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RESEARCH PAPER

Evidence that 5-hydroxytryptamine$_7$ receptors play a role in the mediation of afferent transmission within the nucleus tractus solitarius in anaesthetized rats

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Background and purpose: Central 5-hydroxytryptamine (5-HT)-containing pathways utilizing 5-HT$_7$ receptors are known to be critical for the mediation of cardiovascular reflexes. The nucleus tractus solitarius (NTS) is a site involved in the integration of cardiovascular afferent information. The present experiments examined the involvement of the 5-HT$_7$ receptor in the processing of cardiovascular reflexes in the NTS.
Experimental approach: In anaesthetized rats extracellular recordings were made from 104 NTS neurones that were excited by electrical stimulation of the vagus nerve and/or activation of cardiopulmonary afferents. Drugs were applied ionophoretically in the vicinity of these neurones.
Key results: The non-selective 5-HT$_7$ receptor agonist 5-carboxamidotryptamine maleate (5-CT) applied to 78 neurones increased the firing rate in 18 by 59% and decreased it in 38 neurones by 47%. Similarly, the 5-HT$_{1A}$ agonist 8-OH-DPAT applied to 20 neurones had an excitatory (8), inhibitory (7) or no effect (5) on the 20 neurones tested. In the presence of the 5-HT$_7$ antagonist SB 258719 the 5-CT excitation was attenuated. Furthermore, the excitatory response of NTS neurones evoked by electrical stimulation of the vagus nerve or activation of cardiopulmonary afferents with intra atrial phenylbiguanide was attenuated by SB 258719. The inhibitory action of 5-CT was unaffected by SB 258719 and the 5-HT$_{1A}$ antagonist WAY-100635. WAY-100635 failed to have any effect on 5-CT and vagal afferent-evoked excitations.
Conclusions and implications: Vagal afferent-evoked excitation of NTS neurones can be blocked by SB 258719, a selective 5-HT$_7$ antagonist. This observation further supports the involvement of 5-HT neurotransmission in NTS afferent processing.
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Keywords: nucleus tractus solitarius; 5-hydroxytryptamine; serotonin; 5-HT$_7$ receptors; SB 258719; WAY-100635; 5-HT$_{1A}$ receptors; vagal C-fibre afferents; cardiopulmonary reflex

Abbreviations: 5-CT, 5-carboxamidotryptamine maleate; 8-OH-DPAT, (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide; DLH, DL-homocysteic acid; NTS, nucleus tractus solitarius; PSTHs, peri-stimulus time histograms; SB 258719, 3-methyl-N-[(1R)-1-methyl-3-(4-methyl-1-piperidinyl)pro pyl]-N-methylbenzenesulphonamide hydrochloride; WAY-100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt

Introduction

It has been established that central 5-hydroxytryptamine (5-HT; serotonin) pathways acting through 5-HT$_{1A}$, 5-HT$_3$ and 5-HT$_7$ receptors play a critical role in the regulation of cardiovascular reflexes (see Ramage and Villalón, 2008; nomenclature follows Alexander *et al.*, 2008). 5-HT$_{1A}$ receptors are mainly involved in the reflex regulation of parasympathetic (vagal) control of the heart, and data indicate that the predominant location of these receptors is within the nucleus ambiguus (Wang and Ramage, 2001); the location of myelinated cardiac vagal preganglionic neurones. In contrast, 5-HT$_3$ receptors are involved in afferent processing within the nucleus tractus solitarius (NTS) where their activation appears to involve the release of glutamate, probably to some extent from glial cells (Jeggo *et al.*, 2005). However, the location(s) of

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5-HT$_7$ receptors within the cardiovascular reflex pathways (Kellett *et al*., 2005) is unknown. 5-HT$_7$ receptors may be located on NTS neurones within the reflex pathways (baroreceptor, chemoreceptor and cardiopulmonary; Jordan and Spyer, 1986) as central blockade of 5-HT$_7$ receptors normally interferes with both the sympathetic and the parasympathetic components. The NTS is richly innervated by 5-HT-containing terminals (Steinbusch, 1981) originating centrally from raphé nuclei (Sim and Joseph, 1992; Schaffar *et al*., 1988) and peripherally from vagal afferents (Nosjean *et al*., 1990; Sykes *et al*., 1994). Moreover, binding sites or mRNA for 5-HT$_{1A}$ and 5-HT$_{1B}$ (Manaker and Verderame, 1990; Thor *et al*., 1992), 5-HT$_{2A}$ and 5-HT$_{2C}$ (Pompeiano *et al*., 1994; Wright *et al*., 1995; Cornea-Hébert *et al*., 1999), 5-HT$_3$ (Steward *et al*., 1993), 5-ht$_{5A}$ (Oliver *et al*., 2000) and 5-HT$_7$ receptors (To *et al*., 1995; Gustafson *et al*., 1996) have been localized within the nucleus. The present study was carried out to investigate the effects of the 5-HT$_7$ receptor antagonist, SB 258719 (Forbes *et al*., 1998) and the non-selective 5-HT$_7$ receptor agonist 5-carboxamidotryptamine (5-CT; see Thomas and Hagan, 2004; for the pKi of 5-CT binding to all 5-HT receptor subtypes see Roberts *et al*., 2001) on NTS neuronal activity; ongoing, or that evoked following electrical stimulation of vagal afferents or chemical activation of cardiopulmonary afferents. In addition, the effects of 5-HT$_{1A}$ receptor blockade were examined. A preliminary account of some of these experiments has been published (Oskutyte *et al*., 2007).

Methods

All animal care and experimental procedures complied with the Animals (Scientific Procedures) Act 1986. Experiments were performed on 85 anaesthetized male Sprague-Dawley rats (230–350 g). Anaesthesia was induced with isoflurane (5% in O_2) and maintained with sodium pentobarbital (60 mg·kg^{-1}; i.v. supplemented with 10–15 mg·kg^{-1} i.v. when necessary). After completion of experiments, animals were killed humanely by an overdose of pentobarbital sodium (i.v.). Core body temperature was maintained at 37–38°C by a homeothermic blanket system (Harvard Apparatus, South Natick, MA, USA). The left femoral vein and artery were cannulated (Portex non-sterile tubing, external diameter 0.96 mm, internal diameter 0.58 mm, Scientific Lab. Supplies, Wilford, Nottingham, UK) for administration of drugs and fluids, and for measurement of arterial pressure and collection of arterial blood samples respectively. Arterial and tracheal pressures were measured with pressure transducers (model P23XL, Statham, Hato Rey, Puerto Rico). Two leads were attached to opposite limbs to record electrocardiograph (ECG), and were connected to an amplifier (NL 104, Neurolog, Digitimer Ltd., X 5k, Welwyn Garden City, UK) and filter unit (NL125, Neurolog, 0.5–5.0 kHz). Mechanical ventilation with oxygen-enriched room air was commenced once the animal was in the stereotaxic frame. In all experiments a cannula was pre-filled with phenylbiguanide (PBG; 100–150 μg·mL^{-1}) and inserted into the right atrium. After fixing in a stereotaxic frame, animals were ventilated with oxygen-enriched room air using a positive pressure ventilator (Harvard Rodent ventilator, model 683, Harvard Apparatus, Ltd., Edenbridge, Kent, UK), and subsequently neuromuscular blocked by a single dose of α-bungarotoxin (140 μg·kg^{-1}; i.v.). During neuromuscular blockade the depth of anaesthesia was assessed by monitoring the stability of the arterial blood pressure and heart rate and the cardiovascular responses to pinching of the paw. Additional anaesthetic was administered if required. Animals were constantly infused through the right femoral vein (6 mL·kg^{-1}·h^{-1}) with solution consisting of 10 mL distilled water, 10 mL plasma substitute (Gelofusine), 168 mg sodium bicarbonate and 36 mg glucose. This helped to maintain blood volume and prevent metabolic acidosis. At regular intervals, arterial blood samples were collected in heparinized capillary tubes, and analysed using a pH/blood gas analyser (Siemens Rapidlab® 248, Siemens Healthcare, Camberley, UK). Arterial blood gases were maintained at PO_2 90–120 mmHg, PCO_2 40–50 mmHg, and pH at 7.3–7.4, by adjusting the rate and/or stroke volume of the ventilator.

The rats were fixed in a stereotaxic frame and, using a dorsolateral approach low in the neck, the right cervical vagus nerve was dissected free from the sympathetic trunk. The vagus was placed on bipolar silver wire electrodes for electrical stimulation (50–500 μA, 1ms, 0.3–1 Hz, constant current isolated stimulator (Digitimer DS3) triggered by a programmer (Digitimer 4030). The exposed length of nerve was covered in paraffin wax, and fixed in place with dental impression material (Super-Dent light body dental polyvinylsiloxane, Carlisle Laboratories, Carlisle, UK). To expose the dorsal surface of the caudal brainstem the nuchal muscles were removed from the back of the neck, the occipital bone removed and the dura overlying the brainstem cut and reflected laterally.

Recording protocol

Using multi-barrel microelectrodes, extracellular recordings were made from neurones in an area of the NTS (sites less than 1 mm lateral to midline and between 0.5 mm rostral and 1.5 mm caudal to obex) known to contain neurones receiving cardiopulmonary afferent inputs (Hines *et al*., 1994). Electrodes were constructed by gluing a single-barrelled glass recording electrode (tip diameter 1 μm) to a multi-barrelled (five barrels) glass electrode (tip diameter 3–7 μm; Wang *et al*., 1995). The recording barrel contained 4 M NaCl, and the other barrels contained pontamine sky blue dye, the excitant amino acid DL-homocysteic acid (DLH) and a combination of 5-HT$_{1A/7}$ receptor ligands. Neuronal recordings were amplified 1000–5000 and filtered (0.5–5.0 kHz; NL 125, Neurolog). Electrodes were lowered into the brainstem in 0.5 μm steps (Burleigh, 6000 ULN, Burleigh Instruments (UK) Ltd., Harpenden, UK). Neurones were selected by discharges evoked in response to electrical stimulation of the cervical vagus nerve at 2× threshold for evoking neuronal activity (Jeggo *et al*., 2005). In some experiments recording sites were marked by ionophoretic ejection of pontamine sky blue dye. Ligands were applied to the vicinity of the recorded neurones by ionophoresis (Neurophore, Medical Systems, Digitimer Ltd.). Between drug ejection periods a retaining current of 10–15 nA was applied to each drug barrel. Some neurones with little or no ongoing activity were induced to fire by application of low currents (0–20 nA) of an excitant amino acid DLH. When neuronal firing rate was steady, the effects of agonist and/or antagonist ligands given alone or in combination were tested.

In all experiments possible current artefacts were minimized using the automatic current balancingavailable on the Neurophore system. At the end of the experiment the brainstem was removed and fixed in 10% formal saline. Frozen sections (50 μm) were cut and the location of the recording sites visualized and mapped onto standard sections of a rat brainstem (Paxinos and Watson, 1998).

Analysis of data

Arterial blood pressure, tracheal pressure, ECG and neuronal activity were recorded on a PC hard disk accessed via an A-D interface [Cambridge Electronic Design (CED) 1401 plus, Cambridge, UK]. Off-line analysis of recorded data was made using Spike2 software (CED). Single unit activity was discriminated using a spike processor (D130, Digitimer Ltd.) and displayed as rate histograms. To investigate the effects of ligands on ongoing NTS neuronal activity, baseline and ligand-evoked neuronal firing rates (averaged over a 10–20 s period) were measured and compared. Peri-stimulus time histograms (PSTHs, 20 stimuli) were constructed to investigate the effect of ligands on the vagally evoked response of NTS neurones. The total number of evoked spikes before and during ionophoretic application of the ligands was compared. The response of NTS neurones to cardiopulmonary afferent stimulation by PBG injected into the right atrium was analysed by counting the total number of spikes evoked from the beginning of the excitatory burst until activity returned to pre-injection level. Ligands were classed as evoking excitation or inhibition if activity, respectively, increased or decreased by more than 20% (Wang *et al.*, 1995; Jeggo *et al.*, 2005).

All data are presented as mean ± standard error of the mean except where indicated. Comparisons between means were made with Student's paired *t*-test.

Drugs and solutions

The following drugs, except where stated, were freshly dissolved in 0.9% saline, and their pH adjusted by addition of drops of either 0.1 M HCl or 0.1 M NaOH: DLH (100 mM, pH 8.5), N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate (WAY-100635; 10mM, pH 4) from Sigma-Aldrich, Poole, Dorset, UK. (±)-8-Hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT; 20 mM, pH 4), 5-carboxamidotryptamine maleate (5-CT; 10 mM, pH 4), 3-methyl-N-[(1R)-1-methyl-3-(4-methyl-1-piperidinyl)propyl]-N-methylbenzenesulfonamide hydrochloride (SB 258719; 10 mM, pH 4) from Tocris Cookson Ltd., Bristol, UK. Pontamine sky blue dye (20 mg·mL^{-1}) from BDH, Poole, Dorset, UK) was dissolved in 0.5 M sodium acetate. The following drugs were also used: PBG and α-bungarotoxin, both from Sigma-Aldrich; gelofusine from Braun Medical Ltd., Bucks, UK; sodium pentobarbital (Pentoject) from Animalcare Ltd., York, UK; and isoflurane from Abbott Laboratories Ltd., Kent, UK.

Results

Identification of NTS neurones

A total of 94 NTS neurones were studied that were excited following electrical stimulation of the cervical vagus nerve (100–500 μA, 1 ms, 1 Hz). In 91/94 neurones the response latency ranged from 24 to 46 ms (mean 26.9 ± 1.1 ms) indicating that the conduction velocity fell within the C-fibre range (mean 0.8 ± 0.14 ms^{-1}). The remaining three neurones received short latency inputs with a mean latency of 7.6 ± 1.1 ms indicating they had a conduction velocity of 2.9 ± 0.5 ms^{-1} in the B-fibre range. Forty-six neurones had responses with latency variabilities of <3 ms indicative of a monosynaptic input. The other 48 had a greater variability of onset latency indicating that they probably received a polysynaptic input (see Scheuer *et al.*, 1996). Forty-six neurones showed spontaneous activity whereas the rest were silent. Where neurones had no ongoing discharge, firing was induced by the excitatory amino acid DLH applied by ionophoresis (2–15 nA). Recording sites of 17 neurones that where marked were recovered from the caudal NTS area. It should be noted that there was no overt differences in the responses to 5-HT ligands between those NTS neurones that received a putative mono-synaptic and those receiving a polysynaptic, vagal input.

The effects of 5-CT on NTS neuronal firing rate in the presence and absence of antagonist for 5-HT$_7$ (SB 258719) and 5-HT$_{1A}$ (WAY-100635) receptors

In 18/76 (24%) neurones ionophoretic application of 5-CT (11–100 nA) significantly increased baseline firing rate from a mean of 3.2 ± 0.5 to 5.1 ± 0.5 spikes·s^{-1}. In all of these cases termination of 5-CT application resulted in the immediate return of firing rate to control levels (3.5 ± 0. 6 spikes·s^{-1}; Figures 1 and 2). In nine neurones, ionophoretic application of SB 258719 (100–150 nA) significantly blocked the increase in activity evoked by 5-CT (5.0 ± 0.9 to 4.9 ± 0.7 compared with 4.5 ± 0.8 to 6.3 ± 0.8; Figure 1). SB 258719 given alone did not affect background activity.

In three neurones, tested WAY-100635 (25–75 nA) neither affected the excitations evoked by 5-CT (1.9 ± 0.36 to 4.5 ± 0.772 vs. 2.6 ± 0.7 to 4.8 ± 1.2 spikes·s^{-1}; Figure 2) nor background activity.

In 38/76 neurones (50%), ionophoretic application of 5-CT significantly decreased firing rate from 6 ± 0.8 to 3.4 ± 0.6 spikes·s^{-1}. Again, on termination of 5-CT, activity recovered to 4.7 ± 1.0 spikes·s^{-1} in all 38 neurones tested. Ionophoretic application of SB 258719 (50–100 nA) had no significant effect on 5-CT induced inhibition in 9/9 neurones tested (5.4 ± 1.0 to 3.2 ± 0.8 compared with 4.7 ± 0.9 to 3.2 ± 0.8 spikes·s^{-1} in the presence of SB 258719). On these neurones SB 258719 did not affect background activity.

In two neurones tested, WAY-100635 (25–50 nA) had no effect on the inhibitory action of 5-CT. On these neurones at these currents, WAY-100635 alone did not affect baseline activity, however at higher currents (150–200 nA) WAY-100635 caused inhibition that was not reversed by DLH.

In 20/76 (26%) neurones, 5-CT was without significant effect on neuronal firing rate (3.7 ± 0.5 spikes·s^{-1} compared with 3.5 ± 0.3 spikes·s^{-1}).

Effect of 8-OH-DPAT, a 5-HT$_{1A/7}$ agonist on NTS neuronal activity

In 8/20 (40%) neurones, 8-OH-DPAT (30–80 nA) increased baseline firing rate from 3.5 ± 1.1 to 7.3 ± 1.2 spikes·s^{-1},

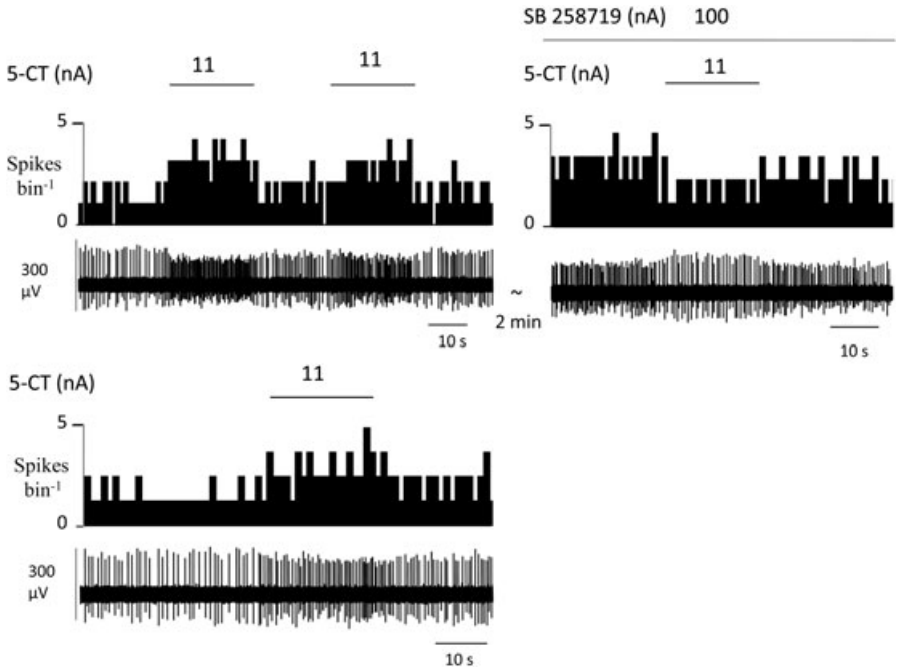

Figure 1 The effect of ionophoretically administered 5-hydroxytryptamine (5-HT)$_7$ antagonist SB 258719 on the excitatory effect evoked by 5-carboxamidotryptamine maleate (5-CT) on a nucleus tractus solitarius (NTS) neurone in an anaesthetized rat. The upper trace shows a rate histogram (spikes·bin^{-1}) and the lower trace, raw data of extracellular recording of activity in this NTS neurone. The time between the top left hand traces and the bottom is 2 min.

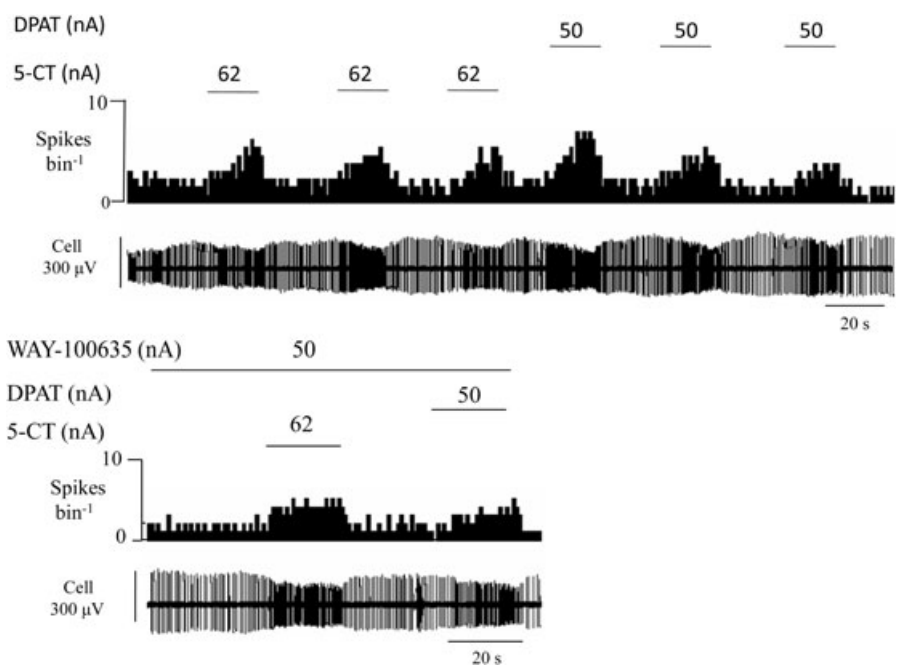

Figure 2 A comparison of the effect of ionophoretically administered 5-carboxamidotryptamine maleate (5-CT) and (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT) on the activity of a nucleus tractus solitarius (NTS) neurone in an anaesthetized rat. The bottom panel shows the effect of these evoked excitations in the presence of the 5-hydroxytryptamine$_{1A}$ antagonist WAY-100635 also ionophoretically applied. The upper trace shows a rate histogram (spikes·bin^{-1}), and the lower trace raw data of extracellular recording of activity in this NTS neurone. The time between the top left hand traces and the bottom is 1 min.

whereas in 7/20 (35%) 8-OH-DPAT decreased firing rate from 6.4 ± 1.2 to 2.3 ± 0.9 spikes·s^{-1} and had no significant effect on the remaining five neurones. This was similar in profile to 5-CT. In 5 neurones tested, 8-OH-DPAT evoked excitation was unaffected by the application of WAY-100635 (Figure 2).

Effects of 5-HT$_{1A/7}$ antagonists on NTS neuronal activity evoked by electrical stimulation of the cervical vagus nerve

Ionophoretic application of SB 258719 (100–150 nA) significantly decreased vagal-evoked activity from 16.9 ± 0.9 to 8 ± 1.3 spikes 20 sweeps^{-1} in 20/35 (57%) tested (Figure 3). Out of

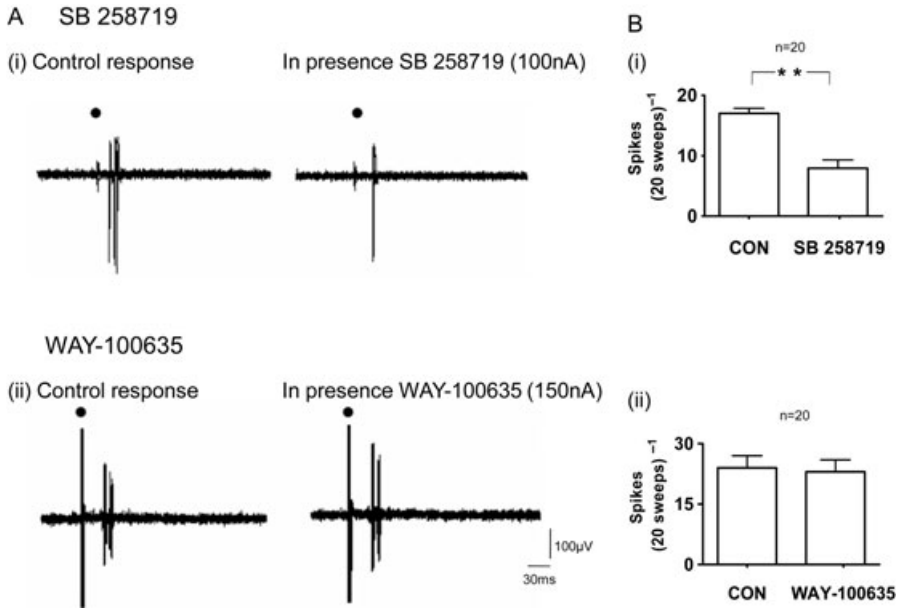

Figure 3 Effect of ionophoretic administration of the 5-hydroxytryptamine (5-HT)$_7$ antagonist SB 258719 and the 5-HT$_{1A}$ antagonist WAY-100635 on vagal afferent-evoked activation of NTS neurones in an anaesthetized rat. In these experiments, the vagus was stimulated electrically (100 μA, 1 ms). (A) Two traces of five superimposed traces of vagal afferent electrically evoked activity in a NTS neurone in the presence and absence of ionophoretically applied (i) SB 258719 or (ii) WAY-100635 at the currents indicated. ● stimulus artefact. (B) Histograms of the mean data of vagal afferent-evoked activity with vertical bars representing standard error of the mean. From left to right: control (CON) evoked activity which is mean response per 20 sweeps and evoked activity during the ionophoretic administration of applied (i) SB 258719 (100–150 nA; n = 20) or (ii) WAY-100635 (50–200 nA; n = 20). Means were compared using Student's paired t-test. $^{**}P < 0.01$.

these 20 neurons, ionophoretic application of 5-CT excited three neurons, inhibited seven and had no effect on the remaining 10 neurons. In two of three neurones excitation evoked by 5-CT was blocked by SB 258719 at the same current that attenuated the evoked activity. The other neuron was not tested with SB 258719. Application of 5-CT to six of the remaining 15 neurones, inhibited four and failed to affect two.

Ionophoretic application of WAY-100635 (50–200 nA) had no effect on the vagal afferent-evoked activity of 20 out 20 neurones tested (Figure 3). 5-CT applied to five of these neurones inhibited four and had no effect on one. 8-OH-DPAT applied to another four neurones inhibited all four.

Effect of the 5-HT$_7$ receptor antagonist SB 258719 on NTS neuronal activity evoked by activation of cardiopulmonary afferents

Activation of cardiopulmonary receptors by injection of PBG (100–150 μg·kg^{-1}) into the right atrium, was investigated in 15 NTS neurones. Six neurones were excited, two neurones were inhibited and seven showed no effect. Ionophoretic application of SB 258719 (150-200 nA) significantly attenuated excitatory responses evoked by PBG in four out of six (66%) neurones tested from 25 ± 2 to 6 ± 3.2 spikes·burst^{-1} (Figure 4). 5-CT was ionophoretically applied to 4 out of 5 of these neurons and caused inhibition. It should be noted that it has previously been shown in the rat (Jeggo *et al.*, 2005) that the hypotension and neuronal excitation evoked by PBG given into the right atrium can be abolished by vagotomy.

Discussion

The data from the present experiments indicate that afferent C-fibre activation of NTS neurones in anesthetized rats involves the activation of 5-HT$_7$ receptors. This is based on the ability of the 5-HT$_7$ receptor antagonist SB 258719, which has pKi 7.5 at 5-HT$_7$ receptors and pKi >5.5 at 5-HT$_{1/2/4/5/6}$ receptors, dopamine D$_2$ and D$_3$ and finally α_{1B}-adrenoceptors (Forbes *et al.*, 1998), to attenuate the excitation evoked by electrical stimulation of vagal afferents and by activation of cardiopulmonary afferent with i.a. PBG. Further, the standard experimental method for determining the effectiveness of an antagonist on a receptor type is to measure the antagonist effect against an agonist response. In the case of 5-HT$_7$ receptors there is no selective agonist and, although 5-CT is often used in studies on 5-HT$_7$ receptors, it has a similar affinity for 5-HT$_{1A/B/D}$ receptors with pKi around 9.0 and for 5-ht$_{5A}$ a pKi of 7.7 (see Roberts *et al.*, 2001). Thus it is not surprising that 5-CT has both excitatory and inhibitory action on NTS neurones. From these affinity values the excitation could be attributed to activation of 5-HT$_7$ receptors, whereas inhibition could be attributed to activation to 5-HT$_{1A/B/D}$ and/or 5-ht$_{5A}$ receptors. In this respect, SB 258719 did block the excitatory action of 5-CT but not the inhibitory action. This is consistent with SB 258719, at the currents applied, being a selective 5-HT$_7$ receptor antagonist, and supports the view that the ability of SB 258719 to attenuate afferent excitation of NTS neurones is due to blockade of 5-HT$_7$ receptors. It should be noted that there are two newer 5-HT$_7$ agonists available AS19 (Leopoldo *et al.*, 2004) and LP44 (see Cordeaux *et al.*, 2009). However there is only limited published data on these

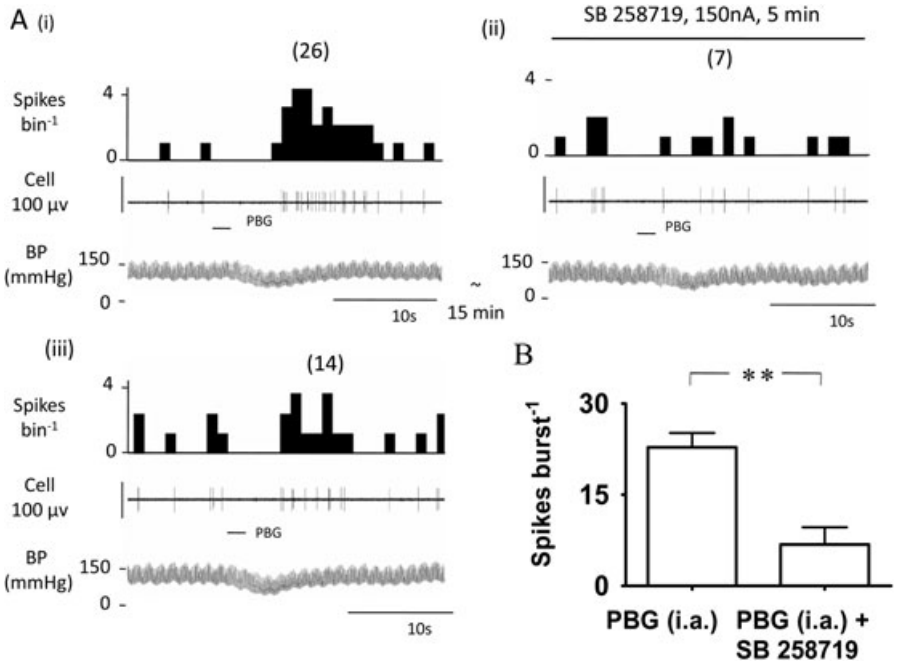

Figure 4 The effects of the 5-hydroxytryptamine (5-HT)$_7$ antagonist, SB 258719, ionophoretically applied to the vicinity of a NTS neurone in an anaesthetized rat that had been excited by cardiopulmonary afferent activation by injecting phenylbiguanide (PBG; 100–150 µg·kg^{-1}) into the right atrium (i.a). (A) Traces of blood pressure (BP), a rate histogram (spikes·bin^{-1}) and raw data of extracellular recording of activity of an NTS neurone. In (i) the effect of PBG on these variables is shown and this is repeated in (ii) in the presence of SB 258719 and again (iii) 10 min after switching off the SB 258719 injection current. (B) Histograms of the mean data (n = 4) of PBG-evoked activity with the vertical lines representing standard error of the mean. From left: activity evoked by PBG (100–150 µg·kg^{-1}) alone given i.a. and activity evoked by PBG (i.a.) in the presence of ionophoretically applied SB 258719 (150–200 nA). Means were compared using Student's paired t-test. **P < 0.01.

compounds and they have not been fully characterized in their overall selectivity for 5-HT receptor subtypes. Further they are insoluble in water, and thus not suitable for ionophoretic application.

In addition to 5-HT$_7$ receptors, the NTS also contains 5-HT$_{1A}$ receptors and these receptors are known to play important role in cardiovascular reflex activation of parasympathetic (vagal) outflow to the heart (see Introduction). In a preliminary report (Ramage and Mifflin, 1998), application of the selective 5-HT$_{1A}$ antagonist WAY-100635 (Forster *et al.*, 1995) failed to have any effect against vagal-evoked excitation of NTS neurones. This observation is now further supported by the present experiments. However WAY-100356 also failed to affect the action of 8-OH-DPAT, the archetypical 5-HT$_{1A}$ agonist (see Alexander *et al.*, 2008), which also has affinity for 5–HT$_7$ receptors (see Barnes and Sharp, 1999). These observations are consistent with those of Wang *et al.* (1997), which showed that ionophoretically applied 8-OH-DPAT inhibited, excited and had no effect in approximately equal numbers of NTS neurones tested. Furthermore, WAY-100635 applied to rat dorsal vagal nucleus preganglionic neurones also failed to block the inhibitory action of 8-OH-DPAT, although the inhibitory action of 8-OH-DPAT on dorsal raphé neurones was attenuated by ionophoretically applied WAY-100635 (Wang *et al.*, 1995). Interestingly in this experimental system, the excitatory action of 8-OH-DPAT was blocked by pindolol but not by WAY-100635. It should be noted that WAY-100635 could block the excitatory action of 5-HT applied ionophoretically, and that pindolol (a 5-HT$_{1A}$ antagonist) could not block the inhibitory action of 8-OH-DPAT on these neurons. However, in the cat, WAY-100635 does block 8-OH-DPAT excitatory action on vagal preganglionic neurones located in the nucleus ambiguus (Wang and Ramage, 2001). This inability of WAY-100635, applied ionophoretically, to block 8-OH-DPAT effects on these neurones in the brainstem of the rat remains to be determined. Further this excitation evoked by 8-OH-DPAT in rats should be tested against SB 258719 to determine if 5-HT$_7$ receptors are also involved. Overall, although the data are negative, they are consistent with other studies suggesting that 5-HT$_{1A}$ receptors are not involved in the control of the reflex activation of cardiac vagal preganglionic neurones at the level of the NTS.

The present data imply that activation of vagal afferents causes the release of 5-HT in the NTS, which in turn activates 5-HT$_7$ receptors to excite the NTS neurones. Furthermore, vagal afferent excitation of NTS neurones has also been shown to be mediated by 5-HT$_3$ receptors (Jeggo *et al.*, 2005). In this respect the overall data on role of central 5-HT$_7$ receptors in the regulation of cardiovascular reflexes compared with 5-HT$_3$ receptors suggest that 5-HT$_7$ receptors play a much more prominent role (see Ramage and Villalón, 2008). However, when directly measuring the effects of these antagonists on NTS neuronal activity evoked by vagal afferents the data indicates that these receptors are similarly effective in mediating this excitation. This difference may simply reflect differences in action of these receptors on NTS output neurones. Nevertheless, the question arises as to how these two different receptors interact in the control of vagal afferent input on the integrative 'input' NTS neurones. Although vagal afferents contain 5-HT, the overall evidence is that they

release glutamate (see Talman, 1997; Machado, 2001). Therefore, 5-HT could be co-released with the glutamate and/or from separate 5-HT-containing vagal afferents. In this respect 5-HT$_3$ receptors are located predominantly on vagal afferents (see Leslie *et al.*, 1994) along with the 5-HT transporter (Huang *et al.*, 2004). This may indicate that certain vagal afferents may be set up to increase the release of glutamate from other afferents by releasing 5-HT. In this respect 5-HT$_3$ receptor activation has been shown to release glutamate in the NTS (Ashworth-Preece *et al.*, 1995; Jeggo *et al.*, 2005). Further, it has been suggested that glutamate can also be released from glial cells by 5-HT$_3$ receptor activation (see Jeggo *et al.*, 2005), and immunoreactivity for the 5-HT$_{3A}$ receptor subunit has been shown on glial profiles within the medial NTS (Huang *et al.*, 2004). In respect to the present study the question arises as to where the 5-HT$_7$ receptors are in this pathway. However, little information exists on the location of 5-HT$_7$ receptors in the NTS. Furthermore, as yet, there is no evidence to indicate whether the excitation of NTS neurones evoked by 5-HT$_7$ receptors also involves the release of glutamate and whether vagotomy affects 5-HT$_7$ receptor binding or mRNA. One possibility is that the vagal afferents may also release 5-HT in the vicinity of 5-HT$_7$ receptors on NTS neuronal cell body and/or dendritic tree as well as increasing neuronal excitability by activating 5-HT$_3$ receptors on glia by increasing the surrounding levels of glutamate, thus depolarizing these neurones so that the 5-HT$_7$ receptor mediated excitation can fully activate these NTS neurones. Another possibility is that vagal afferents are causing the release of 5-HT from the terminals of the central 5-HT projections, which come mainly from the nucleus raphé magnus and the paragigantocellular nucleus (Schaffar *et al.*, 1988), which would involve the activation of 5-HT$_7$ and 5-HT$_3$ receptors or some combination of both possibilities.

In conclusion the present data demonstrate that 5-HT$_7$ receptors play an important role in vagal afferent processing at the level of the NTS along with 5-HT$_3$ receptors. However, how this system operates within the NTS remains to be fully elucidated.

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Conflict of Interest

The authors state no conflict of interest.

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