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Characterization of the *in vitro* effects of 5-hydroxytryptamine (5-HT) on identified neurones of the rat dorsal motor nucleus of the vagus (DMV)

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1 Whole cell patch clamp techniques were used on thin brainstem slices to investigate the effects of 5-hydroxytryptamine (5-HT) on gastrointestinal-projecting dorsal motor nucleus of the vagus (DMV) neurones. Neurones were identified as projecting to the stomach ($n=122$) or intestine ($n=84$) if they contained the fluorescent tracer DiI after it had been applied to the gastric fundus, corpus or antrum/pylorus or to the duodenum or caecum.

2 A higher proportion of intestinal neurones (69%) than gastric neurones (47%) responded to 5-HT with a concentration-dependent inward current which was antagonized fully by the 5-HT_{2A} receptor antagonist ketanserin (1 μM).

3 Stimulation of the nucleus tractus solitarius (NTS) induced inhibitory synaptic currents that were reduced in amplitude by application of the 5-HT_{1A} receptor agonist 8-OHDPAT (1 μM) or the 5-$HT_{1A/1B}$ receptor agonist TFMPP (1 μM) in 61% and 52% of gastric- and intestinal-projecting neurones, respectively. 5-HT also significantly reduced the frequency but not the amplitude of spontaneous inhibitory currents.

4 These data show that 5-HT excites directly a larger proportion of intestinal projecting neurones than gastric-projecting neurones, as well as inhibiting synaptic transmission from the NTS to the DMV. These data imply that the response to DMV neurones to 5-HT may be determined and classified by their specific projections.

Keywords: DMV; brainstem; 5-HT; parasympathetic; preganglionic; gastrointestinal; electrophysiology

Abbreviations: AHP, afterhyperpolarization; AP, action potential; DiI, 1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DMV, dorsal motor nucleus of the vagus; EPSC, excitatory postsynaptic current; HP, holding potential; IPSC, inhibitory postsynaptic current; NAN-190, 1-(2-Methoxyphenyl)-4-[4-(2-phthalminido)butyl]piperazine hydrochloride; NTS, nucleus of the tractus solitarius, 8-OHDPAT, 8-hydroxydipropylaminotetralin; TFMPP, N-(3-trifluoromethylphenyl)piperazine

Introduction

The dorsal motor nucleus of the vagus (DMV) contains the cell bodies of preganglionic parasympathetic neurones that supply the motor innervation to subdiaphragmatic viscera (see Gillis *et al*., 1989). Sensory information (mechanical, osmotic, chemical and caloric) perceived by discrete areas of the gastrointestinal (GI) tract from the oesophagus to the colon are transmitted *via* vagal afferent fibres and are received by cells within different subnuclei of the nucleus of the tractus solitarius (NTS). For example, gastric sensory projections are received principally in the gelatinosus and medialis subnuclei of the NTS, while intestinal sensory projections are received mainly in the commissuralis (comNTS) and medialis subnuclei; oesophageal sensory projections are received in the NTS subnucleus centralis (cenNTS), (Altschuler *et al*., 1989; 1991; Zhang *et al*., 1995; Rogers *et al*., 1999). These distinct subnuclei of the NTS then integrate this information and provide both excitatory and inhibitory inputs to the DMV *via* a monosynaptic pathway (Bertolino *et al*., 1997; Travagli *et al*., 1991; Rogers *et al*., 1999; Fukuda *et al*., 1987; Champagnat *et al*., 1986; Willis *et al*., 1996). Higher CNS centres such as the raphe nuclei and the paraventricular nucleus of the hypothalamus exert modulation at the level of these synaptic connections.

Within the caudal brainstem, a serotonergic input to the dorsal vagal complex (DVC, i.e., DMV and NTS) is provided by both medullary raphe neurones (McCann *et al*., 1989; Hornby *et al*., 1990; Palkovits *et al*., 1986) and vagal afferent neurones (Nosjean *et al*., 1990; Izzo *et al*., 1993; Sykes *et al*., 1994). In addition, autoradiographic studies of the DVC have revealed discrete distributions of 5-HT receptors. In detail, 5-HT_{1A} receptors are localized throughout the NTS, predominantly in the cenNTS, although low quantities are also found in the DMV (Manaker & Verderame, 1990; Thor *et al*., 1992a, b). 5-HT_{1B} receptors are distributed mainly in NTS sunucleus gelatinosus and the DMV (Manaker & Verderame, 1990; Thor *et al*., 1992a, b), while 5-HT_2 receptors are scattered on DMV somata (Wright *et al*., 1995). 5-HT_3 receptors are present throughout the DVC (Steward *et al*., 1993). These studies indicate that a multitude of 5-HT receptor subtypes may be present within the DVC, providing means by which 5-HT may exert a fine modulation of vagal activity.

Vagal preganglionic motoneurones exhibit diverse responses to exogenous application of 5-HT. For example, 5-HT acts on gastrointestinal preganglionic neurones to increase gastric acid secretion (McTigue *et al*., 1992; Krowicki & Hornby, 1995; Yoneda & Tache, 1995; Chi *et al*., 1996; Varanasi *et al*., 1997), while 5-HT, acting through 5-HT_{1A} and

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5-$HT_{1B/1D}$ receptors, exerts opposing effects on bronchoconstrictor preganglionic neurones (Bootle *et al*., 1998) whereas cardiac vagal motorneurones are excited by activation of 5-HT_{1A} receptors (Sporton *et al*., 1991).

Using *in vivo* extracellular recording techniques, approximately 40% of NTS neurones were excited by 5-HT, 30% were inhibited and the remaining 20% of neurones were unaffected (Wang *et al*., 1997). The excitatory effects of 5-HT were mimicked by both 5-HT_{1A} and 5-HT_2 receptor agonists, and, since 5-HT_{1A} receptors are known to be inhibitory (see Bobker & Williams, 1993; Grudt *et al*., 1995), these data may suggest that the 5-HT_{1A} receptor-mediated excitation is obtained as a consequence of disfacilitation of an inhibitory input while the 5-HT_2 receptor-mediated excitation is a consequence of a direct interaction with the recorded units. Within the DMV, both *in vivo* and *in vitro* recordings have shown that 5-HT_2, 5-HT_3 and 5-HT_4 receptor agonists excite between 60 and 80% of DMV neurones (Albert *et al*., 1996; Wang *et al*., 1996; 1998). 5-HT_{1A} receptor agonists, however, excited approximately 20% but inhibited around 66% of DMV neurones (Wang *et al*., 1995) which again may indicate presynaptic actions of 5-HT_{1A} receptor agonists on both excitatory and inhibitory NTS neurones. Hence, these data suggest that the effects of 5-HT on the DMV depend not only on the receptor subtypes present on the DMV cell somata but also on the receptor subtypes present on the terminals of its presynaptic inputs. In addition, our laboratory has reported recently that the intrinsic properties of DMV neurones can be correlated with their target organs (Browning *et al*., 1999).

Unfortunately, despite the increasing evidence for a modulatory role of 5-HT in the DVC, and growing evidence pointing toward an *in vivo* synergistic interaction between 5-HT and TRH in the regulation of gastric acid release (Garrick *et al*., 1994; Yoneda & Tache, 1995), previous studies have not correlated the different responses of DVC neurones and their peripheral target or discrete NTS-DMV circuits. The purpose of this study, therefore, was to investigate the effects of 5-HT on identified DMV neurones that project to discrete areas of the GI tract.

Methods

Retrograde tracing

Sprague-Dawley rats (12 days old) of either sex were anaesthetized deeply with a 6% solution of Halothane® with air (400–600 ml min^{-1}) in accordance with the Animal Care and Use Committee Guidelines, Henry Ford Health System, Detroit, Michigan, U.S.A. The depth of anaesthesia (foot pinch withdrawal reflex) was assessed prior to, and during, surgery. The abdominal and thoracic areas were shaved and cleaned with 70% ethanol, and an abdominal laparotomy performed. During surgery, anaesthesia was maintained by placing the head of the rat in a custom-made anaesthetic chamber through which the halothane/air mixture was perfused. Crystals of the retrograde tracer DiI were applied to the serosal surface of the stomach (along the greater curvature of the gastric fundus and corpus, or to the antrum/pylorus) or to the intestine (the duodenum, at the level of the bifurcation of the hepatic and pancreatico-duodenal arteries, or the caecum at the level of the ileo-caecal junction). The surgical area was embedded in a fast hardening epoxy compound to prevent leakage of the retrograde tracer away from the site of application; the epoxy compound was allowed to dry (3–5 min) before the surgical area was washed with warm sterile saline solution. The wound was then sutured with 5-0 silk and the animal allowed to recover for 10–15 days.

Electrophysiology

The method utilized for the tissue slice preparation has already been described (Travagli *et al*., 1991). Briefly, rats were placed in a transparent, enclosed anaesthetic chamber through which Halothane® bubbled with air was passed. Once a deep level of anaesthesia was attained (abolition of the foot pinch withdrawal reflex), the rat was killed by severing the major blood vessels in the chest. The brainstem was then removed and placed in oxygenated physiological saline at 4°C (see below). Using a vibratome, six to eight coronal slices (200 μm thick) containing the DMV were cut. The slices were incubated for at least 1 h in oxygenated physiological saline at 35 ± 1°C until use. A slice was placed in a perfusion chamber (500 μl volume), held in place by a nylon mesh and maintained at 35°C by continual perfusion with warmed oxygenated physiological saline at a rate of 2.5 ml min^{-1}.

Prior to electrophysiological recordings, retrogradely labelled DMV neurones were identified using a Nikon E600-FS microscope equipped with DIC (Nomarski) optics and TRITC epifluorescent filters. Brief periods of illumination were used to detect the fluorescent neurones; once a labelled cell was localized, electrophysiological recordings were made under brightfield illumination using DIC optics.

Whole cell recordings were made from retrogradely labelled GI-projecting neurones only using patch pipettes filled with potassium gluconate intracellular solution of resistance 3–8 MΩ (see below) and a single electrode voltage clamp amplifier (Axoclamp 2B or Axopatch 1D, Axon Instr., Foster City, California, U.S.A.). Data were filtered at 2 kHz, digitized *via* a Digidata 1200C interface (Axon Instr.), acquired and stored on an IBM PC utilizing pClamp6 software (Axon Instr.). Only those recordings having a series resistance (i.e., pipette + access resistance) <15 MΩ were used. The criteria for accepting a neuronal recording included a membrane that was stable at the holding potential and which returned to baseline after action potential afterhyperpolarization as well as an action potential of at least 60 mV amplitude. Data analysis was performed using pClamp6 software.

Electrical stimulation

Tungsten electrodes (WPI Instr. Ltd. Sarasota, Florida, U.S.A.) were used to electrically stimulate the cenNTS (when recording from gastric-projecting neurones) or the comNTS (when recording from intestinal-projecting neurones). Single stimuli (0.5–1.0 ms, 10–500 μA) were applied using a Master-8 stimulator (AMPI, Jerusalem) every 20 s to evoke submaximal inhibitory postsynaptic currents (IPSC) of amplitude 250–550 pA.

Drug application

Drugs were applied to the bath *via* a series of manually operated valves. 5-HT (30 μM) was applied to all neurones to ascertain whether or not it had an effect *per se* before continuing with the appropriate treatments. To assess the effects of drugs, each neurone served as its own control, i.e., the results obtained after administration of a receptor agonist or antagonist were compared to those before administration using the paired *t*-test. Receptor agonists and antagonists were applied in concentrations demonstrated previously to be effective (Bobker & Williams, 1990; Bobker, 1994; Grudt *et*

al., 1995). Intergroup comparisons were analysed with one-way ANOVA followed by paired *t*-test comparisons or with Chi Square test (χ^2). Results are expressed as mean ± s.e.mean. Significance was defined as $P<0.05$.

Drugs and solutions

Krebs' (in mM): NaCl 126, $NaHCO_3$ 25, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2 and dextrose 11, maintained at pH 7.4 by bubbling with O_2-CO_2 (95% – 5%). Intracellular solution (in mM): K-gluconate 128, KCl 10, $CaCl_2$ 0.3, $MgCl_2$ 1, HEPES 10, EGTA 1, ATP 2, GTP 0.25. Adjusted to pH 7.35 with KOH. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DilC_{18}(3)$; Dil) was purchased from Molecular Probes (Eugene, Oregon, U.S.A.); α-methyl 5-HT, (−)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (R(−)-DOI), 8-hydroxydipropylamino-tetralin (8-OHDPAT), N-(3-trifluoromethylphenyl)piperazine (TFMPP), pindobind-5-HT_{1A}, 3-tropanyl-indole-3-carboxylate hydrochloride (ICS 205-930), ketanserin tartrate and 1-(2-methoxyphenyl)-4-[4-(2-phthalminido)butyl]piperazine hydrochloride (NAN-190) were purchased from RBI (Natick, Massachusetts, U.S.A.); 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane®) and all other chemicals were purchased from Sigma (St. Louis, Missouri, U.S.A.).

Experimental protocols

Single action potential firing: Neurones were current clamped at a holding potential of −55 mV before injection of short (16 ms) duration depolarizing current pulses of intensity sufficient to evoke the firing of a single action potential at its offset. Following superfusion with 5-HT, DC current was injected to return the membrane potential to its pretreatment holding potential before re-running the same protocol. The duration of the action potential at threshold, the amplitude of the afterhyperpolarization (AHP) from resting potential to peak and the AHP duration (AHP-τ) from peak amplitude until it returned to baseline were measured before and after exposure to 5-HT.

I_H and I_{KIR} To evoke I_H and I_{KIR} neurones were voltage-clamped at −50 mV and then step hyperpolarized to −120 mV in 10 mV increments before being returned to the holding potential (Travagli & Gillis, 1994).

I_A The inactivation curve for I_A was constructed using 400 ms long hyperpolarizing steps from a holding potential of −50 mV in 10 mV increments to −120 mV (to remove 1_A inactivation) and repolarization to −50 mV (to activate 1_A).

Results

Dorsal vagal preganglionic motoneurones

Results have been obtained from a total of 206 neurones; 122 were identified as projecting to the stomach and 84 were identified as projecting to the intestine.

Electrophysiology

The basic characteristics of DMV neurones were essentially similar to those already described by this laboratory (Browning *et al*., 1999). Briefly, gastric neurones could be distinguished from intestinal neurones on the basic of their smaller and shorter afterhyperpolarization (19.3 ± 1.0 mV, $n=23$, and 67.7 ± 6.3 ms, $n=20$, vs 25.9 ± 0.8 mV, $n=16$, and 109.3 ± 27.0 ms, $n=14$ for gastric and intestinal neurones, respectively, $P<0.05$; see Figure 2), as well as localization within the DMV columns (i.e. gastric neurones in medial DMV *vs* intestinal neurones in lateral DMV) as well as per their fluorescent labelling.

Effect of 5-HT

In voltage clamp experiments (Holding Potential (HP) = −50 mV), 5-HT (0.3 – 300 μM) was superfused for a period of time sufficient for the induced current to reach a stable plateau, generally between 60 and 150 s, depending on the depth of the neurone within the slice. Superfusion with 5-HT induced a concentration-dependent inward current in 58 out of 122 gastric projecting neurones (i.e. 47.5%) and in 58 out of 84 intestinal projecting neurones (i.e. 69%; $P<0.05$% vs gastric neurones). The induced inward current did not desensitize even at the highest concentrations used. The rate of onset and decay of the inward current was dependent upon the depth of the neurone within the brainstem slice but a wash out period of at least 10 min was allowed between drug applications.

While the maximum current (I_{max}) induced by 5-HT and the concentration that produced the maximum effect was similar for both gastric- and intestinal-projecting neurones (53.8 ± 8.4 pA and 46.4 ± 7.3 pA at 30 μM, for gastric and intestinal neurones, respectively, $P>0.05$); the EC_{50} was shifted rightwards in the intestinal-projecting neurones (10 μM) compared to gastric-projecting neurones (4 μM; $P<0.05$; Figure 1).

Perfusion with 5-HT increased the duration of the action potential in both gastric- and intestinal-projecting neurones. In detail, the action potential duration in gastric-projecting neurones was increased from a control value of 2.6 ± 0.1 – 2.7 ± 0.1 ms in the presence of 5-HT ($n=23$; $P<0.05$) while in intestinal-projecting neurones, the duration of the action potential was increased from a control value of 3.0 ± 0.2 – 3.3 ± 0.3 ms in the presence of 5-HT, respectively ($n=16$; $P<0.05$; Figure 2).

Concomitant to the increase in action potential duration, perfusion with 5-HT decreased the peak amplitude of the AHP in both gastric- and intestinal-projecting neurones. In fact, 5-HT decreased the AHP in gastric-projecting neurones from 19.3 ± 1.0 to 16.3 ± 0.8 mV ($n=23$; $P<0.05$) while in intestinal-projecting neurones, 5-HT decreased the AHP amplitude from 25.9 ± 0.8 to 22.1 ± 0.7 mV ($n=16$; $P<0.05$; Figure 2).

5-HT had no effect on AHP-τ in either gastric or intestinal-projecting neurones. The AHP-τ in gastric-projecting neurones was 67.7 ± 6.3 and 68.6 ± 7.7 ms in control and 5-HT, respectively ($n=20$; $P>0.05$) and in intestinal-projecting neurones it was 109.3 ± 27.0 and 87.8 ± 16.7 ms in control and 5-HT, respectively ($n=14$; $P>0.05$).

5-HT does not affect other voltage-dependent currents (VDC)

We investigated whether 5-HT had any effect on VDC other than I_{AHP}. We studied the non-selective cationic current I_H, the fast-transient potassium current (I_A) and the potassium inward rectifier (I_{KIR}) since these currents are all present in DMV neurones (Browning *et al*., 1999; Travagli & Gillis, 1994) and/or they have been reported to be affected by 5-HT (Andrade & Chaput, 1991b; Bobker & Williams, 1993). Perfusion with 5-HT (30 μM) did not affect the amplitude or activation

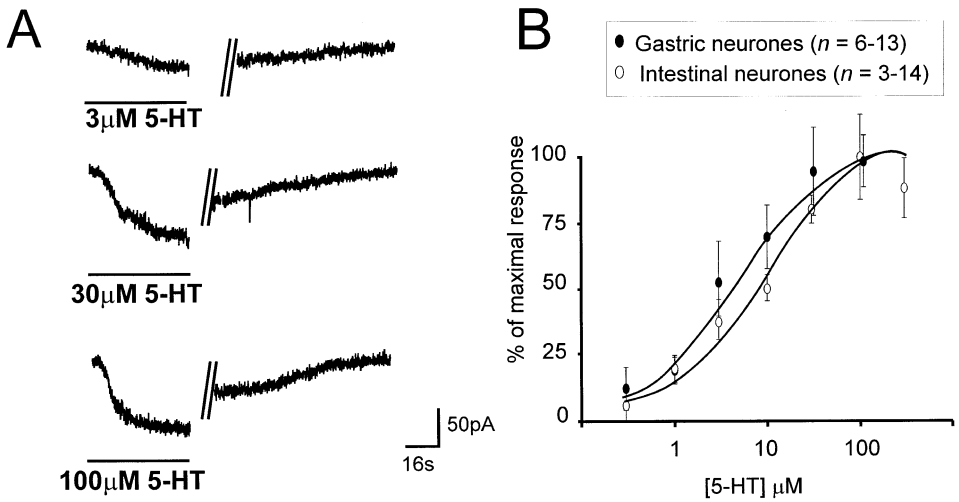

Figure 1 Exogenously applied 5-HT induces a concentration-dependent inward current in gastrointestinal-projecting DMV neurones. (A) Representative traces from one gastric-projecting DMV neurone illustrating the concentration-dependent nature of the inward current induced by superfusion of 5-HT. Neurones were voltage-clamped at −50 mV before application of 5-HT for a period of time sufficient for the induced current to reach a stable plateau (60–150 s). The inward current did not desensitize, even at high concentrations. The vertical lines indicate an interruption in recording of 120, 110 and 135 s for the top, middle and bottom trace, respectively. A recovery period of at least 10 min was allowed between successive applications. (B) Concentration-response curve for gastric- and intestinal-projecting DMV neurones (voltage clamped at −50 mV) in response to exogenously applied 5-HT. The current induced by 5-HT is expressed as a percentage of the maximal response. Note that the EC_{50} value for the inward current in intestinal neurones (10 μM) is significantly larger than that of gastric neurones (4 μM) $^*P<0.05$.

potentials of either currents (I_H: −58±8 pA in control at −120 mV vs −53±7 pA in 5-HT P40.05, $n=11$; I_{KIR} −235±42 pA in control at −120 mV vs −278±42 pA in 5-HT, $P>0.05$; $n=4$). Similarly, perfusion with 5-HT (30 μM) did not affect the absolute amplitude of I_A (617±63 pA in control vs 602±59 pA in 5-HT, $P>0.05$; $n=23$), its activation threshold (−60 mV for both control and 5-HT), its I_{50} (−75 mV for both control and 5-HT) or its kinetics of decay (213±11 ms at −90 mV (see Browning *et al.*, 1999) in control vs 220±11 ms in 5-HT, P.0.05; $n=23$).

Effect of 5-HT$_2$ receptor ligands

In eight neurones (five gastric, three intestinal) the inward current induced by perfusion with 5-HT (30 μM) was unaffected by prior perfusion with the synaptic inhibitor tetrodotoxin (30.9±2.9 pA in control vs 34.1±4.6 pA in the presence of 0.1 μM TTX, $P>0.05$). In ten neurones (six gastric, four intestinal), the 5-HT-induced current was mimicked by application of the 5-HT$_2$ receptor selective agonist, α-methyl-5-HT (31.4±2.7 pA in presence of 5-HT vs 21.9±1.7 pA in the presence of 3 μM α-methyl-5-HT). Furthermore, in ten neurones (six gastric, four intestinal), the inward current induced by 30 μM 5-HT was abolished by prior perfusion with ketanserin (36.2±3.6 pA in control vs 3.22±1.1 pA in 1 μM ketanserin, $P<0.05$). In six neurones (three gastric, three intestinal), the inward current induced by α-Me-5-HT (3 μM) was also abolished by prior perfusion with ketanserin (20.3±2.4 pA in control vs 1.5±0.7 pA in presence of 1 μM ketanserin, $P<0.05$; Figure 3).

In some, but not all, neurones (seven out of ten neurones; 70%) application of ketanserin (1 μM) caused an outward shift in holding current (6±2 pA, $n=6$) which was maintained throughout the duration of ketanserin application.

Effects of 5-HT$_{1A}$ receptor ligands

We also ruled out the involvement of 5-HT$_{1A}$ receptors in the 5-HT-induced inward current with the following experiments:

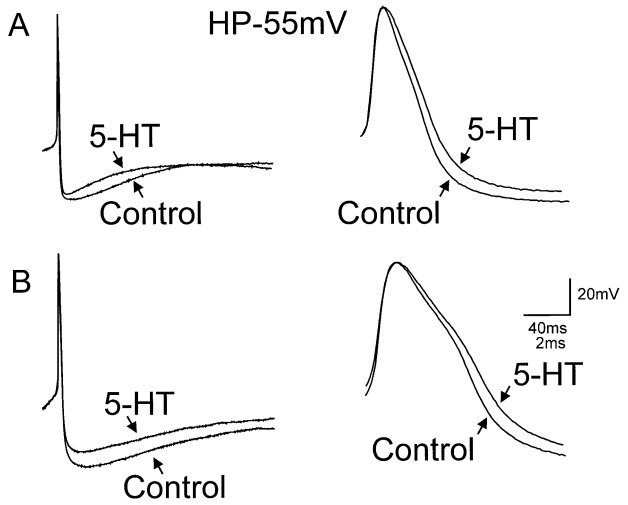

Figure 2 5-HT decreases the duration of the action potential afterhyperpolarization and increases the action potential duration. To evoke a single action potential, neurones were current clamped at −55 mV before passing a short duration (16 ms) depolarizing current pulse of intensity sufficient to evoke the firing of one action potential at its offset. (A) Illustrate the response of a corpus-projecting neurone; (B) Illustrate the response of a caecal-projecting neurone to 5-HT (30 μM). In both gastric-projecting neurones (e.g. A) and intestinal-projecting (e.g. B) DMV neurones 5-HT decreased the amplitude of the afterhyperpolarization (left traces) as well as increasing the duration of the action potential (right traces).

in 18 neurones (eight gastric, ten intestinal) the inward current induced by 30 μM 5-HT was not mimicked by the 5-HT$_{1A}$ receptor agonist, 8-OHDPAT (34.8±3.0 pA current to 5-HT vs 2.3±0.7 pA current to 1 μM 8-OHDPAT; $P<0.05$) while in ten neurones (seven gastric, three intestinal) the 5-HT (30 μM) induced inward current was unaffected by prior perfusion with the 5-HT$_{1A}$ receptor antagonists, NAN-190 (1 μM) or pindobind-5-HT$_{1A}$ (1 μM) (34.8±3.5 pA in control vs 33.1±2.4 pA, $P>0.05$ Figure 3). Application of the 5-HT$_{1A}$

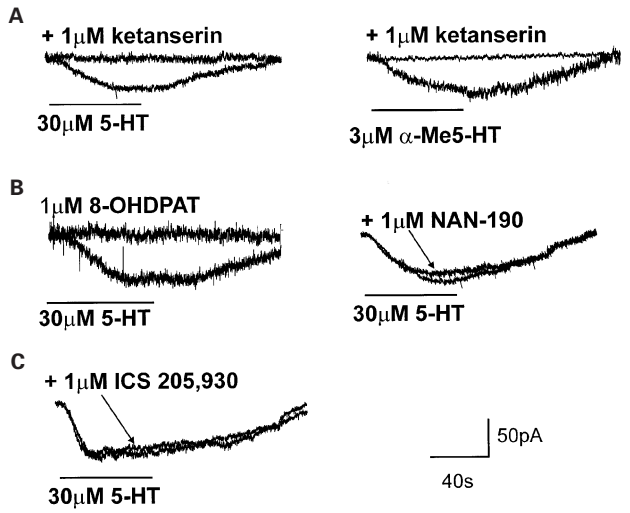

Figure 3 The postsynaptic responses to 5-HT are mediated *via* activation of 5-HT_2 receptors only. All neurones were voltage-clamped at −50 mV before application of 5-HT (30 μM) for periods sufficient to allow the induced inward current to reach a stable plateau. Following complete recovery and a wash-out period of at least 10 min, the response of the same neurone to 5-HT receptor selective agonists or the response to 5-HT in the presence of 5-HT receptor selective antagonists were assessed. Receptor antagonists were superfused for a minimum of 10 min before re-application of receptor agonists. (A) The inward current induced by superfusion with 5-HT (30 μM; left panel) was prevented by prior application of the 5-HT_{2A} receptor antagonist, ketanserin (1 μM). Similarly, the 5-HT_2 receptor agonist α-methyl 5-HT (3 μM; black trace, right panel), mimicked the inward current induced by 5-HT, a response that was also prevented entirely by the 5-HT_{2A} receptor antagonist, ketanserin (1 μM). (B) Unlike 5-HT (30 μM; left panel), the 5-HT_{1A} receptor agonist, 8-OHDPAT (1 μM) had no postsynaptic actions. Similarly, preincubation with the 5-HT_{1A} receptor antagonist, NAN-190 (1 μM, right panel) did not attenuate the 5-HT-induced inward current. (C) Preincubation with the 5-$HT_{3/4}$ receptor antagonist ICS 205-930 (tropisetron, 3 μM) did not attenuate the inward current induced by 5-HT (30 μM).

receptor antagonists themselves caused no change in the membrane holding current.

Effects of 5-HT_3 receptor ligands

Moreover, in six neurones (three gastric, three intestinal) in which 5-HT (30 μM) induced an inward current at a holding potential of −50 mV, perfusion with the non-selective potassium current inhibitor caesium chloride (2 mM) reduced the amplitude of the 5-HT induced inward current (36.7±3.4 pA in control vs 13.7±2.9 pA in presence of CsCl, $P<0.05$), thus arguing in favour of an effect on a potassium-mediated conductance, as with activation of 5-HT_2 receptors.

Finally, pretreatment with the 5-$HT_{3/4}$ receptor antagonist ICS 205−930 (tropisetron; 3 μM did not antagonize the 5-HT induced inward current (32.2±5.7 pA in control vs 33.4±4.9 pA in the presence of ICS 205-930; $n=9$, six gastric, three intestinal; Figure 3). Application of ICS 205-930 alone never caused any change in the membrane holding current.

Effects of 5-$HT_{1A/1B}$, 5-HT_2 and 5-HT_3 receptor ligands on the NTS evoked IPSCs

In gastric-projecting neurones, electrical stimulation of cenNTS evoked IPSC whose amplitude, duration and kinetics of decay were 405.5±23.6 pA, 413±20 ms and 171±19 mV/ms, respectively ($n=44$). Similarly, in intestinal-projecting neurones, electrical stimulation of comNTS evoked IPSC whose amplitude, duration and kinetics of decay were 426±28.9 pA ($P>0.05$ compared with gastric-projecting neurones), 487±37 ms ($P<0.05$ compared with gastric-projecting neurones) and 141±9 mV/ms ($P>0.05$ compared with gastric-projecting neurones), respectively ($n=29$; HP = −50 mV in both gastric and intestinal neurones). The evoked IPSCs were identified as having a GABAergic component since perfusion with the $GABA_A$ receptor antagonists bicuculline (10−30 μM; $n=4$) or picrotoxin (100 μM; $n=2$) attenuated the evoked IPSCs from a control amplitude of 400.8±86.8 pA to 95.1±41.9 pA ($n=6$; $P<0.05$).

In gastric-projecting neurones, perfusion with 5-HT (30 μM) reduced the peak amplitude of the evoked IPSC in 27 out of the 44 neurones tested (i.e. 61%) from 382.7±25.7 pA to 286.7±19.5 pA in control and 5-HT, respectively ($n=27$; $P<0.05$) with an average inhibition of control peak amplitude of 24±2% ($n=27$). Similarly, 5-HT reduced the duration and increased the rate of decay of the IPSC from 414±44 to 367±42 ms and 150±24 to 209±42 mV/ms, respectively ($n=27$, $P<0.05$). In intestinal-projecting neurones, perfusion with 5-HT (30 μM) reduced the peak amplitude of the evoked IPSC in 15 out of the 29 neurones tested (i.e. 52%) from 414.1±40.6 to 334.3±36.2 pA in control and 5-HT, respectively ($n=15$; $P<0.05$) with an average inhibition of the control peak amplitude of 19.1±2% ($n=15$; Figure 4). Similarly, 5-HT reduced the duration and increased the rate of decay of the IPSC from 487±37 to 447±37 ms and 155±17 to 166±19 mV/ms, respectively ($n=15$, $P<0.05$).

The ratio of the amplitude of two postsynaptic currents evoked few milliseconds apart is used to determine the pre- or postsynaptic site of drug action (Travagli & Williams, 1996; Bertolino *et al*., 1997). A change in the ratio is taken as an indication of a presynaptic effect.

When two IPSC were evoked 150−750 ms apart, the second IPSC (C2) was smaller than the first one (C1) in 14 out of the 27 gastric neurones responsive to 5-HT while C2 was larger than C1 in the remaining 13 neurones. When there was a decrease, the paired pulse ratio was reduced from a control value of 0.98±0.01 to 0.89±0.03 in 5-HT ($n=14$; $P<0.05$; Figure 4A). Conversely, in those which had an increase in the paired pulse ratio, its value increased from 0.97±0.01 in control to 1.01±0.01 in 5-HT ($n=13$; $P<0.05$; Figure 4B). In both instances, the alteration of the paired pulse ratio suggested a presynaptic site of action.

In intestinal-projecting neurones, the second IPSC (C2) was smaller than the first (C1) in six out of the 15 neurones responsive to 5-HT, while C2 was larger than C1 in the other nine neurones. When there was a decrease, the paired pulse ratio was reduced from 0.92±0.04 in control to 0.87±0.05 in 5-HT ($n=6$; $P<0.05$). Conversely, in those neurones that had an increase in the paired pulse ratio, its value increased from 0.96±0.01 in control to 1.00±0.01 in 5-HT ($n=9$; $P<0.05$). As for gastric neurones, the alteration of the paired pulse ratio suggested a presynaptic site of action (Figure 4).

Given that both 5-HT_{1A} and 5-HT_{1B} receptors have been identified in the NTS (Thor *et al*., 1992a), we used receptor agonists and antagonists selective for these receptors to identify the subtype(s) involved in mediating the decrease in IPSC amplitude. The results obtained from gastric and intestinal neurones were qualitatively and quantitatively similar, i.e., they responded in a similar fashion only to challenge with 5-HT_{1A} receptor ligands. For this reason, the

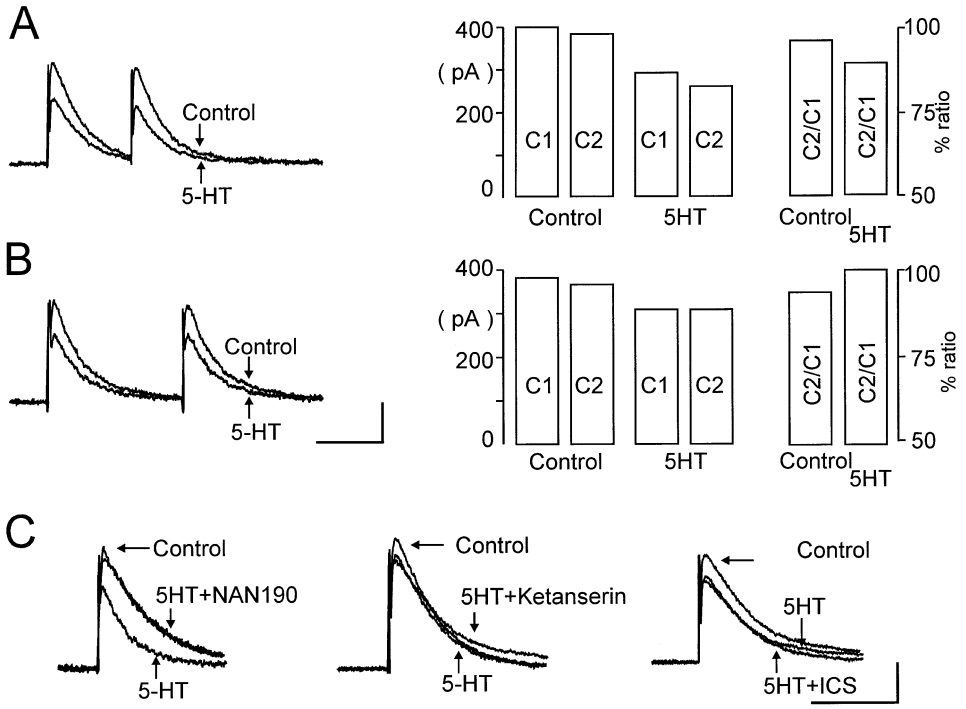

Figure 4 Activation of presynaptic 5-HT_{1A} receptors, decreases the amplitude of evoked IPSC. (A,B) Neurones were voltage-clamped at −50 mV before evoked IPSC were induced in gastrointestinal-projecting DMV neurones by stimulation of the NTS. Pairs of IPSC were evoked 250–750 ms apart (left panel). Following superfusion with 5-HT (30 μM), the amplitude of the evoked IPSC were reduced. This reduction in amplitude of IPSC is highlighted in the bar chart (right panel) which compares the amplitude of the first and second pulse (C1 and C2, respectively) and their ratio (C2/C1) before and after superfusion with 5-HT (30 μM). Calibration bar = 400 ms, 200 pA. (C) The 5-HT-induced reduction in IPSC amplitude was attenuated by prior exposure to 5-HT_{1A} receptor antagonist, NAN-190 (1 μM; left traces) but not by the 5-HT_{2A} receptor antagonist, ketanserin (1 μM; middle traces) or the 5-$HT_{3/4}$ receptor antagonist, ICS 205-930 (tropisetron, 3 μM; right traces). Calibration bar = 400 ms, 200 pA.

data obtained from gastric and intestinal neurones have been pooled with the *n* specified for each subgroup.

The 5-HT_{1A} receptor agonist 8-OHDPAT (1 μM) reduced the peak amplitude of the evoked IPSC from 385.2 ± 35.2 pA to 315 ± 33.1 pA ($n = 14$; eight gastric and six intestinal; $P < 0.05$). Similarly, the 5-$HT_{1A/B}$ receptor agonist TFMPP (1 μM) decreased the peak amplitude of the evoked IPSC from 357 ± 39 to 276.3 ± 28.6 pA ($n = 11$; eight gastric and three intestinal; $P < 0.05$). Pretreatment with the selective 5-HT_{1A} receptor antagonists NAN-190 (1 μM) or pindobind-5-HT_{1A} (1 μM), attenuated the 5-HT (30 μM) induced inhibition of the IPSC from 22.1 ± 2 to 0 ± 2% ($n = 8$; five gastric and three intestinal; $P < 0.05$; Figure 4C). Similarly, pretreatment with NAN-190 or pindobind-5-HT_{1A} completely prevented the inhibition by 8-OHDPAT (from 18.6 ± 2 to 0.6 ± 2%, $P < 0.05$, $n = 6$; three gastric and three intestinal) and TFMPP (from 21.3 ± 3 to 1.2 ± 1.8, $P < 0.05$, $n = 6$; three gastric and three intestinal).

5-HT_3 and 5-HT_4 receptors were not involved in the reduction of evoked IPSC since pretreatment with ICS 205-930 (3 μM, a concentration that affects both 5-HT_3 and 5-HT_4 receptors) did not prevent the 5-HT mediated inhibition (418 ± 42 and 308.8 ± 32.2 pA in ICS 205-930 and in the presence of ICS 205-930 plus 5-HT, respectively, $P < 0.05$; $n = 8$, five gastric and three intestinal; Figure 4C).

Perfusion with the selective 5-HT_2 receptor agonist α-methyl-5-HT (3 μM) did not significantly decrease the peak amplitude of the IPSC (444.5 ± 70.4 pA in control and 399 ± 63.1 pA in 3 μM α-methyl-5-HT, respectively; $n = 8$; five gastric and three intestinal; $P > 0.05$); the failure of 5-HT_2 receptor agonists to decrease the peak amplitude of the IPSC suggests that 5-HT_2 receptors do not modulate this effect.

Effects of 5-HT on spontaneous inhibitory currents (IPSCs)

Without affecting their amplitude, 5-HT (30 μM) decreased the frequency of spontaneous IPSCs in 12 out of 17 neurones and increased IPSC frequency in the remaining five neurones responsive to 5-HT. In the neurones in which 5-HT decreased the frequency of the IPSC, the frequency was reduced from 2.98 ± 1.6 IPSC s^{-1} in control to 0.7 ± 0.3 IPSC s^{-1} in 5-HT ($P < 0.05$). Conversely, in those neurones in which 5-HT had an excitatory effect, the frequency increased from 3.8 ± 3.2 to 7.1 ± 5.1 IPSC s^{-1} in control and 5-HT, respectively ($P < 0.05$). The average amplitude of IPSC was not altered by 5-HT (19.6 ± 0.9 pA compared to 20 ± 0.8 pA in control ($n = 52$) and 5-HT ($n = 54$), respectively ($P > 0.05$). A change in the frequency of spontaneous events without a change in their amplitude has previously been shown to indicate a presynaptic site of action (Travagli & Williams, 1996).

Effects of 5-HT on spontaneous excitatory currents (EPSCs)

5-HT (30 μM) increased the frequency of spontaneous EPSC in six and decreased EPSC frequency in 12 of the 18 neurones responsive to 5-HT without affecting their amplitude. In those neurones in which 5-HT had a stimulatory effect, the frequency increased from 6.9 ± 3.9 to 8.9 ± 3.9 EPSC s^{-1} in control and 5-

HT, respectively ($P<0.05$). Conversely, in those neurones in which 5-HT had an inhibitory effect, the frequency decreased from 6.3 ± 0.7 to 3.2 ± 0.6 EPSC s^{-1} in control and 5-HT, respectively ($P<0.05$). The average amplitude of the EPSC was unaltered by 5-HT (20.9 ± 0.4 pA and 22.3 ± 0.4 pA in control and 5-HT ($n=81$ for both groups), respectively ($P>0.05$), again suggesting a presynaptic site of action.

Discussion

With the present data we report the excitation of GI-projecting neurones of the rat DMV by 5-HT. At the postsynaptic level, 5-HT excited 69% of intestinal-projecting neurones and in 47% of gastric-projecting neurones. In both instances, the excitation was mediated *via* activation of 5-HT_2 receptors only. At the presynaptic level, the 5-HT receptor mediated excitation of DMV neurones was achieved *via* disfacilitation of GABAergic inputs from NTS. The presynaptic effects were achieved *via* a reduction in the peak amplitude of evoked IPSCs (an effect mediated by presynaptic 5-HT_{1A} receptors), a decrease in the frequency of spontaneous IPSCs and an increase in the frequency of EPSCs.

Postsynaptic effects of 5-HT

Recent pharmacological experiments have demonstrated that DMV neurones can be affected by interaction of 5-HT with 5-HT_{1A}, 5-HT_2, 5-HT_3 and 5-HT_4 receptors (Wang *et al*., 1995; 1996; 1998; Yoneda & Tache, 1995; Albert *et al*., 1996). We used ion substitutions as well as 5-HT receptor selective agonists and antagonists to ascertain the receptor subtype(s) involved in the excitatory effects of 5-HT on identified DMV neurones.

With the present data we show that the postsynaptic response of identified DMV neurones to exogenously applied 5-HT is exclusively excitatory. This finding is in agreement with previous reports of 5-HT mediating excitation only (Travagli & Gillis, 1995; Albert *et al*., 1996) but it is in contrast with Wang *et al*. (1995), who showed an inhibition of the firing rate of DMV neurones following iontophoretic application of 8-OHDPAT or 5-HT at high currents. In our study, an outward current was not induced by perfusion with either high concentrations of 5-HT (up to 300 μM) or the 5-HT_{1A} receptor agonist 8-OH-DPAT. In addition, blockade of the 5-HT-induced inward current with the 5-HT_{2A} receptor antagonist ketanserin failed to unmask any outward current, and pretreatment with the selective 5-HT_{1A} receptor antagonists NAN-190 or pindobind-5HT_{1A} failed to prevent 5-HT-induced effects. A plausible explanation for the conflicting results among these groups (Travagli & Gillis, 1995; Wang *et al*., 1995; Albert *et al*., 1996) is that the effects observed following application of 8-OHDPAT or high doses of 5-HT may be a consequence of their action onto the more distant NTS neurones. This hypothesis is indirectly supported by our experiments in which perfusion with 8-OHDPAT inhibited GABA-mediated currents evoked by NTS stimulation. Alternatively, it is possible that in the *in vivo* experiments carried out by Wang *et al*. (1995) there is indeed a tonic GABAergic input present that is not active in the slice preparation.

Our findings show that the postsynaptic response of identified DMV neurones to exogenously applied 5-HT is mediated by 5-HT_2 receptor activation only. Our conclusion stems from the following observations: (i) the 5-HT_2 receptor selective agonist α-methyl-5-HT mimicked the direct effect of 5-HT by eliciting a slow, sustained inward current; (ii) the inward current induced by both 5-HT and α-methyl-5HT was antagonized by pretreatment with the 5-HT_{2A} receptor antagonist ketanserin; (iii) the 5-HT induced inward current was neither mimicked by the 5-HT_{1A} receptor agonist 8-OH-DPAT nor attenuated by the 5-HT_{1A} receptor antagonist NAN-190; (iv) pretreatment with ICS 205–930 at a concentration known to antagonize both 5-HT_3 and 5-HT_4 receptors (Bockaert *et al*., 1989) did not attenuate the 5-HT induced inward current; (v) the response to 5-HT was slow in onset and did not show any desensitization; (vi) the inward current induced by 5-HT was attenuated by pretreatment with the potassium conductance inhibitor caesium; (vii) the 5-HT mediated inward current was still present in the presence of the synaptic transmission blocker tetrodotoxin.

Our conclusions that the excitatory effect of 5-HT is mediated by 5-HT_2 receptors corresponds with recent *in vitro* reports on non-identified DMV neurones (Albert *et al*., 1996) as well as *in vivo* studies on gastrointestinal function (Yoneda & Tache, 1995; Varanasi *et al*., 1997). The inward current induced by 5-HT *via* 5-HT_2 receptors would thus provide a further physiological counterpart to the studies which demonstrated a high level of 5-HT_2 mRNA in the DMV (Wright *et al*., 1995). The finding that, in some neurones, application of the 5-HT_{2A} receptor antagonist ketanserin alone caused a maintained outward shift in holding current suggests that, in a proportion of neurones at least, 5-HT may exert a tonic modulatory role.

Our findings indicate that 5-HT excites DMV neurones directly by antagonizing the afterhyperpolarization that follows a single action potential. This decrease in the afterhyperpolarization amplitude is observed despite a significant increase in the action potential duration, suggesting a direct effect of 5-HT on the calcium-activated potassium conductance (gK-Ca) that underlies the afterhyperpolarization. Similarly, direct inhibition of gK-Ca has been observed in hippocampal neurones, although in that preparation the effect was mediated by 5-HT_4 receptors (Andrade & Chaput, 1991a; Torres *et al*., 1996). The data showing an increase in the duration of the action potential duration would, by consequence, exclude the possibility that the 5-HT mediated reduction in the after hyperpolarization is secondary to calcium current inhibition, an effect recently reported in medullary raphe neurones by activation of 5-HT_{1A} receptors (Bayliss *et al*., 1997).

In this study we report that the majority (i.e. 69%) of intestinal-projecting DMV neurones are excited by exogenous 5-HT while only 47% of gastric-projecting neurones are affected by 5-HT. To the best of our knowledge, there are no published studies that have investigated the effects on intestinal functions of microinjections of 5-HT in the DVC. Conversely, several lines of evidence indicate that microinjections of 5-HT (in combination with TRH) in the DVC affect mainly gastric acid secretion (McTigue *et al*., 1992; Krowicki & Hornby, 1995; Yoneda & Tache, 1995; Chi *et al*., 1996; Varanasi *et al*., 1997). Based on our data reporting a low percentage of DMV gastric-projecting neurones being affected by 5-HT, it is tempting to speculate that gastric-projecting DMV neurones that respond to 5-HT are indeed the ones involved in gastric-acid secretion. Should this speculation be confirmed by *in vivo* experiments, it would further substantiate our previously formulated hypothesis that DMV comprises heterogeneous neuronal populations

that can be distinguished based on their physiological and pharmacological characteristics (Browning *et al*., 1999).

Presynaptic effects of 5-HT

Within the DVC, the NTS provides a robust inhibitory input into the DMV. This prompted us to investigate whether 5-HT had a modulatory effect on evoked inhibitory postsynaptic currents (IPSCs). We thus focused our attention on the inhibitory synaptic connections between the cenNTS and gastric-projecting neurones and on the connections between the comNTS and intestinal-projecting neurones. Our data suggest that the 5-HT-receptor mediated inhibition of IPSC occurs *via* activation of presynaptic 5-HT_{1A} receptors. Our findings are supported by multiple lines of evidence: (i) Perfusion with 5-HT decreased the peak amplitude of the evoked outward current with a concomitant alteration of the paired pulses ratio, and modified the frequency of both spontaneous IPSC and EPSC, both indications of a decreased probability of transmitter release at the presynaptic site (Travagli & Williams, 1996; Bertolino *et al*., 1997; Li & Bayliss, 1998); (ii) The 5-HT_{1A} receptor selective agonist 8-OH-DPAT mimicked the inhibition of 5-HT, in a manner similar to that described previously as a selective 5-HT_{1A} effect (Bobker & Williams, 1993); (iii) The 5-HT receptor-mediated inhibition of the evoked IPSC was mimicked by the 5-$HT_{1A/1B}$ receptor agonist TFMPP, the selective 5-HT_{1A} receptor antagonists NAN-190 and pindobind-5-HT_{1A} attenuated the effects of both 5-HT and TFMPP; (iv) perfusion with 5-HT did not alter the amplitude of spontaneous IPSCs, indicating that they did not affect postsynaptic GABA receptor sensitivity; (v) The clear pharmacological dichotomy between presynaptic (5-HT_{1A}) and postsynaptic (5-HT_2) receptors involved in the 5-HT mediated effects argues against a possible change in the passive properties of the neurones as a reason for IPSC inhibition.

Our data indicating that activation of 5-HT_{1A} receptors in NTS decreases the inhibitory GABAergic input onto DMV neurones suggests that the increase in gastric acid secretion observed by Stephens' group upon administration of the 5-HT_{1A} receptor agonist 5-CT (in combination with TRH) might be due to presynaptic disnhiibition (Varanasi *et al*., 1997), a possibility we put forth recently (Travagli & Gillis, 1995).

In conclusion, our data suggest that DMV neurones are functionally non-homogenous with regard to their serotonergic modulation and indicate that the motoneurones controlling intestinal functions are more likely to be affected by 5-HT than gastric-projecting neurones.

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