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Characterization of the receptors involved in the 5-HT-induced excitation of canine antral longitudinal muscle

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1 We aimed to characterize the 5-HT receptors involved in the 5-HT-induced effect on electrically induced contractions of dog antrum longitudinal muscle *in vitro*.

2 In the presence of L-NOARG (0.1 mM), electrical field stimulation (EFS) induced atropine- and tetrodotoxin-sensitive contractions. Tetrodotoxin or atropine left any agonist tested ineffective. These EFS-induced contractions were on average enhanced by 5-HT (0.3 μM), however, pronounced variation in the response to 5-HT was observed. There were non-significant trends of the selective 5-HT_3 receptor antagonist granisetron (1 μM), and methysergide (1 μM; preventing interactions of 5-HT with 5-HT_1, 5-HT_2, 5-ht_5, 5-HT_6 and 5-HT_7 receptors) to increase the response to 5-HT. The selective 5-HT_4 receptor antagonist GR 113808 (0.1 μM) displayed a non-significant trend to inhibit the 5-HT-induced increase.

3 Combination experiments with methysergide (1 μM), granisetron (1 μM) and GR 113808 (0.1 μM) revealed that the 5-HT (0.3 μM)-induced response consisted of (1) an excitatory component blocked by GR 113808, (2) excitatory and inhibitory components both blocked by methysergide.

4 The selective 5-HT_4 receptor agonist prucalopride (0.3 μM) increased EFS-induced contractions, an effect prevented by GR 113808 (0.1 μM).

5 The increase of EFS-induced contractions by the preferential 5-HT_2 receptor agonist α-Me-5-HT (0.3 μM) was antagonized by 5-HT_{2B} receptor antagonists.

6 The 5-HT_1/5-HT_7 receptor agonist 5-carboxamidotryptamine (5-CT; 0.3 μM) inhibited EFS-induced contractions. This was prevented by methysergide (1 μM), the 5-HT_7 receptor antagonist mesulergine (0.3 μM) and the selective 5-HT_7 receptor antagonist SB-269970 (0.3 μM).

7 In the presence of GR 113808 (0.1 μM), α-Me-5-HT (1 μM) increased EFS-induced contractions. The 5-HT (0.3 μM)-induced inhibition of the stimulation by α-Me-5-HT was prevented by SB-269970 (0.3 μM).

8 In conclusion, dog antral longitudinal muscle is endowed with (1) excitatory neuronal 5-HT_4 receptors and 5-HT_{2B} receptors and (2) inhibitory smooth muscle 5-HT_7 receptors.
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Abbreviations: α-Me-5-HT, α-methyl-5-hydroxytryptamine; 5-CT, 5-carboxamido-tryptamine; EFS electrical field stimulation; GR, GR 113808 (0.1 μM); GRAN, granisetron (1 μM); 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; MET, methysergide (1 μM); TTX, tetrodotoxin

Introduction

The incentive to study involvement of 5-HT receptors in contractile responses of dog isolated stomach tissue was given by results obtained in *in vivo* studies. It was shown that using a Heidenhain pouch set-up, 5-HT administered intravenously induced a contraction that could be prevented by the selective 5-HT_4 receptor antagonist SB 204070 (Bingham *et al*., 1995). Further, compounds with agonistic properties at 5-HT_4 receptors were found to induce an atropine-sensitive gastric contraction (Bermudez *et al*., 1990), and to increase gastric emptying, a process associated with enhanced co-ordinated contractile responses (Gullikson *et al*., 1993). Presence of other 5-HT receptors in the gastric area cannot be excluded, as methysergide (moderate to high affinity for every 5-HT receptor except for the 5-HT_3 and 5-HT_4 receptor) and ketanserin (selective 5-HT_{2A} receptor antagonist) induced in some dogs an ambiguous alteration of gastric contractility. In this manner, these antagonists evoked sometimes an inhibition, but most prominently an increase in contractility was observed (Bingham *et al*., 1995). This study also reported variable effects after administering the selective 5-HT_3 receptor antagonist granisetron. In summary, a mixture of various 5-HT receptor-mediated responses may be observed in studies on dog stomach.

A single *in vitro* study has been performed on the involvement of 5-HT in canine antral contractility using longitudinal muscle strips, where 5-HT induced an increase in electrical field stimulation (EFS)-induced contraction (de Ridder & Schuurkes, 1993). This amplification could not be

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blocked by separate addition of 5-HT_1, 5-HT_2, 5-HT_3 or 5-HT_4 receptor antagonists (ketanserin, methiothepin, granisetron or tropisetron). Nevertheless, in unpublished experiments using this bioassay, an inhibition of the 5-HT-evoked increase in contraction after selective 5-HT_4 receptor antagonism by SB 204070 (10 nM) was observed (mentioned in Briejer *et al*., 1995), however, this effect was not significant. This may be explained by either this tissue simply does not exhibit 5-HT_4 receptors, or other 5-HT receptors in addition to 5-HT_4 receptors mediate enhancement of contractions. Interestingly, the experiment to explore this – testing the effect of a selective 5-HT_4 receptor antagonist on the 5-HT- induced effect under blockade of every 5-HT receptor, with the exception of 5-HT_4 receptor – was not performed. Moreover, in view of the observed clear stimulation of antral contractility by a 5-HT_4 receptor agonist (Bermudez *et al*., 1990), the clear modulatory role that 5-HT_4 receptors play in canine gastric emptying (Bermudez *et al*., 1990; Briejer *et al*., 1998) and the recent demonstration of 5-HT_4 receptors present on cholinergic nerves in gastric corpus longitudinal and circular muscle (Prins *et al*., 2001), we hypothesized that these receptors should be demonstrable also in the antrum *in vitro*.

This discrepancy between the *in vitro* and *in vivo* results prompted us to re-investigate *in vitro* the effect of 5-HT on canine antral motility. Using the same tissue but a slightly modified bioassay as compared to that reported by de Ridder & Schuurkes (1993), we performed cross-antagonism experiments to explore possible heterogeneity of 5-HT receptors and studied the effects of some tryptamine analogues and selective 5-HT receptor agonists.

Methods

Preparation

Beagle dogs (5–12 kg) of both sexes were used. They were decerebrated and, subsequently, exsanguinated through the carotid artery. The stomach was excised and cut open along the greater curvature. Luminal contents were rinsed out with De Jalon solution (composition in mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.8) and the mucosa and adhering omentum were removed. From the antropyloric canal 1 cm proximal to the pylorus, eight longitudinal muscle strips of approximately 2–3 cm length and 2–3 mm width were cut and anchored to organ bath hooks and suspended in a classical organ bath set-up for isotonic measurement (2 g load). The 20 ml organ baths were filled with De Jalon solution, kept at 37°C and gassed with carbogen (95% O_2, 5% CO_2).

Experimental protocol:

After a 30-min period of stabilization, the strips were contracted with carbachol (10 μM) to test their viability and responsiveness. After wash-out, L-NOARG (0.1 mM) was added to the organ bath solution in order to prevent relaxation due to nitric oxide. After 30 min of incubation, the muscle strips were electrically stimulated (initial parameters: 1-ms pulses in trains of 10 s, at an interval of 3 min, at 20 Hz and 20 V), where each pulse train resulted in a contraction. After 4–5 consecutive pulse trains, contractions were maximal. The voltage was then reduced until reproducible contractions approximating 30–50% of the contraction observed at 20 V was obtained. The current measured ranged from 1.0 to 2.5 A at 20 V (response always maximal) and from 0.25 to 0.45 A at reduced voltage (3–8 V). This was allowed for at least 15 min, followed by addition of treatment, which, in turn, was left to incubate for 15 min, while EFS was continued. Then, a single-dose of agonist was added to each organ bath under continued electrical stimulation and the response was followed for another 15 min. Each muscle strip was used to test the effect of one treatment on the effect of a single-dose of agonist. Treatments included solvents, single antagonists or various combinations of antagonists.

Data analysis

For each individual muscle strip, the average contraction to 5 EFS pulse trains before addition of treatment or solvent was taken as 100% (called the initial value) and all contractions of this strip were related to this initial value. Repeated measures over time were analysed using PROC MIXED (SAS v PC 6.12) for data with an unbalanced covariance structure. A level of $P<0.05$ was considered to be significant and the number of dogs used was denoted by n.

Compounds

The following compounds were used (with their pharmaceutical names and respective suppliers given in parentheses): [1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate (GR 113808), 4-amino-5-chloro-2,3-dihydro-N-(1-[3-methoxypropyl]-4-piperidinyl)-7-benzofurancarboxamide HCl (prucalopride; R093877), ((R)-3-(2-(2-(4-methyl-piperidin-1-yl)ethyl)-pyrrolidine-1-sulphonyl)-phenol; SB-269970), granisetron HCl, ketanserin tartrate, 2-methyl-5-HT, m-chlorophenylbiguanide HCl, mesulergine HCl, N-(1-methyl-5-indolyl)-N′-(3-methyl-5-isothiazolyl)urea (SB-204741) (Janssen Research Foundation, Belgium), atropine sulphate, carbachol, (Janssen Chimica, Belgium), tetrodotoxin, 5-HT creatinine sulphate, (Serva, Germany), methysergide maleate (RBI, USA), α-methyl-5-HT, 5-carboxamidotryptamine (5-CT; Tocris Cookson, U.K.).

All compounds were dissolved in 0.9% NaCl solution, except for GR 113808, which was dissolved in 0.9% NaCl acidified with tartaric acid in the stock solution. Solutions were prepared freshly on the day of the experiment and all dilutions were made using 0.9% NaCl solution. The solvents did not affect EFS-induced contractions.

Results

Bioassay characteristics

Carbachol (10 μM) induced a rapid contraction, and, after wash-out, the muscle strips returned to basal muscle length within 5 min. The muscle strips were not spontaneously active during the experiment, although addition of α-Me-5-HT or 5-HT, but not prucalopride, 2-Me-5-HT or 5-CT, resulted sometimes in subordinate spontaneous short-lasting

contractility (roughly $<10\%$ of contraction to maximal stimulation). This effect of α-Me-5-HT or 5-HT was not influenced by any antagonist or tetrodotoxin (TTX). As the focus of this study was to characterize the effect of agonists on neuronal EFS-induced contractions, these effects were not further studied. Each electrical pulse train in the presence of L-NOARG (0.1 mM) resulted in a contraction with appreciable reproducibility, although decay of the contractile response was observed in time-matched control experiments ($1.9\pm0.4\%$ per pulse train, $n=26$ dogs, depicted in most figures showing mean results). Atropine and tetrodotoxin prevented EFS-induced contractions, therefore, these were entirely due to stimulation of cholinergic nerves followed by release of acetylcholine stimulating muscarinic cholinoceptors.

Experiments with 5-HT

Pre-liminary experiments revealed that mainly excitatory responses to 5-HT were observed at low concentrations (3 nM: increase of initial value by $17\pm5\%$, 30 nM: $110\pm51\%$, $n=6$). 5-HT (0.3 μM) induced excitatory as well as inhibitory responses, whereas at a high concentration of 5-HT (i.e. 3 μM), inhibitory responses pre-dominated ($48\pm27\%$ inhibition of initial value, $n=6$). We chose 5-HT (0.3 μM) to perform further experimentation with, since at this concentration the heterogenous nature of the 5-HT-induced effect was most pronounced. This could be particularly helpful in testing the hypothesis that multiple mechanisms are involved in the 5-HT-induced response in this bioassay. Single-dose addition of 5-HT was preferred above cumulative addition since time-dependent decay of EFS-evoked contractions would limit reproducibility of the 5-HT curve.

The effect of 5-HT (0.3 μM) on EFS-evoked contractions was subject to pronounced across-animal variation. In this manner, inhibition, stimulation, a biphasic response consisting of initial stimulation followed by inhibition as well as no response to 5-HT was observed (individual data presented in Figure 1A). The mean response was significant stimulation (average maximal increase by $53\pm10\%$; Figure 1B). In the presence of methysergide (1 μM), 5-HT induced enhancement of EFS-induced contractions only. In the presence of the selective 5-HT_3 receptor antagonist granisetron (1 μM), 5-HT induced a stimulation in six out of seven antral preparations and was ineffective in one. However, these tendencies of methysergide (1 μM) and granisetron (1 μM) to augment the 5-HT-evoked effect (increases by $70\pm26\%$ and $58\pm15\%$, respectively) were not significant. Atropine or tetrodotoxin blocked EFS-evoked contractions, in their presence 5-HT induced no effect (tetrodotoxin) or a marginal, non-significant, re-appearance of contractions (atropine).

Pre-incubation with the selective 5-HT_4 receptor antagonist GR 113808 (0.1 μM) did not modify the heterogenous nature of the 5-HT-evoked response, as an inhibition (in 1/7 dog preparations), no effect (in 1/7 preparations) as well as an increase (in 5/7 preparations) was observed (mean response: Figure 2A). On average, GR 113808 tended to inhibit the 5-HT-induced response ($22\pm13\%$), but this effect was not significant. The significant increase in EFS-evoked contractions by 5-HT that was still observed in the presence of methysergide (1 μM; Figure 2B), or granisetron (1 μM; Figure 2C), or methysergide (1 μM) plus granisetron (1 μM; Figure 2D), was antagonized by GR 113808 (0.1 μM). This suggested

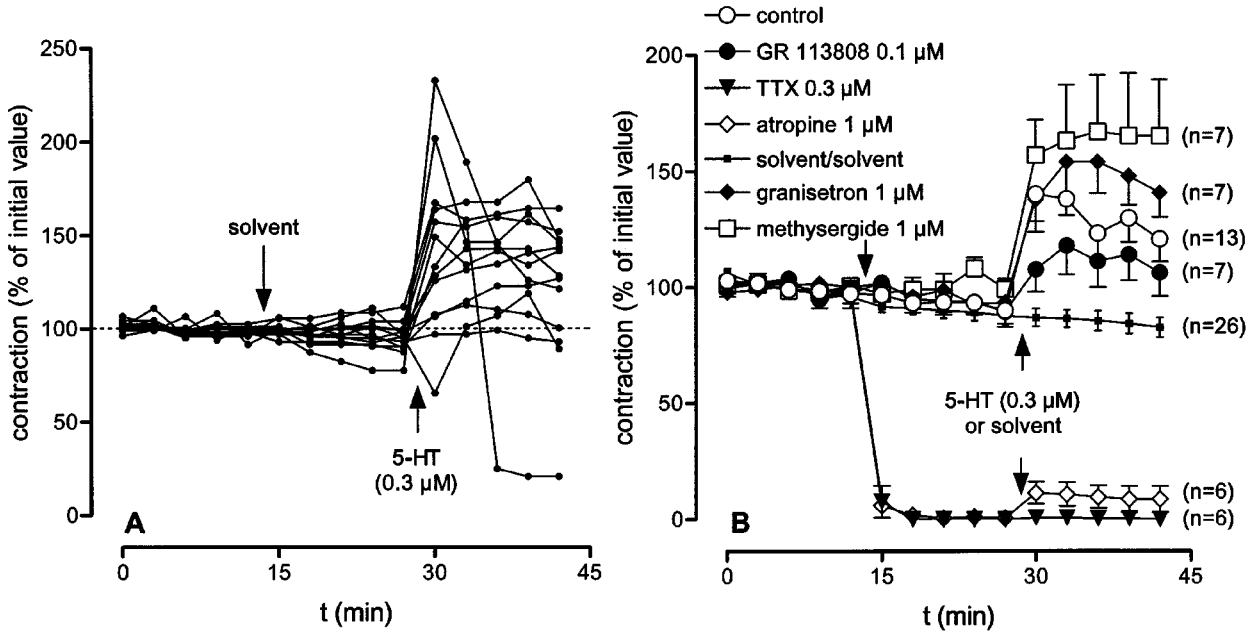

Figure 1 (A) The effect of 5-HT on electrical field stimulation-induced contractions of canine antral longitudinal muscle. Data points represent $n=13$ individual experiments, and are within one dog specimen connected by a line. Experiments were carried out in the presence of L-NOARG (0.1 mM). (B) Mean effect of pre-incubation with methysergide, granisetron, GR 113808, tetrodotoxin (TTX) and atropine on the 5-HT-induced effect on electrical field stimulation-evoked contraction of dog isolated antrum longitudinal muscle. Experiments were carried out in the presence of L-NOARG (0.1 mM). Data points are presented as mean contraction ± s.e.mean, calculated as percentage of the mean contraction to 5 EFS pulse trains immediately prior to any addition of compound (initial value).

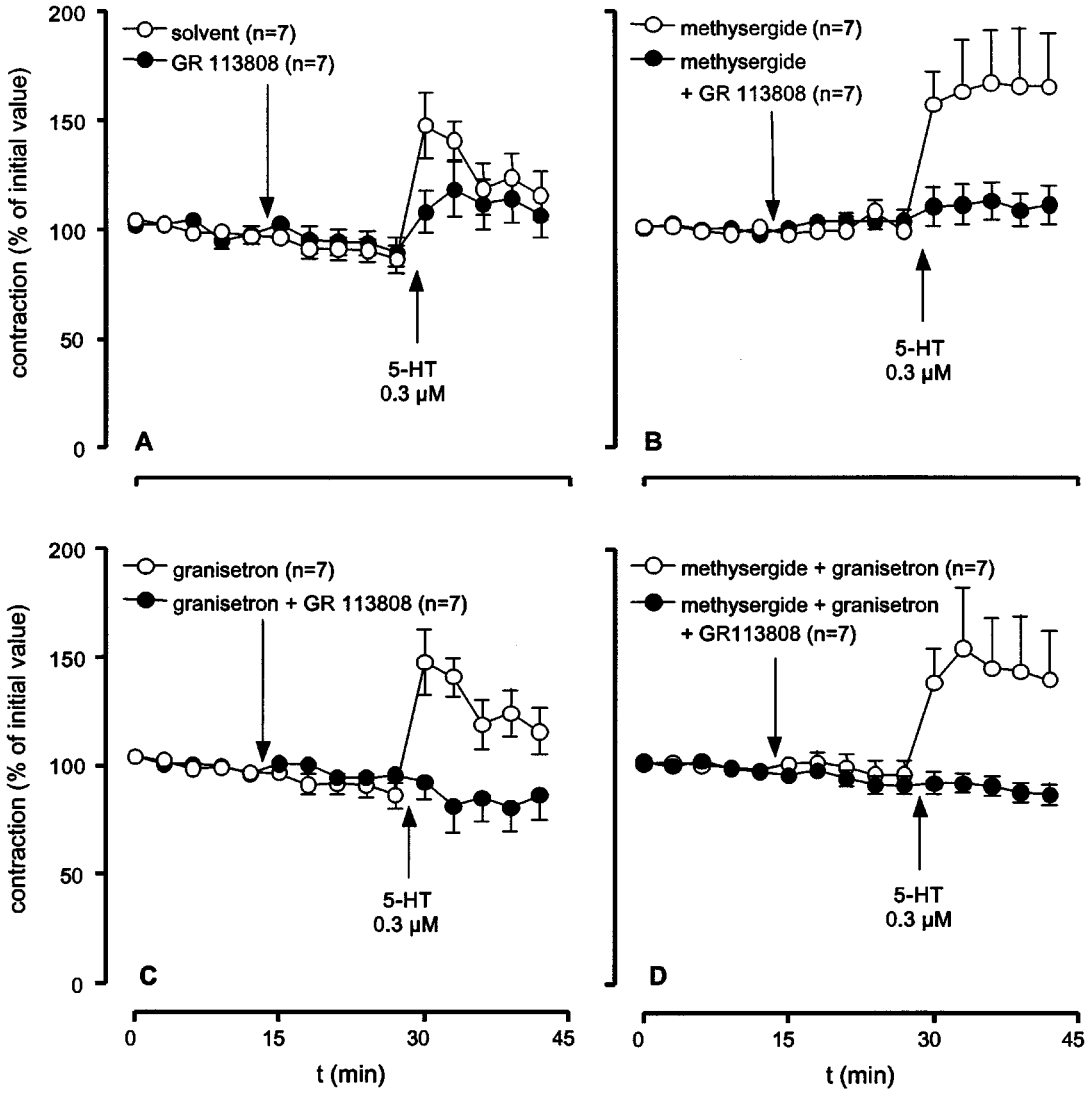

Figure 2 Mean effect of pre-incubation with GR 113808 (0.1 μM) on the effect of 5-HT on electrical field stimulation-induced contractions of canine antral longitudinal muscle in solvent conditions (A), in the presence of methysergide (1 μM) (B), in the presence of granisetron (1 μM) (C), or in the presence of granisetron (1 μM) plus methysergide (1 μM) (D). All experiments were carried out in the presence of L-NOARG (0.1 mM). Data points are presented as mean contraction $\pm$s.e.mean, calculated as percentage of the mean contraction to 5 EFS pulse trains immediately prior to any addition of compound (initial value).

that in the presence of methysergide and granisetron, the 5-HT-induced effect was 5-HT_4 receptor-mediated.

When granisetron (1 μM) plus GR 113808 (0.1 μM) were included, 5-HT caused an inhibition in 3/7 dog preparations, a stimulation in one preparation, whereas 5-HT was without effect in the other three (individual responses: Figure 3A). No effect to 5-HT was observed after inclusion of methysergide (1 μM; Figure 3B). Thus, under the conditions applied, methysergide prevented a heterogenous response to 5-HT.

Up to this point, these data suggested that the 5-HT-induced increase of EFS-induced contraction is the net result of activation of a heterogenous (stimulatory and inhibitory) 5-HT receptor population, of which one population with excitatory characteristics resembles the 5-HT_4 receptor. To characterize which 5-HT receptors are involved, we applied in further experiments tryptamine analogues and selective agonists in combination with antagonists.

Exploration of 5-HT_4 receptor involvement

The selective 5-HT_4 receptor agonist prucalopride increased contractions elicited by EFS (64$\pm$21%, $P<0.05$ vs initial value; Figure 4). The selective 5-HT_4 receptor antagonist GR 113808 (0.1 μM) antagonized this effect to prucalopride. In the presence of atropine or tetrodotoxin the EFS-contractions were blocked, leaving prucalopride without effect.

Exploration of 5-HT_3 receptor involvement

2-methyl-5-HT (2-Me-5-HT), a preferential 5-HT_3 receptor agonist, did not affect EFS-evoked contractions ($n=5$; results not shown). In preparations of two dogs, the 5-HT_3 receptor agonist m-chlorophenylbiguanide (1 μM) was tested, but this failed to affect the contractions elicited by EFS as well.

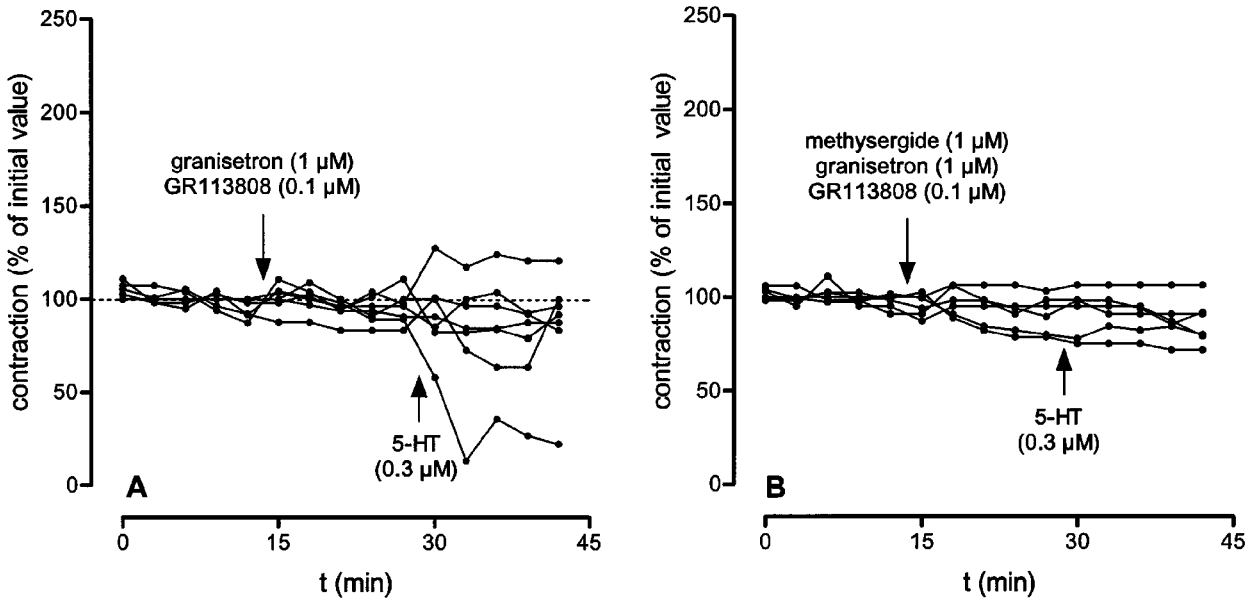

Figure 3 The effect of pre-incubation with granisetron and GR 113808 (A) and further inclusion of methysergide (B) on the effect of 5-HT on electrical field stimulation-induced contractions of canine antral longitudinal muscle. Data points represent $n=7$ individual experiments, and are within one dog specimen connected by a line. All experiments were carried out in the presence of L-NOARG (0.1 mM).

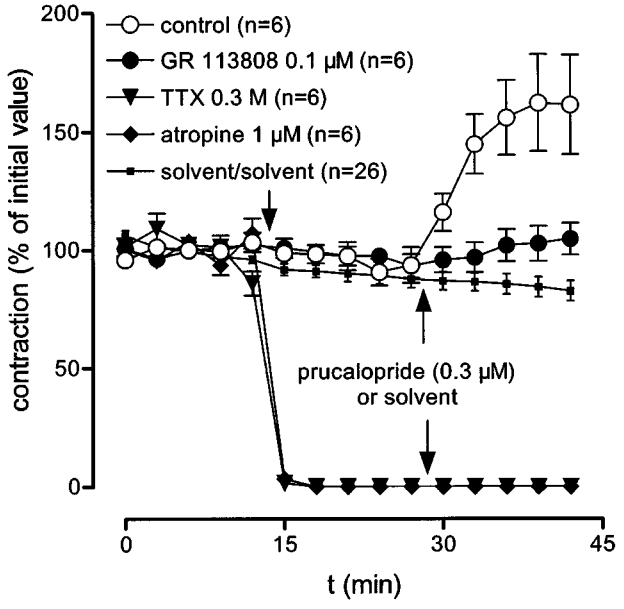

Figure 4 Mean effect of pre-incubation with tetrodotoxin (TTX), atropine or GR 113808 on the effect induced by prucalopride on electrical field stimulation-evoked contractions of dog isolated antrum longitudinal muscle. Experiments were carried out in the presence of L-NOARG (0.1 mM). Data points are presented as mean contraction ± s.e.mean, calculated as percentage of the mean contraction to 5 EFS pulse trains immediately prior to any addition of compound (initial value).

Exploration of 5-HT$_2$ receptor involvement

The preferential 5-HT$_2$ receptor agonist α-methyl-5-HT (α-Me-5-HT) induced a marked increase in contractions elicited by EFS (74 ± 14%, $P<0.05$ vs initial value; Figure 5). The selective 5-HT$_{2A}$ receptor antagonist ketanserin (0.1 μM) increased the EFS-induced contractions (increase by 24 ± 7%, $P<0.05$ vs initial value) and additionally, failed to antagonize the effect to α-Me-5-HT (increase of 55 ± 11%, $P>0.05$ vs control effect of α-Me-5-HT). Ketanserin (10 nM) did not increase EFS-evoked contractions and also failed to antagonize the response to α-Me-5-HT. Addition of the 5-HT$_2$ and 5-HT$_7$ receptor antagonist mesulergine, the 5-HT$_1$, 5-HT$_2$, 5-HT$_5$, 5-HT$_6$ and 5-HT$_7$ receptor antagonist methysergide (1 μM) or the selective 5-HT$_{2B}$ receptor antagonist SB-204741 (0.3 μM) did not modify the EFS-induced contractions, but antagonized the increase to α-Me-5-HT (increase by 19 ± 12, 15 ± 10 and 17 ± 6%, respectively, all $P<0.05$ vs control effect of α-Me-5-HT). Inclusion of tetrodotoxin blocked the EFS-contractions leaving α-Me-5-HT ineffective. In the presence of atropine, the EFS contractions were blocked (−97 ± 2% inhibition of initial value), but addition of α-Me-5-HT resulted in a re-appearance of contractions upon EFS in 3/6 dogs (average effect 26 ± 15% of initial value). Similar to that observed with 5-HT (Figure 1B), this effect was not significant.

Investigation of 5-HT$_1$ or 5-HT$_7$ receptor involvement

5-carboxamidotryptamine (5-CT; 0.3 μM), preferentially stimulating 5-HT$_1$ and 5-HT$_7$ receptors, induced an inhibition of EFS-induced contraction (−91 ± 4%; $P<0.05$ vs initial value; Figure 6). In the presence of methysergide (1 μM), that in 4/6 dog preparations marginally augmented the contractions to EFS (average effect 20 ± 8%, $P<0.05$), 5-CT induced stimulation in all dogs tested. The methysergide-induced contraction increments in this experimental group of dogs contrasted with the non-significant effect of methysergide on EFS-induced contractions in the experimental groups where either 5-HT or α-Me-5-HT was the agonist. The 5-HT$_2$ and

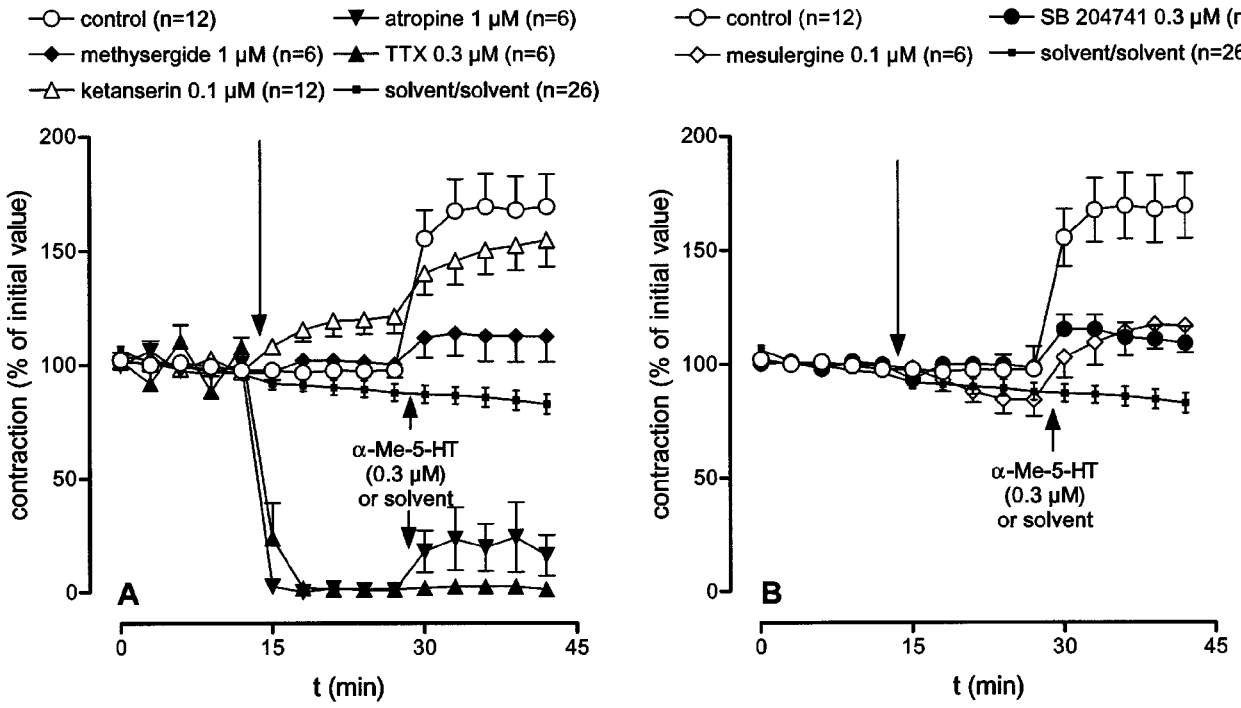

Figure 5 (A) Mean effect of pre-incubation with tetrodotoxin (TTX), atropine, methysergide and ketanserin on the effect induced by α-methyl-5-HT (α-Me-5-HT) on electrical field stimulation-evoked contractions of dog isolated antrum longitudinal muscle. (B) Mean effect of pre-incubation with mesulergine and SB-204741 on the α-Me-5-HT-induced effect on electrical field stimulation-evoked contraction of dog isolated antrum longitudinal muscle. All experiments were carried out in the presence of L-NOARG (0.1 mM). Data points represent mean contraction ± s.e.mean, calculated as percentage of the mean contraction to 5 EFS pulse trains immediately prior to any addition of compound (initial value).

5-HT_7 receptor antagonist mesulergine (0.3 μM) prevented any effect of 5-CT (0.3 μM), as illustrated by the EFS-induced contractions in this treatment group being nearly superimposed on those in the solvent – solvent group. SB-269970 (0.3 μM), a compound reported to be a selective 5-HT_7 receptor antagonist (Hagan *et al*., 2000) left 5-CT (0.3 μM) ineffective.

Under 5-HT_4 receptor blockade by GR 113808 (0.1 μM), α-Me-5-HT (1 μM) induced an increase in EFS-evoked contractions (Figure 7). 5-HT induced an inhibition of these contractions, an effect prevented by SB-269970 (0.3 μM).

In order to characterize the nature of the agonist-induced effect on EFS-induced contractions, the agonist effect on cumulative concentration – contraction curves to acetylcholine was assessed. The acetylcholine curves were non-monophasic and did not reach their maximal response up to 100 μM, hence, curve fitting was not performed. Pre-treatment with 5-HT (0.3 μM), prucalopride (0.3 μM) or α-Me-5-HT (0.3 μM) did not modify the curve to exogenously added acetylcholine. On average, the contraction to acetylcholine was reduced, albeit non-significantly, by 5-CT (0.3 μM) by 26% ($n=4$, results not shown).

Discussion

The data presented here strongly suggest presence of excitatory 5-HT_4 and 5-HT_{2B} receptors and inhibitory 5-HT_7 receptors in dog antral longitudinal muscle. The simultaneous stimulation of this heterogenous 5-HT receptor population by 5-HT may serve as an explanation for the variable 5-HT-evoked responses observed.

The primary indication for a heterogenous receptor population involved in the actions of 5-HT was provided by the first experiment, in which separate incubation of methysergide, granisetron or GR 113808 could not significantly affect the response to 5-HT. These three antagonists together shield interactions of 5-HT with every currently known 5-HT receptor (5-HT_1, 5-HT_2, 5-HT_5, 5-HT_6 and 5-HT_7) receptors by methysergide (Gommeren *et al*., 1998); 5-HT_3 receptors by granisetron (Sanger & Nelson, 1989), 5-HT_4 receptors by GR 113808 (Gale *et al*., 1994), thus absence of antagonism by these compounds may appear indicative of 5-HT activating receptors not belonging to the 5-HT receptor family. However, this suggestion was overruled by the blockade of the response by the combination of these three antagonists. This outcome did also favour the hypothesis that more than one 5-HT receptor mediated the response to 5-HT. The second indication for 5-HT receptor heterogeneity was the observation that the 5-HT-evoked increase was considerably variable in the sub-set of dogs used. The further increase in variation of the response to 5-HT upon 5-HT_4 receptor antagonism by GR 113808, either with or without inclusion of granisetron (blocking 5-HT_3 receptors), also corroborated heterogeneity. In this manner, the varying 5-HT-induced effect at this concentration may be explained by across-animal and/or within-animal variation in expression of both stimulatory and inhibitory 5-HT receptors.

Upon blockade of every 5-HT receptor with the exception of 5-HT_4 receptors (by inclusion of granisetron and

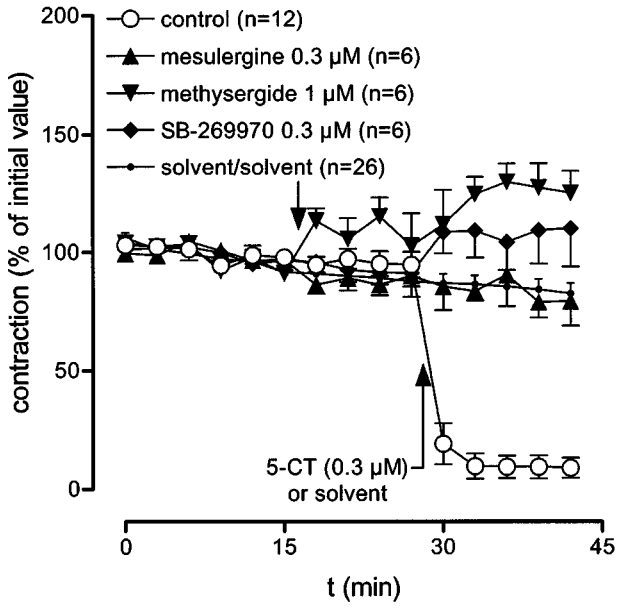

Figure 6 Mean effect of pre-incubation with mesulergine, methysergide and SB269970 on the effect induced by 5-carboxamidotryptamine (5-CT) on electrical field stimulation-evoked contractions of dog isolated antrum longitudinal muscle. All experiments were carried out in the presence of L-NOARG (0.1 mM). Data points are presented as mean contraction ± s.e.mean, calculated as percentage of the mean contraction to 5 EFS pulse trains immediately prior to any addition of compound (initial value).

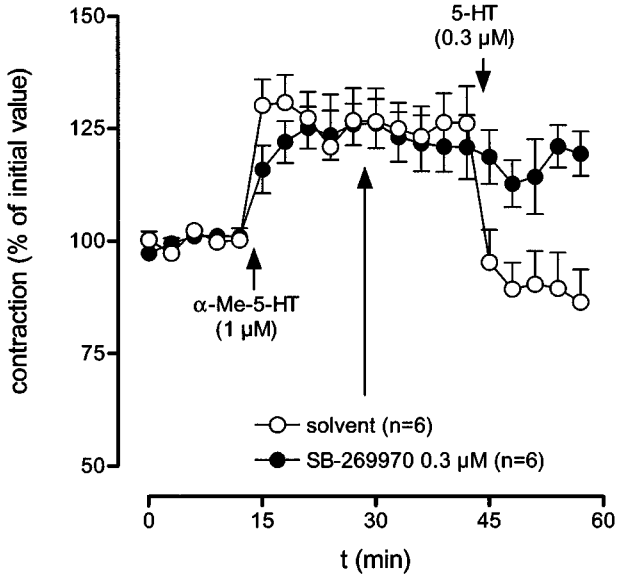

Figure 7 Mean effect of pre-incubation with SB269970 on the effect induced by 5-HT on α-Me-5-HT (1 μM)-enhanced electrical field stimulation-evoked contractions of dog isolated antrum longitudinal muscle. All experiments were carried out in the presence of GR 113808 (0.1 μM) and L-NOARG (0.1 mM). Data points are presented as mean contraction ± s.e.mean, calculated as percentage of the mean contraction to 5 EFS pulse trains immediately prior to any addition of compound (initial value).

methysergide), the remaining potentiating effect of 5-HT was blocked by addition of GR 113808, suggesting involvement of excitatory 5-HT_4 receptors. Indeed, the markedly increased EFS-evoked contractions due to the selective 5-HT_4 receptor agonist prucalopride were also antagonized by GR 113808, hereby emphasizing that indeed 5-HT_4 receptors are involved. In line with that observed in the dog corpus *in vitro* (Prins *et al*., 2001), the absence of effect of either 5-HT or prucalopride in the presence of atropine or tetrodotoxin, suggested localization of excitatory 5-HT_4 receptors on cholinergic nerves. This demonstration of excitatory 5-HT_4 receptors on antral cholinergic fibres of the dog, a phenomenon already described for humans (Schuurkes *et al*., 1991) and guinea-pigs (Takada *et al*., 1999), suggests that dogs may not be that different with respect to 5-HT_4 receptor-mediated antral motility as previously suggested (de Ridder & Schuurkes, 1993). The latter study suggested the 5-HT-induced antral muscle excitation was not due to 5-HT_4 receptor stimulation.

In the presence of GR 113808 and granisetron, there was still a variable response to 5-HT. As additional pre-incubation with methysergide blocked any response to 5-HT, this outcome was suggestive for a methysergide-sensitive mechanism of 5-HT-evoked inhibition and stimulation. The presence of an inhibitory 5-HT receptor was stressed by the experiments with the preferential 5-HT_1/5-HT_7 receptor agonist 5-CT. 5-CT induced an inhibition that was prevented by methysergide, that for this particular experiment was used to block 5-HT_1 and 5-HT_7 receptors. This implies that methysergide can indeed be utilized to prevent 5-HT from stimulating the inhibitory 5-HT receptor in this preparation. This was corroborated by the effect of 5-HT in the presence of methysergide, in which no 5-HT-induced inhibition was observed. The antagonism of the 5-CT response by mesulergine, a 5-HT_7 receptor antagonist that lacks relevant affinity at 5-HT_1 receptors, suggested involvement of 5-HT_7 receptors. The selective 5-HT_7 receptor antagonist SB-269970 (0.3 μM; Lovell *et al*., 2000) prevented the inhibitory effects due to 5-CT, evidently demonstrating that this effect by 5-CT was indeed mediated by 5-HT_7 receptors. Moreover, the inhibition to 5-HT under combined 5-HT_4 receptor blockade and firm 5-HT_2 receptor stimulation was prevented by SB-269970. This emphasized 5-HT_7 receptor involvement and demonstrated that 5-HT stimulates these receptors at 0.3 μM.

It is highly likely that these 5-HT_7 receptors are located directly on the smooth muscle. In a previous study using the same tissue, the 5-HT- or 5-CT-induced relaxation of methacholine-evoked precontraction were both insensitive to TTX but antagonized by mesulergine (Prins *et al*., 1999). 5-HT_7 receptors are positively coupled to adenylate cyclase (Bard *et al*., 1993), hence a location on smooth muscle is in full agreement with a relaxation. As in this study the EFS-induced contractions were observed on a stable basal muscle length, a direct relaxation was not observed. Thus, smooth muscle 5-HT_7 receptor-mediated relaxation presumably functionally antagonized EFS-evoked contraction. This is also corroborated by the reduced contractions to exogenously added acetylcholine after 5-CT (0.3 μM) addition.

As indicated above, under 5-HT_4 receptor blockade, there was still a heterogenous response to 5-HT to be observed. The inhibitory component was accounted for by smooth muscle 5-HT_7 receptors. The remaining stimulatory response to 5-HT might involve 5-HT_2 receptors, as suggested by the marked contraction increase due to the preferential 5-HT_2 receptor agonist α-Me-5-HT. This effect was mediated by

receptors located on nerves, as demonstrated by the lack of effect of α-Me-5-HT in the presence of TTX. In the presence of atropine, contractions were blocked, but the subsequent re-appearance of contractions after α-Me-5-HT was remarkable and also seen to some extent with 5-HT, but not with the other agonists tested. Although this effect by either α-Me-5-HT or 5-HT was not significant, it may suggest that either the increase of acetylcholine release due to these agonists was so great, that it partly surmounted the antagonism of muscarinic cholinoceptors by atropine (1 μM), or that 5-HT_2 receptors are located on primarily cholinergic but to some extent also on non-cholinergic fibres. Alternatively, a non-5-HT-receptor mechanism may be involved. Involvement of 5-HT_{2A} receptors in the α-Me-5-HT-evoked contraction increase is ruled out by the failure of ketanserin at selective 5-HT_{2A} receptor concentrations to antagonize the response to α-Me-5-HT. Antagonism observed with the selective 5-HT_{2B} receptor antagonist SB-204741 (0.3 μM; Forbes *et al*., 1995), and the 5-HT_2 receptor antagonists methysergide (1 μM) and mesulergine (0.1 μM) suggested interactions with 5-HT_{2B} receptors.

Interestingly, ketanserin at 0.1 μM induced an increase of contractions in its own right. The observation that methysergide itself increased contractions only in the experiment with 5-CT, and was ineffective in other experiments (i.e. with 5-HT and α-Me-5-HT), suggest that there is across-animal variation in stimulation by these antagonists. We do not have a clear explanation for these observed effects. This property is most likely not related to 5-HT_{2A} receptor antagonism, since ketanserin (10 nM, a concentration that selectively antagonizes dog 5-HT_{2A} receptors; Prins *et al*., 1997) did not increase these contractions. The mechanisms involved, therefore, may be associated with other than 5-HT receptor-related mechanisms. Notwithstanding the putative relevance to stimulation of antral motility, this property did not appear to contribute to elucidation of the action of 5-HT in this bioassay, therefore, it was not further studied.

Involvement of 5-HT_3 receptors in the 5-HT-evoked excitation is unlikely, as the 5-HT_3 receptor agonists 2-Me-5-HT and m-chlorophenylbiguanide did not affect EFS-induced contractions. Nevertheless, 5-HT did induce a subordinate increase in contractions in the presence of methysergide and GR 113808 (blocking every 5-HT receptor except 5-HT_3 receptor) as compared to solvent, and this effect to 5-HT was not observed when granisetron was included as well. It is more likely that the excitation due to activation of 5-HT_{2B} receptor and 5-HT_4 receptors in the muscle strips tested was so strong, that the applied concentrations of methysergide and GR 113808 were insufficient to completely prevent the response to 5-HT. Alternatively, if 5-HT_3 receptors are indeed involved, their response is so weak, that it may be anticipated that they play a role of minor importance in antral longitudinal muscle motility.

In conclusion, this study demonstrates that canine isolated antrum longitudinal muscle exhibits excitatory 5-HT_4 receptors on cholinergic nerves, excitatory 5-HT_{2B} receptors on primarily cholinergic nerves, and inhibitory (smooth muscle) 5-HT_7 receptors. The outcome of this study may seem contradictive to our previous study of this tissue showing that 5-HT-increased EFS-induced contraction cannot be blocked by antagonism of 5-HT_1, 5-HT_2, 5-HT_3 or 5-HT_4 receptors (de Ridder & Schuurkes, 1993). In that study, separate addition of ketanserin (5-HT_{2A} receptor antagonist), methiothepin (5-HT_1, 5-HT_2 and -contemporarily unknown-5-HT_7 receptor antagonist), granisetron (5-HT_3 receptor antagonist) or tropisetron (5-HT_3 and 5-HT_4 receptor antagonist), did not affect the response to 5-HT. That study paralleled our finding that separate addition of antagonists was ineffective. Pharmacologically knocking out 5-HT_4 receptor involvement only, might as such not prevent 5-HT from increasing EFS contractions *via* 5-HT_{2B} receptor activation. *Vice versa*, under 5-HT_{2B} receptor blocking conditions, the 5-HT-induced increase will be taken over by 5-HT_4 receptor activation. Hence, the conclusion that these receptors are not involved in the 5-HT-induced response appears understandable, however, is not true. Additionally, operational 5-HT_7 receptors mediating relaxation and the across- and within-dog variation in 5-HT responses displayed in our study have made the characterization even more difficult. Here, the combination of antagonists and the use of more selective 5-HT receptor ligands at appropriate concentrations, allowed us to characterize the heterogenous 5-HT response in this tissue.

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