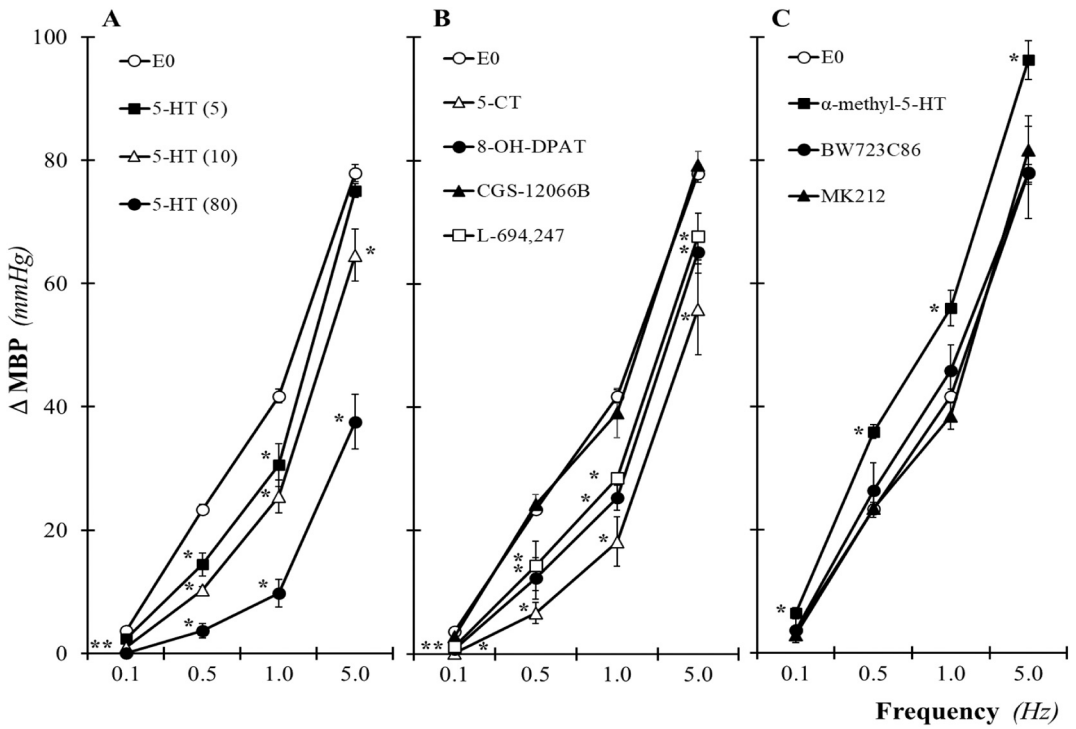

Fig. 2. Increases in mean blood pressure (Δ MBP; mmHg) evoked by noradrenergic electrical stimulation in the course of i.v. continuous perfusions ($n = 5$ each) of nothing (control; E0), and (A) increasing doses of 5-HT (5, 10 and 80 μg/kg/min), (B) 8-OH-DPAT, L-694,247, CGS-12066B and 5-CT (5 μg/kg/min each), or (C) α-methyl-5-HT, BW723C86 and MK212 (5 μg/kg/min each) in fluoxetine-treated diabetic rats. *$p < 0.05$ *vs* E0 curve.

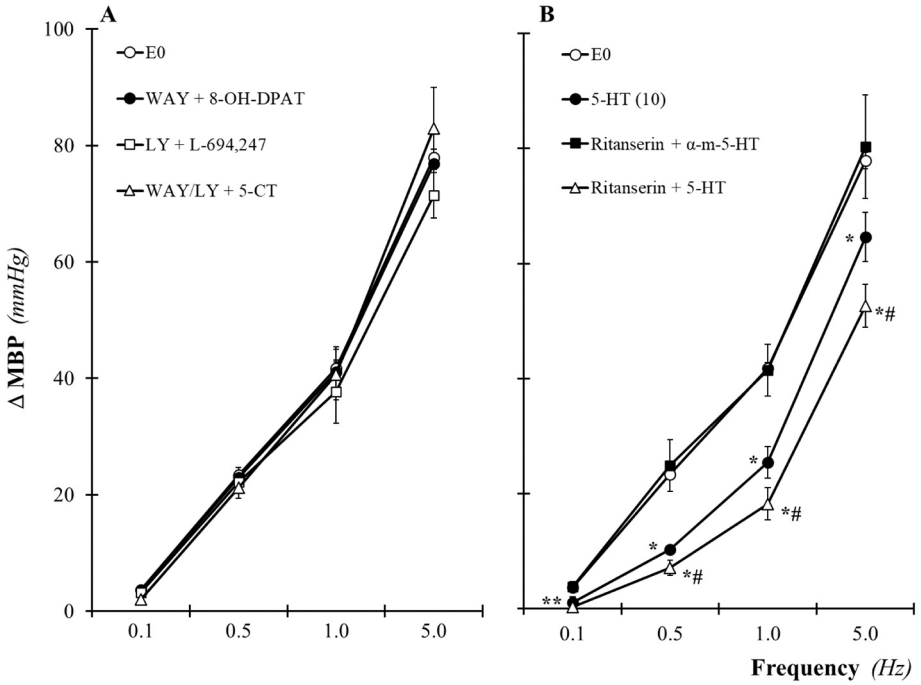

Fig. 3. Variations on increases in mean blood pressure (Δ MBP; mmHg) by electrical stimulation of noradrenergic outflow after (A) i.v. perfusion of L-694,247, 8-OH-DPAT, or 5-CT (5 μg/kg/min each) after i.v. bolus of LY310762 (LY; 1 mg/kg), WAY-100,635 (WAY; 100 μg/kg), or a combination of WAY plus LY, respectively; or (B) i.v. perfusion of 5-HT (10 μg/kg/min) in the absence or presence of i.v. bolus of ritanserin (1 mg/kg), or ritanserin plus α-methyl-5-HT infusion (α-m-5-HT; 5 μg/kg/min) in fluoxetine-treated diabetic rats ($n = 5$ each group). *p < 0.05 *vs* E0; #p < 0.05 *vs* the corresponding agonist.

shows, continuous i.v. perfusion of 5 μg/kg/min of 5-CT decreased the vasoconstrictor responses to NE, while α-methyl-5-HT (5 μg/kg/min) augmented the NE-produced vasopressor responses ($p < 0.05$ each agonist) compared to E0 curve.

3.7. Vascular expression of 5-HT receptors in diabetic rats

The expression of 5-HT receptors was examined in thoracic aorta from control and fluox-t diabetic rats by Western blot ($n = 4$ for each group of animals). The aortic expression of 5-HT_{1A} receptor protein was substantially higher in fluox-t compared with control diabetic rats (Fig. 4), whereas there are no differences in the 5-HT_{1D} and 5-HT_{2A}

Table 3
Impact of 5-$HT_{1/7}$ or 5-HT_2 agonists on the vasoconstrictions evoked by norepinephrine in fluoxetine-treated diabetic animals.

Infusions (μg/kg/min; i.v.)	Norepinephrine (μg/kg; i.v. bolus)			
	0.01	0.05	0.1	0.5
E0 (nothing)	11.0 ± 1.0	16.8 ± 1.4	23.7 ± 1.9	46.2 ± 3.3
5-CT (5)	4.5 ± 0.9*	9.5 ± 0.7*	11.9 ± 3.3*	34.3 ± 3.0*
α-methyl-5-HT (5)	24.4 ± 3.8*	29.0 ± 1.8*	40.5 ± 3.7*	67.8 ± 5.7*
	Δ MBP (mmHg)			

Impact of i.v. perfusions of nothing (control group; E0), 5-CT (5-$HT_{1/7}$ receptor agonist; 5 μg/kg/min) or α-methyl-5-HT (5-HT_2 receptor agonist; 5 μg/kg/min) on the ascents in mean blood pressure (Δ MBP; mmHg) induced by exogenous norepinephrine (0.01–0.5 μg/kg) in fluoxetine-treated diabetic rats ($n = 5$ each group).
* $p < 0.05$ *vs* E0.

receptor expressions between non-treated and fluox-t diabetic animals (Fig. 4).

4. Discussion

Diabetes shares some etiologic factors with depression, leading to a strong interconnection where several neurohumoral disturbances negatively influence on their evolution [2,23]. It is known that one of the markedly altered systems within depressive disorders is the serotonergic (being also an important player in pathologies such as diabetes mellitus), which makes SSRI drugs the treatment of choice for depression [15]. Nevertheless, the cardiovascular influence of SSRIs has not been clarified in diabetes situation, showing controversial peripheral effects utilizing fluoxetine (the most widely used SSRI) [24,25].

In this line, our current results demonstrate that fluoxetine treatment for 14 days modifies the vascular sympathetic neurotransmission in type 1 diabetic rats, as well as the modulation by the peripheral serotonergic system. In fluox-t diabetic rats, there is a decrease in NE release at vascular level, and sympathetic inhibitory and excitatory 5-HT receptors (both of pre and/or postjunctional in nature) co-occur: the vascular sympatholytic effect is mainly provoked by 5-$HT_{1A/1D}$ receptors, whereas the 5-HT_{2A} subtype is the sympatho-excitatory receptor mainly involved in the increased vasopressor responses. Additionally, the chronic fluoxetine treatment reduced the hyperglycaemia, and improved the blood renal parameters in diabetic rats.

As previously demonstrated [19], the 14-day fluoxetine administration (at dose 10 mg/kg/day; p.o.) seeks to reproduce the oral treatment in patients, excluding the distress connected to other administration methods. This fluoxetine dose causes concentrations in rat plasma [26] which can be normally found in subjects suffering from depressive disorders receiving fluoxetine therapy.

In the present research, 14-day fluoxetine treatment (p.o.) did not alter SBP and HR in awake diabetic rats, therefore it did not affect the hypertensive state of non-treated diabetic rats; other different studies described dissimilarities in cardiovascular variables by fluoxetine in normoglycaemic rats, although the treatment period was different comparing with our current report [27]. However, the non-fasting glycaemia and the body weight gain were significantly diminished in fluox-t compared to control diabetic rats. These data agree with other studies where fluoxetine induced these metabolic changes, in addition to others, improving the chronic complications derived from diabetes [28,29]; these effects have been also observed in patients with T1D treated with fluoxetine [30]. Furthermore, altered blood markers of renal function (BUN and creatinine) in diabetic rats were significantly reduced with 14-day fluoxetine treatment. Although we cannot disregard the possibility that the improvement in renal parameters is due to the reduction of hyperglycaemia by fluoxetine, according to our results, Aksu and colleagues [31] demonstrated that 3-day fluoxetine treatment improved kidney functionality in a rat model of acute kidney injury. Therefore, it seems that this drug does induce kidney benefits in different experimental models independently of glycaemia.

In anesthetized pithed rats (where only the peripheral nervous system is operative), baseline MBP and HR in fluox-t hyperglycaemic rats were comparable to non-treated group, as it happened in normoglycaemic rats treated with fluoxetine [19]. These variables were modified by 5-HT (80 μg/kg/min) and 5-HT agonists (1-PBG, α-methyl-5-HT and

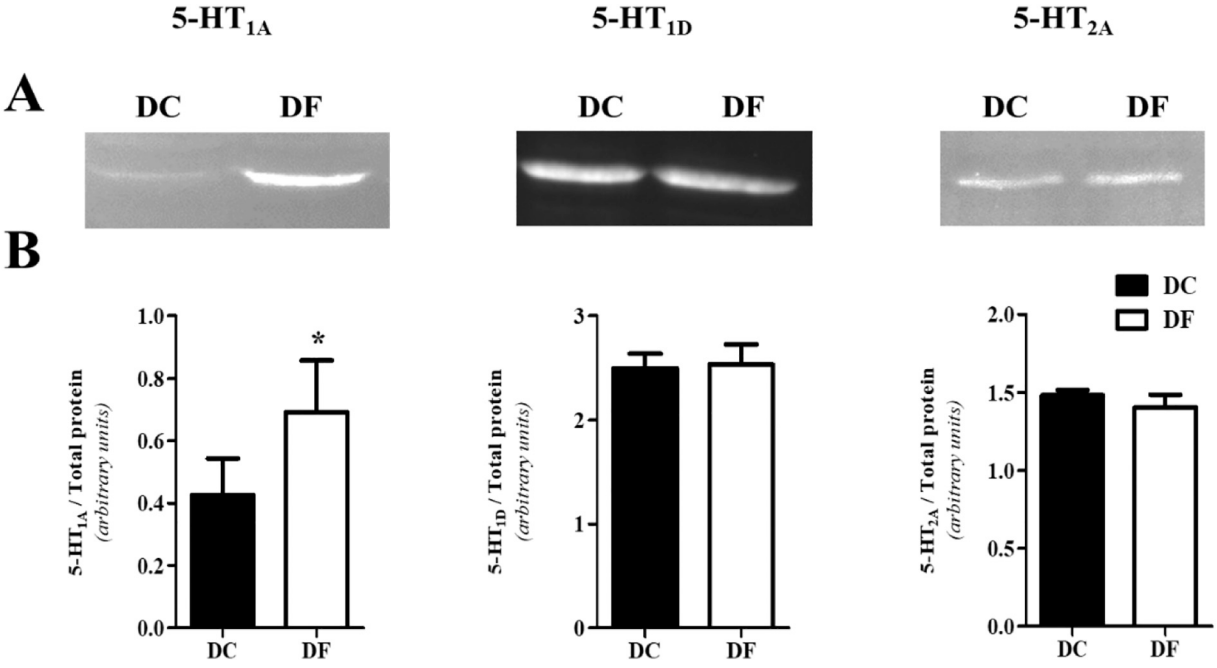

Fig. 4. 5-$HT_{1A/1D/2A}$ receptor expression in thoracic aortas from control (DC) and fluoxetine-treated (DF) diabetic rats by Western blot. A representative blot of total protein extracts from four independent experiments is shown, using total amount of protein as a loading control (A). Blots were assessed by densitometric analysis. The ratio of 5-HT receptors expression *vs* total protein is outlined in the graph (B). All values are represented as mean ± SEM. *$p < 0.05$ *vs* DC values.

5-CT) likewise it happened in normoglycaemic animals treated with fluoxetine [19]. In fluox-t diabetic rats, 5-HT produced vasorelaxant effect under ritanserin (5-HT_{2A} antagonist) pretreatment, whereas the 5-CT-induced vasodilation was blocked by the cocktail of LY310762 + WAY-100,635 (5-HT_{1D} and 5-HT_{1A} antagonists, respectively), which confirms the involvement of these 5-HT_1 family subtypes in the serotonergic vasodilator response as well as the vasopressor effect mediated by 5-HT_{2A} receptor activation, as previously established in non-diabetic animals [19].

The outcomes found by us reveal that 5-HT always exhibits a vascular sympatholytic effect [9–14,19], and since the rats of this research must have augmented the 5-HT levels by fluoxetine treatment, this increase in 5-HT levels at the neuroeffector union could be related to the lesser discharge of vascular NE, and, then, to the decreased vasoconstrictions in diabetic rats treated with fluoxetine compared to untreated diabetic set (current findings). In accordance with these data, the acute fluoxetine administration reduced the rat renal sympathetic nerve activity [32], as well as 14-day fluoxetine treatment decreased of acetylcholine release after vagal stimulation in rats [22]. In fluox-t diabetic rats, 5-HT evoked a reduction of the electrical-induced vasoconstrictions in a dose-dependent manner; this sympatho-inhibition was higher at low stimulation frequencies as already confirmed by us [9–14,19]. The 5-$HT_{1/7}$, 5-HT_{1A} and 5-HT_{1D} receptor agonists (5-CT, 8-OH-DPAT and L-694,247, respectively) mimicked the 5-HT sympatholytic effect. These outcomes are partly in line with our preceding data in normoglycaemic fluox-t rats [19], since 5-$HT_{1A/1D}$ subtypes participate in the 5-HT sympatho-inhibition but they are pre and/or postjunctional in nature by also reducing the vasopressor responses induced by exogenous NE. In some way, it could thus enhance sympathetic inhibition by the involvement of the 5-HT_1 family in diabetic rats under fluoxetine treatment, because only prejunctional 5-HT_{1A} receptors are implicated in non-treated diabetic rats [13].

The participation of 5-HT_{1A} and 5-HT_{1D} subtypes in diabetic rats treated with fluoxetine was confirmed using a) the antagonists WAY-100,635 and LY310762, which totally reversed 8-OH-DPAT- and L-694,247-produced sympathetic inhibition, respectively, and b) the mixture of LY310762 + WAY-100,635, which abolished the 5-CT-induced sympatho-inhibition. The latter also rules out the possible involvement of 5-HT_7 receptors, since 5-CT also displays affinity for these receptors [33]. All these results are reinforced by the fact that the effector mechanism of 5-HT_{1A} and 5-HT_{1D} subtypes consist of inhibiting adenylyl cyclase activity, which is correlated with the decrease of the neuromodulators discharge [34]. Our Western blot study demonstrated that 5-HT_{1A} as well as 5-HT_{1D} receptor subtypes were expressed in vascular tissue in both diabetic groups, with a significant increase in 5-HT_{1A} subtype in diabetic animals receiving fluoxetine treatment.

As recently found in normoglycaemic rats treated with fluoxetine [19], the 5-HT_{2A} subtype activation is implicated in the increase of the vasoconstrictions, since a) α-methyl-5-HT (5-HT_2 agonist) produced the sympatho-excitatory effects, b) the activation of 5-HT_{2B} or 5-HT_{2C} subtypes did not modify the electrical-evoked vasoconstrictor responses, and c) the antagonism of 5-HT_{2A} subtype with ritanserin [35] eliminated the sympatho-enhancer response by α-methyl-5-HT. This effect is pre and/or postjunctional in nature, since α-methyl-5-HT also enhanced vasoconstrictions induced by i.v. NE. According to our results, it has been established that 5-HT_{2A} subtype is located at spinal sympathetic neurons in rats exhibiting a dual function (pre- and postsynaptic) [36]. In addition, our current data show that 5-HT_{2A} subtype is expressed in aortic arteries of untreated and fluox-t diabetic rats.

In view of these results, fluoxetine treatment seems to dictate the involvement of the different receptor subtypes (5-$HT_{1A/1D/2A}$) modifying vascular noradrenergic neurotransmission in diabetic rats, since the same receptors are implicated in normoglycaemic rats treated with fluoxetine [19]. The difference lies in the fact that all sympatho-modulatory receptors exert their effects pre and/or postjunctionally, as well as that the sympatholytic 5-HT_{1A} receptors are overexpressed in diabetic rats treated with fluoxetine, being the only shared receptor that is functionally involved in non-treated [13] as well as fluox-t diabetic rats (current results).

Some studies have correlated the prolonged administration of fluoxetine with harmful cardiovascular consequences (hypertensive and tachycardic effects) [24,37]; the manifestation of these cardiovascular unsafe responses could be elicited by the sympathetic potentiating receptors, such as 5-HT_{2A} subtype. Opposed to the possible harmful effects of sympathetic enhancement, it has been shown that chronic treatment with fluoxetine in patients suffering from T1D can amplify the sympathetic activity as a counterregulatory response during moderate hypoglycaemia, which could be useful in clinical practice to combat and diminish the appearance of risky hypoglycaemia [38].

Given that diabetes and depression have several pathophysiological associations such as serotonergic alteration and noradrenergic overactivation [39,40] that increase the cardiovascular risk of this comorbidity, a more in-depth study of how antidepressant treatment with SSRIs could influence the therapy of these patients is necessary, reducing cardiovascular disorders related to the alteration of vascular sympathetic innervation.

Some limitations of our current results should be considered. We used pithed rats which is a proper model to evaluate peripheral cardiovascular responses; however, it eliminates all the influence of central nervous system, which may be important in the clinical practice (under depression and/or T1D). Also, all the procedures were carried out in males, considering our experience and preceding outcomes. Still, it is remarkable that *in vivo* studies (as present) remain the last and necessary step prior to clinical research, and this work contributes to the knowledge about the complex influence of fluoxetine at cardiovascular level under T1D situation.

5. Conclusion

Fourteen-day oral treatment with fluoxetine (10 mg/kg/day) ameliorates glycaemic and renal profiles, decreases the vascular noradrenergic outflow, and changes the 5-HT regulation on sympathetic tone in type 1 diabetic rats: activation of pre and/or postjunctional 5-HT_{2A} receptors enhances sympathetic-evoked vasopressor responses, whereas 5-HT inhibits electrical-induced vasoconstrictions mainly by pre and/or postjunctional 5-HT_{1A} (which is overexpressed) and 5-HT_{1D} receptors. Hence, the 5-HT modulation of the vascular sympathetic innervation might become a potential pharmacological strategy to approach cardiovascular disorders related to chronic hyperglycaemia.

CRediT authorship contribution statement

Alicia Rodríguez-Barbero: Western blot studies. Asunción Morán: Designed the experiments, Analyze the data, Writing- Reviewing and Editing. Claudia Alarcón-Torrecillas: Western blot studies. Cristina López: *In vivo* experiments, Analyze the data. José Ángel García-Pedraza: *In vivo* experiments, Analyze the data, Writing- Reviewing and Editing. Juan Francisco Fernández-González: *In vivo* experiments, Analyze the data. María Luisa Martín: Designed the experiments. Mónica García-Domingo: Designed the experiments, Analyze the data, Writing-Reviewing and Editing. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by University of Salamanca (project number 18.KD7N/463AC01). The authors would like to thank Mrs. Ana Isabel Barrios for her assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.120335.

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