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# DIABETES-INDUCED CHANGES IN 5-HYDROXYTRYPTAMINE MODULATION OF VAGALLY INDUCED BRADYCARDIA IN RAT HEART

**Mónica García, Asunción Morán, M Luisa Martín, A Vega Ortiz de Urbina and Luis San Román**

*Department of Physiology and Pharmacology, University of Salamanca, Salamanca, Spain*

## SUMMARY

**1. In the present study, we investigated how alloxan-induced diabetes affects the ability of 5-hydroxytryptamine (5-HT) to modulate bradycardia induced *in vivo* by electrical stimulation of the vagus nerve in pithed rats. We also analysed the type and/or subtype of 5-HT receptors involved.**

**2. Diabetes was induced in male Wistar rats with a single injection of alloxan (150 mg/kg, s.c.). Four weeks later, rats were anaesthetized, pretreated with atenolol and pithed. Electrical stimulation (3, 6 and 9 Hz) of the vagus nerve resulted in frequency dependent decreases in heart rate (HR).**

**3. In diabetic rats, intravenous bolus administration of high doses of 5-HT (100 and 200 μg/kg) increased the bradycardia induced by vagal electrical stimulation. Similarly, low doses (10 μg/kg) of the 5-$HT_{1/7}$ receptor agonist 5-carboxamidotryptamine (5-CT), increased vagally induced bradycardia. However, at high doses (50, 100 and 150 μg/kg), 5-CT reduced the bradycardia. Attenuation of the vagally induced bradycardia evoked by the higher doses of 5-CT was reproduced by L-694,247 (50 μg/kg), a selective agonist for the non-rodent 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors. Enhancement of the vagally induced bradycardia elicited by low doses of 5-CT was reproduced by the selective 5-$HT_{1A}$ receptor agonist 8-hydroxydipropylaminotretalin hydrobromide (8-OH-DPAT; 50 μg/kg). These stimulatory and inhibitory actions on vagal stimulation-induced bradycardia in diabetic rats were also observed after administration of exogenous acetylcholine.**

**4. Vagally induced bradycardia in diabetic rats was not affected by administration of the selective 5-$HT_2$ receptor agonist α-methyl-5-HT (150 μg/kg), the selective 5-$HT_3$ receptor agonist 1-phenylbiguanide (150 μg/kg) or the selective 5-$HT_{1B}$ receptor agonist CGS-12066B (50 μg/kg).**

**5. Enhancement of the electrical stimulation-induced bradycardia in diabetic rats caused by 5-CT (10 μg/kg) or 8-OH-DPAT (50 μg/kg) was abolished by the selective 5-$HT_{2/7}$ receptor antagonist mesulergine (1 mg/kg) and the selective 5-$HT_{1A}$ receptor antagonist WAY-100,635 (100 μg/kg), respectively. Similarly, pretreatment with the non-selective 5-$HT_1$ receptor antagonist methiothepin (0.1 mg/kg) blocked the inhibitory effect of 5-CT (50 μg/kg) on the bradycardia induced by vagal electrical stimulation in diabetic rats. BRL-15572 (2 μg/kg), a selective 5-$HT_{1D}$ receptor antagonist, inhibited the action of L-694,247 (50 μg/kg), a selective agonist for the non-rodent 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors, on the vagally induced bradycardia.**

**6. In conclusion, in the present study, experimental diabetes evoked changes in both the nature and 5-HT receptor types/subtypes involved in vagally induced bradycardia.**

**Key words: alloxan-induced diabetes, 5-$HT_1$ receptors, 5-hydroxytryptamine, vagally induced bradycardia.**

## INTRODUCTION

The effect of 5-hydroxytryptamine (5-HT) on cholinergic neurotransmission and parasympathetic cardiovascular effects has received considerable attention over many years. In fact, central 5-HT-containing neurons contribute to the reflex activation of parasympathetic outflow in many species.[1,2] However, the findings reported to date are controversial. Some studies have proposed potent depolarizing actions, with an increase in vagal activity mediated by 5-$HT_3$ and 5-$HT_2$,[3,4] 5-$HT_4$[5] or 5-$HT_{1A}$ receptor activation in different animal species, including rats,[6] cats[7] and rabbits.[8] Other reports have suggested an inhibitory serotonergic effect on acetylcholine (ACh) release via 5-$HT_1$ receptor activation.[9,10] In addition, 5-HT can induce the Bezold–Jarisch reflex, which consists of marked hypotension and bradycardia leading to myocardial ischaemia or infarction through activation of serotonin receptors on vagal afferent nerves. We have shown previously that, in pithed rats, the serotonergic mechanisms involved in cardiac cholinergic neurotransmission are presynaptic in nature and that they can dually inhibit or facilitate ACh release via 5-$HT_2$ or 5-$HT_3$ receptor activation, respectively.[11]

Diabetes is a disease currently considered to be an important public health problem owing to its increasing prevalence and the fact that it causes and exacerbates macro- and microvascular complications. Autonomic neuropathy is a common and severe complication of diabetes mellitus that leads to dysfunction in the cardiovascular system. The reduced ability to finely regulate heart rate (HR) in long-term diabetes is attributed to profound disturbances in autonomic function,[12] which are probably due to parasympathetic dysfunction.[13] In agreement with this hypothesis, early autonomic nerve dysfunction has normally been attributed to vagal damage and affects the control of HR.[14]

Several studies performed under different conditions have supported the theory that during the development of diabetes there are impaired myocardial responses to muscarinic receptor activation by ACh. These responses are related to hyper- or hyposensitization and

Correspondence: María Luisa Martín Calvo, Laboratorio de Farmacognosia y Farmacología, Departamento de Fisiología y Farmacología, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, ES-37007 Salamanca, Spain. Email: marisam@usal.es

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may include changes in muscarinic receptor expression.[15–18] Clinical studies in diabetic patients have suggested that changes in the autonomic nervous system control of cardiovascular function may be associated with insulin-dependent diabetes mellitus. Beat-to-beat HR variation is reduced at rest and during deep breathing, demonstrating that parasympathetic nervous control of the heart is diminished in diabetes.[19,20]

In previous studies, we demonstrated that experimental diabetes induces significant changes in the serotonergic modulation of the autonomic sympathetic nervous system.[21,22] Other studies have suggested that diabetes induces changes in cholinergic transmission.[13,17,20] Thus, the aim of the present study was to determine whether diabetes is able to modify the effects of 5-HT on vagally induced bradycardia in the rat heart, which we demonstrated previously in normoglycaemic rats.[11] We investigated the effects of 5-HT on the *in vivo* cardiac response to parasympathetic nerve activation during experimental alloxan-induced hyperglycaemia by examining the serotonergic receptors involved in the cholinergic cardiac responses to vagal electrical stimulation. We also compared the effects of exogenous administration of ACh in hyperglycaemic–pithed rats with those in normoglycaemic rats.

## METHODS

### Animal preparation

One hundred and eighty male Wistar rats (250–350 g) were used in the present study. Rats were kept and supplied by the Animalarium of the Faculty of Pharmacy at the University of Salamanca (Spain; PAE-SA001). Housing conditions and experimental procedures were in accordance with European Union regulations regarding the use of animals for scientific purposes (86/609/EEC, Article 5, Appendix II; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31986L0609:EN:HTML) and enacted by Spanish legislation.

Diabetes was induced in 145 rats by a single injection of alloxan (150 mg/kg, s.c.) dissolved in 0.9% NaCl. Rats were maintained on tap water and regular food available *ad libitum* for 4 weeks. A second group of 35 rats was maintained under the same conditions for the same time period and served as the age-matched control. Weight and blood glucose levels were determined before and 2, 7, 14, 21 and 28 days after alloxan administration. Only rats with elevated blood glucose levels (> 11 mmol/L) at all time points were considered diabetic.

Rats, both diabetic and normoglycaemic, were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). After cannulation of the trachea, rats were pithed by inserting a stainless steel rod through the orbit and foramen magnum,[23] then artificially respirated with room air using a Harvard respiratory pump (1 mL air/100 g, 50 strokes/min; Harvard Apparatus. South Natick, MA, USA). The right and left jugular veins were cannulated for the infusion of agonists and antagonists, respectively.

Arterial blood pressure was monitored from the left carotid artery cannula using a pressure transducer connected to a PRS 206 amplifier (Cibertec, Madrid, Spain). Heart rate was measured by analysing the blood pressure data with a CAR 306 cardiotachograph (Cibertec). Both the blood pressure and HR data were shown on a Letica Polygraph 4000 (Cibertec).

Both vagus nerves were isolated, ligated at the cervical level and cut rostrally to the ligature to prevent afferent and efferent vagal reflexes. Electrical stimulation was applied as square-wave pulses from a Cibertec stimulator (CS-9; supramaximal intensity: 15 ± 3 V; 1 msec; 3, 6 and 9 Hz for 15 s at 5 min intervals), with a platinum bipolar electrode connected to the caudal stump of the right cervical vagus nerve.

Before electrical stimulation, rats were treated with heparin (1000 IU/kg). Then, they were given D-tubocurarine (2 mg/kg, i.v.), to avoid electrically induced muscular twitching, and atenolol (1 mg/kg, i.v.), to prevent sympathetic effects. Rats were kept warm (37.5 ± 0.5°C) with a heating lamp.

### Experimental protocols

After the haemodynamic status of the animals had been stable for at least 10 min, baseline values for mean blood pressure (MBP) and HR were determined. A platinum bipolar electrode was applied to the caudal stump of the right cervical vagus nerve and electrical stimulation was applied as square-wave pulses from a Cibertec stimulator, applying trains of 15 s, which consisted of pulses of 1 msec duration and supramaximal intensity (15 ± 3 V) at increasing frequencies (3, 6 and 9 Hz). Thus, the control stimulation–response curve was completed in approximately 15 min. At this point, rats were divided into six different agonist or antagonist treatment groups ($n = 5$ rats/group).

The first series of experiments was performed in normoglycaemic control rats to confirm previous data from our laboratory.[11] This group was subdivided into two. In the first subgroup ($n = 25$), rats received an i.v. bolus of saline solution (1 mL/kg; $n = 5$), as a control for this group of experiments, or 5-HT (10, 15, 30 and 50 μg/kg; $n = 5$ for each dose). Five minutes after the treatment, a new stimulation–response curve was obtained. In the second subgroup ($n = 10$), the effects of saline solution (1 mL/kg; $n = 5$) or 5-HT (30 μg/kg; $n = 5$) on the bradycardia induced by exogenous ACh (1, 5 and 10 μg/kg) were investigated. In this group, the ACh was administered before (control curve) and 5 min after pretreatment with either the saline solution or 5-HT.

In the first alloxan-treated diabetic group ($n = 75$), rats received an i.v. bolus of one of the following: (i) saline solution (1 mL/kg; $n = 5$; control group for all agonist treatments); (ii) 5-HT at 10, 30, 50, 100 or 200 μg/kg ($n = 25$); (iii) 5-carboxamidotryptamine maleate (5-CT; a selective 5-$HT_{1/7}$ receptor agonist) at 10, 50, 100 and 150 μg/kg ($n = 20$); (iv) 150 μg/kg α-methyl-5-HT (a selective 5-$HT_2$ receptor agonist; $n = 5$); (v) 150 μg/kg 1-phenylbiguanide (a selective 5-$HT_3$ receptor agonist; $n = 5$); (vi) 50 μg/kg 8-hydroxydipropylaminotretalin hydrobromide (8-OH-DPAT; a selective 5-$HT_{1A}$ receptor agonist; $n = 5$); (vii) 50 μg/kg CGS-12066B (an agonist at rodent 5-$HT_{1B}$ receptors; $n = 5$); or (viii) 50 μg/kg L-694,247 (a selective agonist for non-rodent 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors; $n = 5$). Five minutes after each treatment, a new stimulation–response curve was obtained.

The second alloxan-treated diabetic group ($n = 20$) was run in parallel with the group described above and examined, in the presence of saline solution (1 mL/kg), the effects of 1 mg/kg methiothepin ($n = 5$), a non-selective 5-$HT_1$ receptor antagonist, 100 μg/kg WAY-100,635 ($n = 5$), a selective 5-$HT_{1A}$ receptor antagonist, 1 mg/kg BRL-15572 ($n = 5$), a selective 5-$HT_{1D}$ receptor antagonist, and 1 mg/kg mesulergine ($n = 5$), a selective 5-$HT_{2/7}$ receptor antagonist.

The third alloxan-treated diabetic group ($n = 20$) was used to determine which receptor subtype was involved in regulating the effects of 5-HT on HR. Methiothepin (1 mg/kg), WAY-100,635 (100 μg/kg), BRL-15572 (1 mg/kg) or mesulergine (1 mg/kg) were administered 5 min before 5-CT (50 μg/kg; $n = 5$), 8-OH-DPAT (50 μg/kg; $n = 5$), L-694,247 (50 μg/kg; $n = 5$) or 5-CT (10 μg/kg; $n = 5$), respectively. Five minutes after each treatment, a new S-R curve was obtained.

In the fourth group, rats received an i.v. bolus of atropine (0.3 or 0.5 mg/kg; $n = 5$ for each dose) before electrical stimulation to confirm that cholinergic responses were induced by electrical stimulation.

In the last group of animals ($n = 20$), the effects of saline solution (1 mL/kg; $n = 5$), 5-CT (10 or 50 μg/kg; $n = 5$ for each dose), 8-OH-DPAT (50 μg/kg; $n = 5$) and L-694,247 (50 μg/kg; $n = 5$) were examined on the bradycardia induced by exogenous ACh (1, 5 and 10 μg/kg), which was administered before (control curve) and 5 min after drug pretreatment.

### Drugs used

In addition to the anaesthetic (pentobarbital sodium; Sigma Chemical, St Louis, MO, USA), the drugs used in the present study were as follows: heparin sodium (Roche, Madrid, Spain); alloxan monohydrate, 5-HT creatinine sulphate, D-tubocurarine HCl, atenolol (Sigma Chemical); atropine sulphate (Scharlau, Barcelona, Spain); methiothepin mesylate, 5-CT maleate, 8-OH-DPAT, CGS-12066B, L-694,247, 1-phenylbiguanide, α-methyl-5-HT, mesulergine (Research Biochemicals International, Natick, MA, USA); WAY-100,635 ((*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride); Pharmacia, Milan, Italy) and BRL-15572 hydrochloride (Tocris, Bristol, UK). All drugs were dissolved in distilled

water at the time of the experiments, with the exception of BRL-15572, which was dissolved in 20% propylene glycol.

### Statistical analysis

All data are expressed as the mean±SEM of at least five experiments. Comparisons of results between different experimental groups and their corresponding control group were performed using ANOVA, followed by the Newman–Keuls' multiple comparison test. Differences were considered to be statistically significant when $P < 0.05$.

## RESULTS

### Systemic haemodynamic variables

Alloxan-induced diabetes elicited a marked increase in serum glucose levels. Diabetic rats failed to increase their bodyweight compared with control rats. Table 1 shows the mean values of bodyweight and glycaemia before and 4 weeks after the induction of diabetes in control and diabetic rats.

Resting MBP and HR was 40.5 ± 1.5 mmHg and 290 ± 5 b.p.m., respectively, in diabetic anaesthetized pithed rats and 45.6 ± 2.3 mmHg and 282 ± 7 b.p.m. in normoglycaemic anaesthetized rats. These values were not altered significantly by the bolus i.v. administration of saline, the 5-HT receptor agonists (5-HT, 5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, α-methyl-5-HT and 1-phenylbiguanide) or the 5-HT receptor antagonists (methiothepin, mesulergine, BRL-15572 and WAY-100635; data not shown).

### Effects of physiological saline or 5-HT on vagally induced bradycardia in normoglycaemic rats

Electrical stimulation of the right vagus nerve in normoglycaemic rats resulted in frequency dependent bradycardia. The decreases in HR remained stable after bolus i.v. administration of saline solution (1 mL/kg; $n = 5$).

Administration of the lowest dose of 5-HT (10 μg/kg; $n = 5$; Fig. 1a) did not modify the vagally induced bradycardia at the frequencies tested, whereas administration of higher doses of 5-HT (15, 30 and 50 μg/kg; $n = 5$ for each dose) resulted in a dose-dependent attenuation of the vagally induced bradycardia (Fig. 1a). This attenuation was significant at a stimulation frequency of 3 Hz for the higher doses of 5-HT (15, 30 and 50 μg/kg). After administration of 30 and 50 μg/kg 5-HT, the attenuation was also significant at stimulation frequencies of 6 and 9 Hz ($P < 0.05$).

### Effects of physiological saline or 5-HT on vagally induced bradycardia in diabetic rats

Electrical stimulation of the right vagus nerve in diabetic rats resulted in frequency dependent bradycardia. This electrically induced bradycardia in diabetic rats was less pronounced than the bradycardia obtained under the same conditions in normoglycaemic rats. These differences were statistically significant at stimulation frequencies of 3 and 6 Hz ($P < 0.05$). The decreased HR remained stable after bolus i.v. administration of saline solution (1 mL/kg; $n = 5$). However, the effects caused by electrical stimulation of the vagus nerve were completely blocked by prior administration of atropine (0.3 and 0.5 mg/kg; $n = 10$; data not shown), confirming the cholinergic nature of the responses to electrical stimulation.

**Table 1** Bodyweight and glycaemia in control and diabetic rats

| | Bodyweight (g) | Glycaemia (mmol/L) | *n* |
|---|---|---|---|
| Control rats | | | |
| Initial | 315 ± 3 | 5.3 ± 0.5 | 25 |
| 4 weeks later | 426 ± 15 | 5.4 ± 0.3 | 25 |
| Diabetic rats | | | |
| Initial | 337 ± 6 | 4.8 ± 0.4 | 145 |
| 4 weeks later | 364 ± 5* | 27.2 ± 0.5* | 145 |

Data are the mean±SEM for *n* rats. *$P < 0.05$ compared with control rats.

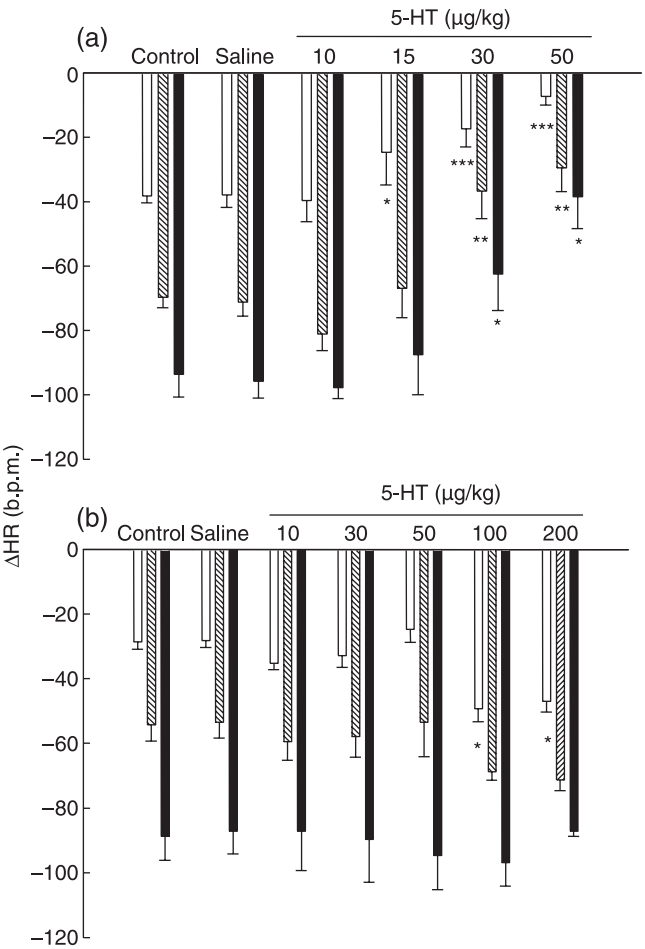


**Fig. 1** Changes in heart rate (ΔHR) evoked by electrical stimulation of the peripheral end of the right vagus in atenolol (1 mg/kg)-pretreated (a) normoglycaemic and (b) diabetic pithed rats before (control; $n = 5$) and after bolus i.v. administration of 1 mL/kg saline solution or 10, 15, 30 or 50 μg/kg 5-hydroxytryptamine (5-HT) to normoglycaemic rats ($n = 5$) or 10, 30, 50, 100 and 200 μg/kg 5-HT to diabetic rats ($n = 5$). (□), 3 Hz stimulation; (▨), 6 Hz stimulation; (■), 9 Hz stimulation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with saline ($n = 5$ for normoglycaemic rats; $n = 70$ for diabetic rats).

Low doses of 5-HT (10, 30 and 50 μg/kg; $n = 15$) did not significantly modify the vagally induced bradycardia across the stimulation frequency range tested (Fig. 1b). However, high doses of 5-HT (100 and 150 μg/kg; $n = 10$) caused an increase in the bradycardia,

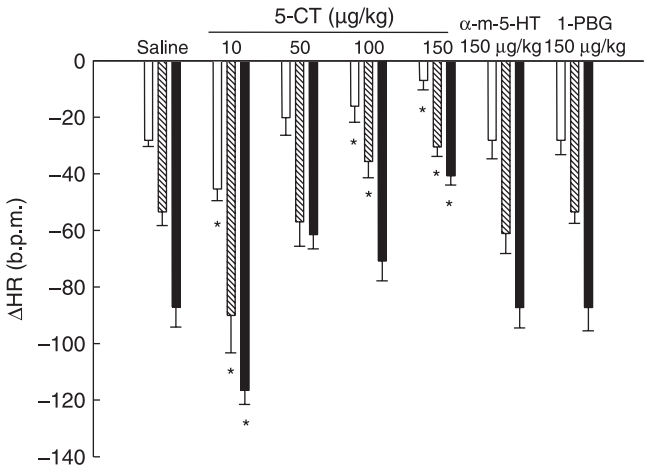


**Fig. 2** Changes in heart rate (ΔHR) evoked by electrical stimulation of the peripheral end of the right vagus in atenolol (1 mg/kg)-pretreated diabetic pithed rats after bolus i.v. administration of 1 mL/kg saline solution, 10, 50, 100 and 150 μg/kg 5-carboxamidotryptamine (5-CT; $n = 5$ for each dose), α-methyl-5-hydroxytryptamine (α-m-5-HT; 150 μg/kg; $n = 5$) or 1-phenylbiguanide (1-PBG; 150 μg/kg; $n = 5$). (□), 3 Hz stimulation; (▨), 6 Hz stimulation; (■), 9 Hz stimulation. *$P < 0.05$ compared with saline ($n = 70$).

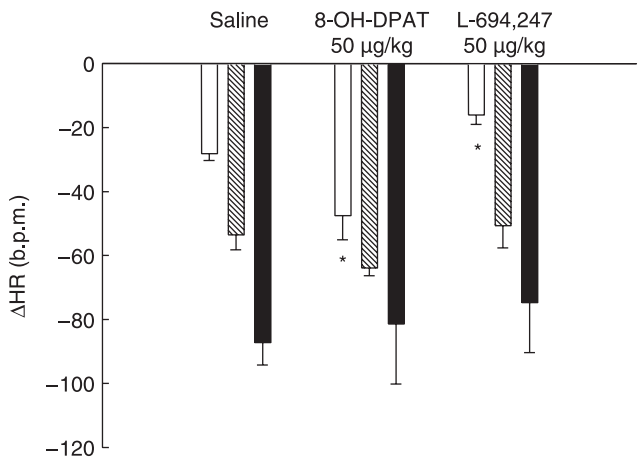


**Fig. 3** Changes in heart rate (ΔHR) produced by electrical stimulation of the peripheral end of the right vagus in atenolol (1 mg/kg)-pretreated diabetic pithed rats after bolus i.v. administration of 1 mL/kg saline solution, 8-hydroxydipropylaminotretalin hydrobromide (8-OH-DPAT; 50 μg/kg, $n = 5$) or L-694,247 (50 μg/kg; $n = 5$). (□), 3 Hz stimulation; (▨), 6 Hz stimulation; (■), 9 Hz stimulation. *$P < 0.05$ compared with saline ($n = 70$).

although this was only significant at a stimulation frequency of 3 Hz (Fig. 1b; $P < 0.05$).

## Effects of bolus i.v. administration of 5-HT receptor agonists (5-CT, 8-OH-DPAT, CGS-12066B, L-694,247, α-methyl-5-HT and 1-phenylbiguanide) on vagally induced bradycardia in diabetic rats

In diabetic rats, the bradycardic effects induced by vagal electrical stimulation were dually modified by 5-CT (a selective 5-$HT_{1/7}$ receptor agonist) depending on the dose administered. At low doses (10 μg/kg), 5-CT enhanced the electrically induced bradycardia (Fig. 2), whereas at high doses (50, 100 and 150 μg/kg) the electrically induced bradycardia was inhibited (Fig. 2).

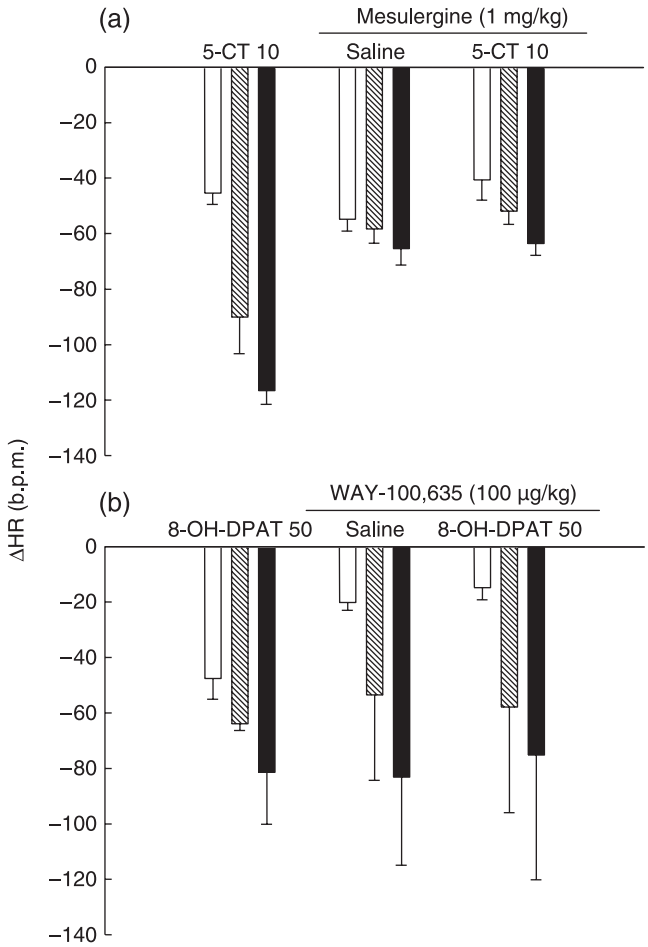


**Fig. 4** Effect of i.v. administration of (a) mesulergine (1 mg/kg) on the enhanced effect of 5-carboxamidotryptamine (5-CT; 10 μg/kg; $n = 5$) or (b) WAY-100,635 (0.1 mg/kg) on the enhanced effect of 8-hydroxydipropylaminotretalin hydrobromide (8-OH-DPAT; 50 μg/kg; $n = 5$) on bradycardia induced by vagal electrical stimulation in diabetic pithed rats. (□), 3 Hz stimulation; (▨), 6 Hz stimulation; (■), 9 Hz stimulation. *$P < 0.05$ compared with saline ($n = 5$).

The enhanced bradycardic effect induced by low doses of 5-CT in diabetic rats was reproduced by administration of the selective 5-$HT_{1A}$ receptor agonist 8-OH-DPAT (50 μg/kg). However, this effect was only observed at a stimulation frequency of 3 Hz (Fig. 3). The inhibitory effect of high doses of 5-CT on the vagally induced bradycardia was reproduced by administration of the selective 5-$HT_{1D}$ receptor agonist L-694,247 (50 μg/kg; Fig. 3).

Administration of the selective 5-$HT_2$ receptor agonist α-methyl-5-HT (150 μg/kg) or the selective 5-$HT_3$ receptor agonist 1-phenylbiguanide (150 μg/kg) did not modify the decreases in HR induced by vagal electrical stimulation (Fig. 2). In addition, i.v. bolus administration of the selective 5-$HT_{1B}$ receptor agonist CGS-12066B (50 μg/kg) had no effect on the bradycardia elicited by electrical stimulation of the vagus nerve (data not shown).

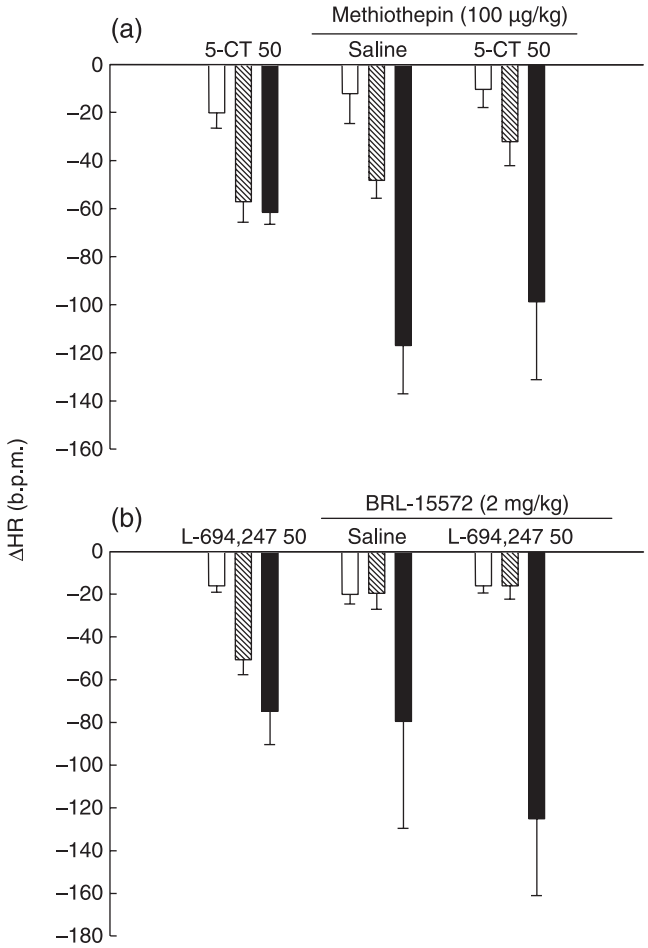


**Fig. 5** Effect of i.v. administration of (a) methiothepin (0.1 mg/kg) on the inhibitory effect of 5-carboxamidotryptamine (5-CT; 50 μg/kg; $n = 5$) or (b) BRL-15572 (2 mg/kg, $n = 5$) on the inhibitory effect of L-694,247 (50 μg/kg; $n = 5$) on bradycardia induced by vagus electrical stimulation in diabetic pithed rats. (□), 3 Hz stimulation; (▨), 6 Hz stimulation; (■), 9 Hz stimulation. *$P < 0.05$ compared with saline ($n = 5$).

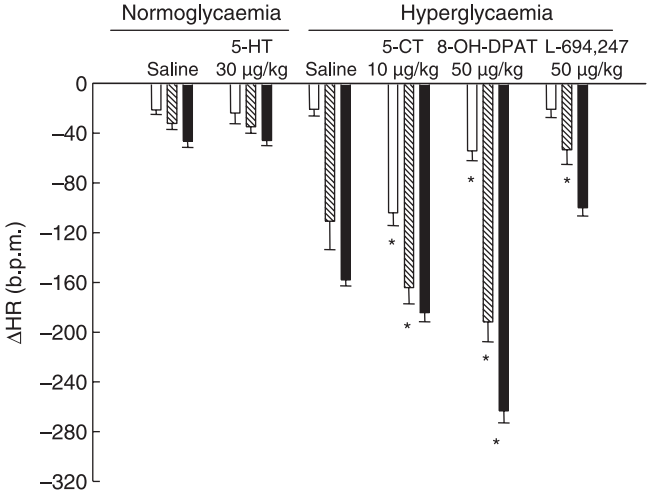


**Fig. 6** Changes in heart rate (ΔHR) evoked by exogenous administration of 1 (□), 5 (▨) and 10 μg/kg (■) acetylcholine in atenolol (1 mg/kg)-pretreated in normoglycaemic pithed rats after bolus i.v. administration of saline solution (1 mL/kg; $n = 5$) or 5-hydroxytryptamine (5-HT; 30 μg/kg; $n = 5$) or in diabetic pithed rats after bolus i.v. administration of saline solution (1 mL/kg; $n = 5$), 5-carboxamidotryptamine (5-CT; 10 μg/kg; $n = 5$), 8-hydroxydipropylaminotretalin hydrobromide (8-OH-DPAT; 50 μg/kg; $n = 5$) or L-694,247 (50 μg/kg, $n = 5$). *$P < 0.05$ compared with saline ($n = 5$).

## Effects of bolus i.v. administration of 5-HT receptor antagonists (methiothepin, mesulergine, WAY-100,635 and BRL-15572) on vagally induced bradycardia in diabetic rats

In diabetic pithed rats, administration of the selective 5-$HT_{1A}$ receptor antagonist WAY-100,635 (100 μg/kg; $n = 5$) or the selective 5-$HT_{1D}$ receptor antagonist BRL-15572 (2 mg/kg; $n = 5$) did not modify the decreased HR induced by vagal electrical stimulation (data not shown). The non-selective 5-$HT_1$ receptor antagonist methiothepin (100 μg/kg; $n = 5$) slightly, but not significantly, enhanced the bradycardia induced by vagal stimulation at high frequencies (data not shown), whereas mesulergine (1 mg/kg; $n = 5$), a selective 5-$HT_{2/7}$ receptor antagonist, significantly enhanced the bradycardia induced by electrical stimulation at a frequency of 3 Hz compared with control (–54.8 ± 4.3 *vs* –28.2 ± 2.2 b.p.m., respectively; $P < 0.05$).

Pretreatment with mesulergine (1 mg/kg) reduced the enhanced effect of 5-CT (10 μg/kg; $n = 5$; Fig. 4a). Similarly, prior administration of WAY-100,635 (100 μg/kg) abolished the effects of 8-OH-DPAT (50 μg/kg; $n = 5$; Fig. 4b). Pretreatment with methiothepin (0.1 mg/kg) abolished the inhibitory effect of 5-CT (50 μg/kg; $n = 5$) on the bradycardia induced by vagal electrical stimulation in diabetic rats (Fig. 5a). The effects of L-694,247 (50 μg/kg), a selective agonist for non-rodent 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors, on the vagally induced bradycardia were not apparent after pretreatment with BRL-15572 (2 μg/kg; $n = 5$; Fig. 5b).

## Effects of saline or 5-HT on the bradycardia induced by exogenous ACh in normoglycaemic rats

In some normoglycaemic animals, bradycardia was induced by exogenous administration of the muscarinic agonist ACh at doses of 1, 5 and 10 μg/kg. These bradycardic effects were dose dependent and remained stable after bolus i.v. administration of saline (1 mL/kg; $n = 5$). Administration of 5-HT (30 μg/kg; $n = 5$) had no effect on the bradycardia induced by exogenous ACh (Fig. 6).

## Effects of saline, 5-CT, 8-OH-DPAT and L-694,247 on the bradycardia induced by exogenous ACh in diabetic rats

In some rats, bradycardia was induced by exogenous administration of the muscarinic agonist ACh (1, 5 and 10 μg/kg), resulting in a dose-dependent decrease in HR. These bradycardic effects remained stable after bolus i.v. administration of saline (1 mL/kg; $n = 5$). However, administration of 5-CT (10 μg/kg; $n = 5$) and 8-OH-DPAT (50 μg/kg; $n = 5$) enhanced the ACh-induced bradycardia, whereas L-694,247 (50 μg/kg; $n = 5$), a selective agonist for non-rodent 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors, inhibited this response (Fig. 6).

## DISCUSSION

In the present study, we examined the role of hyperglycaemia on the effects of 5-HT in heart parasympathetic innervation in pithed rats. We investigated the 5-HT receptors involved in the bradycardia induced by vagal electrical stimulation or by the administration of

exogenous ACh during experimental alloxan-induced diabetes. Alloxan induces a syndrome resembling Type 1 diabetes mellitus, which is characterized by hyperglycaemia, hypercholesterolaemia, glycosuria and elevated levels of glycosylated haemoglobin in erythrocytes.[24–27]

Herein, we showed that there was no significant difference in basal HR between normoglycaemic and diabetic rats, as reported previously by Akiyama *et al*.[28] This is in contrast with other reports that describe a reduced intrinsic HR[29] and parasympathetic tonus in diabetes.[30]

In the present study, we showed that, in diabetic rats, the bradycardic effect produced by electrical stimulation of the vagus nerve was impaired compared with responses in normoglycaemic rats. Vagal electrical stimulation at frequencies of 3 and 6 Hz induced a significantly less pronounced decrease in HR in diabetic rats compared with controls. However, a recent study in diabetic rats has shown that the responsiveness of HR to cardiac vagal electrical stimulation is enhanced at higher stimulation frequencies (16, 32 and 64 Hz).[31]

We also demonstrated that exogenous administration of ACh (10 μg/kg) to hyperglycaemic rats produced a more pronounced and statistically significant bradycardia than in normoglycaemic rats. We and others have suggested previously that chemically induced diabetes may elicit functional defects in cardiac cholinergic nerves.[11,32–34] The increased inhibition of ACh release by inhibitory neuronal $M_2$ muscarinic receptors during experimental diabetes has been described extensively in different experimental models, including rat lungs,[35] the ileum and trachea from diabetic rats,[36] the urinary bladder[37,38] and even in human heart atrium.[39] Therefore, in the present study, we examined the role of diabetes on serotonergic receptors involved in the cardiac responses to vagal electrical stimulation.

Although 5-HT, the well-characterized endogenous ligand for all 5-HT receptors, had no inhibitory effect on the bradycardia induced by electrical stimulation in diabetic rats, the selective 5-$HT_{1/7}$ receptor agonist 5-CT[40] had a dual effect: at low doses (10 μg/kg), 5-CT enhanced bradycardia, whereas higher doses (50, 100 and 150 μg/kg) of 5-CT decreased the bradycardic effect at all stimulation frequencies. In contrast, administration of either α-methyl-5-HT (a selective 5-$HT_{2A/2B/2C}$ receptor agonist[41]) or 1-phenylbiguanide (a selective 5-$HT_3$ receptor agonist[42,43]) had no effect on the vagally induced bradycardia in diabetic rats. These findings suggest that, in the diabetic rat, the serotonergic effects on bradycardia are mediated through the 5-$HT_1$ receptor and not through 5-$HT_2$ or 5-$HT_3$ receptors. In contrast, we have demonstrated previously that in normoglycaemic pithed rats 5-$HT_2$ receptors are involved in the inhibition of vagally induced bradycardia and that 5-$HT_3$ receptors are required for stimulatory effects.[11] We propose that in the diabetic rat, as in normoglycaemic Wistar rats,[11] the serotonergic system interferes with cholinergic cardiac transmission, producing both an enhancement and inhibition of the bradycardic effect induced by vagal stimulation. Previous studies hypothesized that, at the gastrointestinal level in normoglycaemic animals, 5-$HT_1$ receptors are involved in the reduction of ACh release.[9,10] In order to determine the 5-$HT_1$ receptor subtype responsible for the action observed here, we used different antagonists and selective 5-$HT_1$ agonists in our experimental model of diabetes.

In the diabetic rat, we observed that the increase in vagally induced bradycardia after administration of 5-HT and low doses of 5-CT (the selective 5-$HT_{1/7}$ receptor agonist) is mimicked at low stimulation frequencies by the selective 5-$HT_{1A}$ receptor agonist 8-OH-DPAT.[44] In contrast, L-694,247, a selective agonist for non-rodent 5-$HT_{1B}$ and 5-$HT_{1D}$ receptors,[45] was able to mimic the inhibitory action on vagally induced bradycardia elicited by high doses of 5-CT. However, CGS-12066B, the selective agonist for rodent 5-$HT_{1B}$ receptors,[46] had no effect on vagally induced bradycardia. These findings suggest that the 5-$HT_{1A}$ receptor is involved in regulating the stimulatory effects observed and that the 5-$HT_{1D}$ receptor mediates the inhibitory actions.

The selective 5-$HT_{1A}$ antagonist WAY-100,635[47] and the selective 5-$HT_{1D}$ antagonist BRL-15572[48] did not affect the vagally induced bradycardia in diabetic rats. However, the vagally induced bradycardia was enhanced by both methiothepin (a non-selective 5-$HT_1$ receptor antagonist[40]) and mesulergine (a selective 5-$HT_{2/7}$ receptor antagonist that is commonly used as a 5-$HT_7$ receptor antagonist in view of its affinity for this receptor type[49,50]). Significant enhancement of the vagally induced bradycardia was observed only after treatment with mesulergine. These findings are consistent with earlier reports describing an intrinsic effect on ACh release in rat striatal slices for both mesulergine and methiothepin.[51]

Interestingly, in diabetic rats, the enhancing effect of 5-CT at the cardiac parasympathetic level was partially inhibited by mesulergine, indicating that the hypotensive and bradycardic action of 5-CT may be due, at least in part, to its direct interaction with 5-$HT_7$ receptors. The stimulatory effect of 5-CT on the enhancement of the bradycardia induced by vagal stimulation in diabetic rats has been reported before.[52] Further, in the present study we showed that, in diabetic rats: (i) methiothepin[40] (a non-selective 5-HT receptor antagonist) blocked the inhibitory effect of 5-CT; (ii) 8-OH-DPAT (a selective 5-$HT_{1A}$ receptor agonist) was able to enhance the bradycardia produced by vagal stimulation; and (iii) that 8-OH-DOPAT action was abrogated by WAY-100,635[47] (a selective 5-$HT_{1A}$ antagonist). These findings suggest a possible peripheral role for serotonergic receptors in modulating cholinergic transmission in addition to the well-known central 5-$HT_{1A}$ receptor regulation in cardiovascular effects.[53,54] Similarly, the action produced on the vagally induced bradycardia by the selective 5-$HT_{1D}$ agonist L-694,247[45] was abolished after pretreatment with BRL-15572, a selective 5-$HT_{1D}$ antagonist,[48] indicating that 5-$HT_{1D}$ receptors may also be involved.

We also showed that the dual effect (stimulatory and inhibitory actions) of the vagally induced bradycardia caused by the selective 5-$HT_1$ serotonergic agonists persisted when the bradycardia was elicited by exogenous ACh in diabetic rats. These results confirm the pre- and post-junctional nature of these serotonergic actions during diabetes. Nevertheless, these findings are in contrast with results described previously by our group[11] in normoglycaemic rats.

In conclusion, experimental diabetes elicits changes in both the nature and the 5-HT receptor type/subtype involved in vagal electrically stimulated bradycardia. Activation of 5-$HT_{1A}$ receptors induces enhancement of vagally induced bradycardia, whereas the attenuation of this bradycardia is due to the activation of 5-$HT_{1D}$ receptors. Such responses induced by 5-HT occur at both the pre- and post-junctional levels in diabetic pithed rats.

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