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Neurotransmitters mediating the intestinal peristaltic reflex in the mouse

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Abbreviations:

5-HT, 5-hydroxytryptamine, serotonin; CGRP, calcitonin gene-related peptide; DC32-87 (D-Phe-Cys-Tyr-DTrp-Lys-Abu-Cys-Nal-NH_2); DC32-97 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-DNal-NH_2); DC32-92 (D-Phe-Phe-Phe-DTrp-Lys-Thr-Phe-Thr-NH_2); GR113808A (1-[2-methylsulfonylamino)ethyl]-4-piperidinyl)methyl-1methyl-1H-indole3-carboxylate maleate salt); LY278584, (1-methyl-N-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1H-indazole-3-carboxamide maleate); L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NKA, neurokinin A; NKB, neurokinin B; PACAP, pituitary adenylate cyclase-activating peptide; PHI, peptide histidine, isoleucine; PRL-2903, (9H-Fpa-cyclo[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2); SP, substance P; SST, somatostatin; SST3-ODN-8 (carbamoyl-des-$AA^{1,2,4,5,12,13}$[D-Cys^3,Tyr^7,D-Agl^8(Me,2-naph-thyoyl)]-somatostatin-14); SSTR, somatostatin type receptor; VIP, vasoactive intestinal peptide; ELISA, enzyme-linked immunosorbant assay; RIA, radioimmunoassay; IC_{50}, concentration causing 50% inhibition of radioligand binding in RIA.

Key Words: neuropeptides, enteric nervous system, myenteric plexus, colon, gastrointestinal motility, serotonin.

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ABSTRACT

The motor, modulatory, and sensory neurotransmitters that mediate the peristaltic reflex in the mouse colon were identified by direct measurement and their involvement in various pathways determined by selective receptor antagonists. Mucosal stimulation in the central compartment of a three-compartment flat sheet preparation of mouse colon elicited ascending contraction and descending relaxation in the orad and caudad compartments, respectively. Ascending contraction was accompanied by SP release, a marker for excitatory neurotransmitter release, into the orad compartment and was partly inhibited by atropine and spantide, and abolished by a combination of the two antagonists. Descending relaxation was accompanied by VIP release, a marker for inhibitory neurotransmitter release, into the caudad compartment, and was partly inhibited by VIP_{10-28} and L-NNA, and abolished by a combination of the two agents. Somatostatin release increased during descending relaxation: immunoneutralization of somatostatin or blockade of its effect with a selective SSTR2 antagonist inhibited descending relaxation. The δ opioid receptor antagonist, naltrindole, augmented descending relaxation and ascending contraction. CGRP release increased in the central compartment and was mediated by concurrent release of 5-HT since its release was blocked by a $5\text{-}HT_4$ receptor antagonist. Both the latter and the CGRP antagonist, $CGRP_{8-37}$, inhibited ascending contraction and descending relaxation. Thus, the reflex in mouse like that in rat and human intestine is initiated by mucosal release of 5-HT and activation of $5\text{-}HT_4$ receptors on CGRP sensory neurons, and is relayed via somatostatin and opioid interneurons to VIP/NOS inhibitory motor neurons, and via cholinergic interneurons to ACh/tachykinin excitatory motor neurons.

The intestinal peristaltic reflex is one of very few functions where the role of enteric peptide neurotransmitters has been elucidated. Several advances including precise immunocytochemical mapping of enteric neurons, sensitive radioimmunoassay, and the availability of peptide antibodies and receptor antagonists have facilitated the identification of these neurotransmitters and their roles in the regulation of the peristaltic reflex. However, molecular tools, e.g., non-lethal knockout animals, have yet to be used extensively in identifying the neurotransmitters and receptors that mediate the peristaltic reflex, making it essential to develop techniques to characterize the reflex in mice similar to those used successfully in rats and guinea pigs.

The topography of enteric neurons is generally well conserved. In the mouse, as in other mammalian species, a third of the myenteric neurons in the small intestine and colon contain vasoactive intestinal peptide (VIP) and its homologue, *p*eptide *h*istidine *i*soleucine/*m*ethionine (PHI or PHM in humans), which is derived from the same precursor: 75% of these neurons contain NOS, and 50% contain NOS and neuropeptide Y (NPY) (Sang and Young, 1996; Sang et al., 1997). These neurons project caudad either as interneurons or as inhibitory motor neurons innervating circular smooth muscle. Two thirds of myenteric neurons contain acetylcholine (ACh): 50% of these contain the tachykinins, substance P (SP) and neurokinin A (NKA), which are derived from the same precursor (Sang and Young, 1998; Sang and Young, 1996; Sang et al., 1997). Neurons containing ACh and the tachykinins project orad as excitatory motor neurons that innervate both circular and longitudinal smooth muscle. Other cholinergic myenteric neurons act as interneurons in ascending pathways (Sang and Young, 1998). Few enteric neurons, about 1% of the total in the mouse, contain 5-HT (Sang and Young, 1996; Sang

et al., 1997). Somatostatin and opioid neurons synapse mainly with other neurons in the myenteric plexus, consistent with a role for somatostatin and opioid peptides as neuro-neural modulators in various interneuronal pathways (Heinicke and Kiernan, 1990).

The motor limbs of the peristaltic reflex in human, rat, and guinea pig intestine are regulated by excitatory (ACh, SP/NKA) and inhibitory (VIP/PACAP/NOS) motor neurons that act orad and caudad to the site of stimulation (Grider and Makhlouf, 1990). Mechanical or chemical stimuli emanating from the mucosa activate intrinsic sensory neurons that contain calcitonin gene-related peptide (CGRP) (Grider, 1994a; Pan and Gershon, 2000). The sensory neurons relay the stimuli to excitatory and inhibitory motor neurons via a set of coupled interneurons that contain ACh, somatostatin, or [Met]enkephalin (Grider, 1994b). The mucosal stimuli (passage of chyme or acidic pH) that activate the sensory neurons do so indirectly by releasing 5-hydroxytryptamine (5-HT) from intestinal enterochromaffin cells, which, in turn, acts on 5-HT_4 receptors located on sensory nerve terminals, except in the guinea pig where 5-HT acts on both 5-HT_3 and 5-HT_4 receptors (Grider et al., 1996; Foxx-Orenstein et al., 1996; Kadowaki et al., 1996). Indirect evidence based on measurement of intestinal propulsion in the mouse suggests involvement of 5-HT_4 receptors, although some studies have raised the possibility that both 5-HT_3 and 5-HT_4 receptors are involved (Sanger et al., 1998; Nagakura et al., 1997a; Nagakura et al., 1997b; Hegde et al., 1994). None of these pathways has been characterized in the mouse. The present study provides the first detailed analysis of the motor, modulatory, and sensory neurotransmitters that regulate the peristaltic reflex in the mouse, and provides evidence for the selective involvement of 5-HT_4 receptors in mediating the reflex.

METHODS

Measurement of peristaltic reflex in compartmented flat-sheet segments of mouse colon. The peristaltic reflex was measured in a miniaturized three-compartment, flat-sheet preparation of mouse colon similar to that previously described for rat colon (Grider, 1994a; Grider and Jin, 1994). A 5-cm segment of distal colon was opened and pinned mucosal side up in a tissue bath. The segment was divided into three compartments by vertical partitions sealed with vacuum grease and 1 ml of a Krebs-bicarbonate medium was added to each compartment. The dimensions of each compartments was identical: 8 mm deep, 10 mm long and 18 mm wide. The composition of the medium was (in mM) 118 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 NaH_2CO_3, and 11 glucose. In experiments where neuropeptide release was measured, the medium contained also 0.1 % bovine serum albumin, 10 μmol/L amastatin, and 1 μmol/L phosphoramidon. For measurement of 5-HT release, the medium contained in addition 10 μmol/L pargyline.

The peristaltic reflex was initiated by stroking the mucosa with a fine brush (2 to 8 strokes at a rate of 1 stroke/s). Ascending contraction of circular muscle was measured in the orad peripheral compartment and descending relaxation was measured in the caudad peripheral compartment using force-displacement transducers attached to the muscle layers. In separate experiments, various receptor agonists or antagonists, inhibitors, and antibodies were added either to the central compartment or to both peripheral compartments. Those added to both peripheral compartments included the non-specific tachykinin receptor antagonist, spantide; the muscarinic antagonist, atropine;

the VIP receptor antagonist, VIP_{10-28}; the NOS inhibitor, L-NNA; the opioid δ receptor antagonist, naltrindole; the somatostatin type 2 receptor (SSTR2) antagonist; PRL-2903; the SSTR3 antagonist, SST3-ODN-8; the SSTR2 agonist, DC32-87; the SSTR3 agonist, DC32-97; the SSTR4/5 agonist, DC32-92; and SST antibody #775. Those added to the central compartment only included the $5\text{-}HT_3$ receptor antagonist, LY278584; the $5\text{-}HT_4$ receptor antagonist, GR113808A; and the CGRP receptor antagonist, $hCGRP_{8-37}$.

For measurement of neurotransmitter release, the segment was incubated with medium for a 15 min period without stimulation. At the end of this period, the medium was collected from each compartment separately, rapidly frozen, and stored for measurement of basal transmitter release. Fresh medium was added to each compartment and mucosal stimuli (i.e., 4 or 8 strokes) were applied to the central compartment five times, each one applied at a 3-min interval, during a 15 min-period; the medium from the central and both peripheral compartments was collected and frozen for subsequent radioimmunoassay for peptides and ELISA for 5-HT. Release during the 15 min period in which the peristaltic reflex was stimulated 5 times was compared to the release during the 15 min period in which the colon was not stimulated (i.e. the period of basal release). Previous studies in rat, guinea pig and human intestine and colon indicated that this collection paradigm yielded concentrations of neuropeptides and 5-HT in the medium that were in the optimum range for measurement by the assays used in this study.

At the end of the experimental period, the whole colonic segment was divided into the three sections representing the portion in each compartment by cutting along the position of the vertical dividers. The tissues were blotted dry and weighted. The weights in each of the three compartments were similar; the overall mean wet weight of tissue in a

compartment was 85.3±6.1 mg. The tissue wet weight in each compartment was used to normalize the release of neuropeptide and 5-HT into the medium of that compartment.

Measurement of peptide neurotransmitters. SP was measured using antibody RAS 7451 as previously described (Grider, 1989). The limit of detection of the assay was 3 fmol/ml and the IC_{50} was 15±4 fmol/ml of original sample. The antibody reacted with SP but did not cross-react with NKA, NKB, NPY, somatostatin, VIP, or [Met]-enkephalin.

VIP was measured using antibody RAS 7161 as previously described (Grider, 1989). The limit of detection of the assay was 3 fmol/ml and the IC_{50} was 20±3 fmol/ml of original sample. The antibody reacted with VIP but did not cross-react with VIP10-28, PACAP, PHI, secretin, glucagon, NPY, somatostatin, or [Met]-enkephalin.

Somatostatin (SST) was measured using antibody RAS-8001 as previously described (Grider, 1994b). The limit of detection of the assay was 6 fmol/ml and the IC_{50} was 110±12 fmol/ml of original sample. The antibody reacted with SST but did not cross-react with NPY, pancreatic polypeptide, SP, NKA, VIP, or [Met]-enkephalin.

CGRP was measured by radioimmunoassay using antibody RIK 6006 as previously described (Grider, 1994a). The limit of detection of the assay was 3 fmol/ml and the IC_{50} was 43±7 fmol/ml of original sample. The antibody reacted with CGRP but did not cross-react with amylin, calcitonin, SP, NKA, somatostatin, VIP, [Met]-enkephalin, 5-HT, LY278584, or GR13808A.

Measurement of 5-HT. 5-HT was chemically derivatized to N-acetyl-5-HT and measured by ELISA. The limit of detection of the assay was 0.5 nmol/ml and the IC_{50} was 6.1±0.8 nmol/ml. The N-acetyl-5-HT antibody reacted fully with N-acetyl-5-HT but

did not react with 5-hydroxytryptophan, 5-hydroxy-3-indole acetic acid, 5-hydroxytryptophol, melatonin, SP, VIP, CGRP, LY278584, or GR113808A.

Data analysis. Ascending contraction and descending relaxation of circular muscle were measured as grams force and normalized as the percent of the response to a maximal stimulus. The mean overall maximal response from all preparations was 0.68 ± 0.03 g for ascending contraction and 0.46 ± 0.03 for descending relaxation. Stastically significant difference between control responses and responses in the presence and absence of agonists and antagonists was tested by ANOVA followed by Student t tests (GraphPad software, San Diego, CA).

Release of neuropeptide and of 5-HT were measured as concentration (nmol or fmol/ml) in original sample and normalized for the duration of the collection period (15 min) and the wet weight of the colonic tissue in the compartment to yield values as nmol/100mg.min and fmol/100 mg.min. The release was calculated as the amount above or below basal levels measured in the absence of stimulation of the peristaltic reflex. Stastically significant change in release from basal level, and difference between release in the presence and absence of antagonist was tested by ANOVA followed by Student t tests (GraphPad software, San Diego, CA). Values are plotted as percent change from basal levels.

Materials. Spantide, SP, VIP, SST, CGRP, SP antibody RAS 7451, CGRP antibody RIK 6006, VIP antibody RAS 7161, ^{125}I-VIP, ^{125}I-SP, ^{125}I-SST, and ^{125}I-CGRP were purchased from Bachem-Peninsula (Torrance, CA). The 5-HT ELISA kit was purchased from ICN (Costa Mesa, CA). Naltrindole and the 5-HT_3 receptor antagonist, LY278584 were purchased from Research Biochemicals Inc. (Natick, MA).

Somatostatin antiserum #775 was purchased from Dr. A. Arimura (Tulane University New Orleans, LA). Atropine, L-NNA, amastatin, phosphoramidon, pargyline, and all other chemicals and reagents were purchase from Sigma Chemicals (St. Louis, MO). GR113808A was a gift of Drs. G. Kilpatrick and B. Bain (Glaxo-SmithKline Research and Development, (Middlesex, UK). The somatostatin agonists DC32-87, DC32-97, DC32-92 and the SSTR2 antagonist, PRL-2903 were gifts of Dr. David H. Coy, Tulane University, (New Orleans, LA). The SSTR3 antagonist, SST3-ODN-8 was a gift of Dr. Jean Rivier, The Salk Institute, (La Jolla, CA).

RESULTS

Pattern of 5-HT and peptide neurotransmitter release during the peristaltic reflex in the mouse colon. Mucosal stroking in the central compartment elicited stimulus-dependent contraction of circular muscle in the orad compartment (ascending contraction) and relaxation in the caudad compartment (descending relaxation). The phases of the reflex were qualitatively similar to those described for rat and guinea pig colon and for human small intestine (Grider and Makhlouf, 1990; Grider, 1989; Grider and Foxx-Orenstein, 1998). Maximal responses elicited at 8 strokes in mouse colon were 0.68±0.03 g for ascending contraction and 0.46±0.03 g for descending relaxation.

In order to determine the pattern of neurotransmitter release during the peristaltic reflex, basal measurements of release were obtained in each compartment and again after the reflex was triggered by mucosal stroking in the central compartment. There was a precise pattern of release for each neurotransmitter: SP release increased only in the orad compartment in conjunction with ascending contraction (Fig. 1B), and VIP release increased only in the caudad compartment in conjunction with descending relaxation (Fig.2B). Somatostatin release increased in the caudad compartment and decreased in the orad compartment (Fig. 3B). CGRP and 5-HT release increased only in the central compartment (Fig 6).

Neurotransmitters mediating the ascending phase of the peristaltic reflex. Addition of the muscarinic antagonist, atropine, to the orad compartment abolished ascending contraction elicited by 2 strokes and inhibited contraction elicited by 8 strokes by 55±3% ($p<0.001$). In contrast, the tachykinin antagonist, spantide (10 μM), had no effect on ascending contraction elicited by 2 strokes and inhibited contraction elicited by

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8 strokes by 38±2% ($p<0.001$). The combination of atropine and spantide virtually abolished contraction elicited by all levels of stimulation (Fig. 1A). It should be noted that spantide blocks the effects of both SP and NKA, which are synthesized by the same precursor and co-released during stimulation..

Basal release of SP into the three compartments was similar (39±2, 42±4, and 38±2 fmol/100mg^{-1}.min^{-1}). Mucosal stroking in the central compartment caused a stimulus dependent release of SP exclusively into the orad compartment (66±14% and 102±8% increase above basal with 4 and 8 strokes, respectively) (Fig 1B). Addition of atropine and spantide alone or in combination to the caudad compartment had no effect on descending relaxation (data not shown).

Neurotransmitters mediating the descending phase of the peristaltic reflex. Addition of the VIP/PACAP (VPAC) receptor antagonist, VIP_{10-28} (10 μM), to the caudad compartment abolished the response to 2 strokes and inhibited the response to 8 strokes by 52±5% ($p<0.001$). Addition of the NOS inhibitor, L-NNA (100 μM), to the caudad compartment inhibited the response to 2 and 8 strokes by 66±9% and 22±1%, respectively (Fig 2A.). The combination of VIP_{10-28} and L-NNA abolished descending relaxation elicited by 2, 4, and 6 strokes and inhibited the response to 8 strokes by 77±4 % (Fig. 2A), implying participation of NO as well as VIP and its homologues, PHI and PACAP. PHI is co-synthesized and co-released with VIP from the same precursor, PACAP is co-released with VIP from the same or adjacent terminals (Sundler et al., 1992), and all three peptides are blocked by VIP_{10-28} (Katsoulis et al., 1996).

Basal release of VIP was similar in the three compartments (49±5, 41±6, and 46±4 fmol/100mg^{-1}.min^{-1}). Mucosal stroking in the central compartment caused a

stimulus dependent release of VIP exclusively into the caudad compartment (22±7% and 68±9% increase above basal at 4 and 8 strokes, respectively) (Fig. 2B). Addition of VIP_{10-28} and L-NNA alone or in combination to the orad compartment had no effect on ascending contraction (data not shown).

Modulatory role of somatostatin in the peristaltic reflex. Addition of somatostatin antibody #775 (1:100) for 60 min or the SSTR-2 antagonist, PRL-2903 (0.1 μM) (Rossowski et al., 1998) for 10 min to the caudad compartment inhibited descending relaxation elicited by all levels of stimulation, abolishing the response to 2 strokes and partly inhibiting the maximal response (51±6% with antibody and 33±2% with antagonist) (Fig. 3A). Addition of the SSTR-3 receptor antagonist, SST3-ODN-8 (1.0 μM) (Rivier et al., 2002) to the caudad compartment had no effect on descending relaxation (Fig 3A). Consistent with the inhibitory effect of the SSTR-2 antagonist, addition of the preferential SSTR-2 agonist, DC32-87 (0.1 μM) (Raynor et al., 1993), augmented descending relaxation elicited by all levels of stimulation, increasing the response to 2 strokes by 120±13% ($p<0.01$) and the maximal response by 35±10% ($p<0.01$) (Fig. 3A). The preferential SSTR-3 agonist, DC32-97 (0.1 μM), and the preferential SSTR-4/5 agonist, DC32-92 (0.1 μM), had no effect (data not shown). The somatostatin antiserum, SSTR2 and SSTR3 antagonists, and SSTR2, SSTR3., and SSTR4/5 agonists had no effect on ascending contraction (data not shown).

Basal release of somatostatin was similar in all three compartments (1.0±0.2, 1.1±0.2, and 0.9±1 fmol/100mg^{-1}.min^{-1}). Mucosal stroking caused a stimulus dependent increase in somatostatin release into the caudad compartment (72±8% and 124±18% above basal at 4 and 8 strokes, respectively); the decrease in the orad compartment

(32±6% and 30±7% below basal level at 4 and 8 strokes, respectively) was not stimulus dependent (Fig. 3B). Somatostatin release did not change in the central compartment.

Modulatory role of opioid peptides in the peristaltic reflex. Previous studies in rat, guinea pig and human, have shown that endogenous opioids, chiefly [Met]enkephalin and its derivatives, interact preferentially with δ opioid receptors to exert a continuous restraint on inhibitory and excitatory motor neurons; elimination of this restraint augments the release of inhibitory and excitatory neurotransmitters and increases descending relaxation and ascending contraction (Grider and Foxx-Orenstein, 1998; Grider, 1994b). Consistent with this pattern, addition of the δ opioid receptor antagonist, naltrindole (10 μM), augmented the response to 2 strokes by 56±12% (ascending contraction) and 50±8% (descending relaxation) and the response to 8 strokes by 33±7% (ascending contraction) and 41±6% (descending relaxation) (Fig 4.) .

Role of CGRP in the regulation of the peristaltic reflex. Studies in other species have shown that CGRP acts as neurotransmitter in sensory neurons mediating the peristaltic reflex (Grider, 1994a; Grider et al., 1996; Pan and Gershon, 2000). Addition of the CGRP antagonist, $hCGRP_{8-37}$ (10 μM) to the central compartment inhibited ascending contraction and descending relaxation at all levels of stimulation. The responses elicited by 2 strokes were abolished and those elicited by 8 strokes were inhibited by 58±5 (ascending contraction) and 72±5 % (descending relaxation) (Fig. 5). Addition of the antagonist to the caudad or orad compartments had no effect (data not shown).

Basal release of CGRP was similar in all three compartments (7.4±0.6, 8.2±0.6, and 7.6±0.8 fmol/100mg^{-1}.min^{-1}). Mucosal stroking in the central compartment caused a

stimulus dependent release of CGRP into the central compartment (68±4 % and 117±8 % increase above basal level at 4 and 8 strokes, respectively) but not into the orad or caudad compartments (Fig. 6B).

Role of mucosal 5-HT release in initiating the peristaltic reflex. Previous studies had shown that the peristaltic reflex elicited by mucosal stimulation is initiated by release of 5-HT from enterochromaffin cells, which then acts on 5-HT receptors located on intrinsic sensory CGRP-containing neurons (Pan and Gershon, 2000; Grider et al., 1996; Foxx-Orenstein et al., 1996). The pattern of 5-HT release in mouse colon paralleled that of CGRP release. Basal release of 5-HT was similar in all three compartments (21.2±2.4, 24.6±6.1, and 22.7±3.4 pmol/100mg^{-1}.min^{-1}). Mucosal stroking caused a stimulus dependent release of 5-HT (315±29% and 502±85% increase above basal levels at 4 and 8 strokes, respectively) in the central compartment, but not in the orad or caudad compartments (Fig. 6A).

The coupling of 5-HT to CGRP was determined by evaluating the effect of 5-HT receptor antagonists on CGRP release elicited by mucosal stimulation. Addition of the 5-HT_4 receptor antagonist, GR113808A (1 μM), to the central compartment inhibited CGRP release elicited by mucosal stimulation. CGRP release elicited by 4 strokes decreased from 68±4% to 11±3% above basal level ($p<0.001$ for the difference); CGRP release elicited by 8 strokes decreased from 117±8% to 61±14% above basal levels ($p<0.01$ for the difference) (Fig.7). Addition of the 5-HT_3 receptor antagonist, LY278584 (10 μM), had no effect on CGRP release elicited by 4 or 8 strokes (61±5 % and 135±14 % increase above basal levels, respectively) (N.S. different from control levels).

In accordance with the dependence of CGRP release on 5-HT release and activation of 5-HT_4 receptors, addition of GR113808A (1 μM) to the central compartment abolished ascending contraction and descending relaxation elicited by 2 strokes and inhibited ascending contraction elicited by 8 strokes by 50±7% and descending relaxation by 53±13% (Fig. 8). As expected, addition of LY278584 (10 μM) had no effect on ascending contraction or descending relaxation (Fig. 8).

DISCUSSION

The present study provides the first detailed analysis of the motor, modulatory, and sensory neurotransmitters that mediate the intestinal peristaltic reflex in the mouse, and shows that it closely resembles the reflex in other mammalian species, including human (Fig. 9). This is not unexpected, since the topography of enteric neurons in mammalian species is generally well conserved, including the relative proportions of excitatory and inhibitory motor neurons, their projections within the myenteric plexus and into the smooth muscle layers, and the pattern of co-localization of neurotransmitters (Sang and Young, 1998; Sang and Young, 1996; Sang et al., 1997; Ekblad et al., 1985). As noted above, excitatory motor neurons usually co-express ACh and the tachykinins, SP and NKA, and inhibitory motor neurons co-express VIP and its homologs, PHI and PACAP, as well as NOS. Additional features shared by mammalian species include the low density 5-HT and somatostatin neurons, and the presence of CGRP in enteric neurons, probably primary sensory neurons that relay the reflex initiated by mucosal stimuli.

A miniature version of a three-compartment preparation enabled measurement of neurotransmitter release in relation to the ascending and descending phases of the reflex, as well as precise application of agonists, antagonists, and antibodies to corroborate the participation of specific neurotransmitters. SP release was used as a marker for excitatory neurotransmitter release: in the mouse as in other species, SP and NKA are co-localized with ACh in motor neurons innervating intestinal smooth muscle (Sang and Young, 1998). A combination of atropine and the NK_1/NK_2 receptor antagonist, spantide, abolished ascending contraction suggesting that no other excitatory

neurotransmitter participates in causing contraction. As in other species, the tachykinins were more effective in blocking the response to higher levels of stimulation, suggesting differential release of ACh and tachykinins from nerve terminals (Grider and Makhlouf, 1990; Grider and Foxx-Orenstein, 1998).

VIP was used as a marker for inhibitory neurotransmitter release. VIP is co-synthesized with PHI and is usually co-localized with PACAP (Sundler et al., 1992) and NOS in inhibitory motor neurons (Sang and Young, 1996; Sang et al., 1997). Furthermore, a functional linkage exists between NO and VIP/PACAP in gastrointestinal smooth muscle in all mammalian species including the mouse (Murthy et al., 1996; Jin et al., 1996; Jin et al., 1997; Grider and Jin, 1993b). NO formed in nerve terminals regulates the release of VIP and its homologs (Grider and Jin, 1993a); in turn, VIP and PACAP stimulate sequentially NO and cGMP formation and induce muscle relaxation by interacting with natriuretic peptide receptor C (NPR-C) and activating eNOS expressed in gastrointestinal smooth muscle cells (Murthy et al., 2000). In addition, VIP and PACAP interact with $VPAC_2$ receptors to stimulate cAMP formation and induce further muscle relaxation (Murthy et al., 2000; Murthy et al., 1993). In the present study, both L-NNA and VIP_{10-28} inhibited descending relaxation. VIP_{10-28} appeared to be more potent, since it blocked the interaction of VIP and PACAP with both NPR-C and $VPAC_2$ receptors, thereby inhibiting the two main pathways (NO/cGMP and cAMP) mediating smooth muscle relaxation (Murthy et al., 1997; Murthy et al., 1993; Murthy et al., 2000). The combination of L-NNA and VIP_{10-28} abolished relaxation by inhibiting neuronal and smooth muscle NO formation and VIP/PACAP release, thereby eliminating all pre- and post-junctional pathways mediating muscle relaxation.

Although myenteric somatostatin neurons are relatively sparse in all mammalian species including the mouse (Ekblad et al., 1985; Heinicke and Kiernan, 1990), they play an important role as interneurons in relaying sensory signals to inhibitory motor neurons (Grider, 1994b). Somatostatin release increased during the descending phase of the reflex in a stimulus dependent fashion. Immunoneutralization of somatostatin or blockade of SSTR2 receptors inhibited descending relaxation, whereas activation of SSTR2 receptors augmented relaxation. Neither agonists nor antagonists of SSTR3, SSTR4/5 had any effect on relaxation. The small decrease in somatostatin during the ascending phase did not appear to be functionally relevant, since immunoneutralization of somatostatin and application of agonists and antagonists of various somatostatin receptor subtypes had no effect on ascending contraction. The participation of SSTR2 receptors in the peristaltic reflex is consistent with immunohistochemical studies demonstrating the presence of SSTR2 receptors on enteric neurons containing VIP and NO in rat and mouse (Sternini et al., 1997; Allen et al., 2002). These findings raise the possibility of an alternative parallel pathway in which the projection of SST is directly to the inhibitory motor neuron. Indeed, it is likely that there are multiple descending interneuronal pathways that regulate the activity of the VIP.PACAP/NOS inhibitory motor neurons. SSTR2 receptors have also been shown to mediate the role of somatostain in the regulation of gastric acid secretion (Kawakubo et al., 1999; Martinez et al., 1998; Prinz et al., 1994) .

A recent studies by Abdu et al (2002) examined the role of somatoatatin and SST receptors in mediating the intestinal migrating motor complex (MMC) of mice and rat. The authors identified an inhibitory role for somatostatin mediated by SSTR2 and a non-SSTR2 receptor in mouse where SST and SST analogs prolonged the interval between

MMCs rather than affecting the amplitude of the muscle responses. The MMC is a more complex motor pattern than the peristaltic reflex and it is likely to involve multiple interactive pathways. Abdu et all suggest that in their study, the site of action of SST and the SST receptor population identified in their study is likely to be in the interneuronal circuitry regulating the timing of motor activity. It is highly likely that SST affects gut motility at a variety of sites, considering its ability to regulate the release of many other endogenous substances including acetylcholine. As also indicated by these authors, it is possible that there are differences between jejunum and colon with regard to the actions of SST in the peristaltic reflex and the MMC. It is also interesting in their study, that the MMC was not affected in SSTR2 knockout mice in spite of profound effect of SST, SST analogs, and SSTR antagonists. The authors attribute this finding to possible redundancy in SST receptors, but this may also reflect the multiple sites of action of SST as well as redundancy in pathways mediating the complex motor patterns of intestine and colon.

Studies in other species have shown that the effect of somatostatin on descending relaxation was indirectly mediated by its ability to suppress the continuous restraint exerted by opioid neurons on inhibitory motor neurons (Grider, 1994b). In these studies, somatostatin inhibited [Met]enkephalin release and augmented VIP/PACAP/NO release and descending relaxation, whereas somatostatin antibody had the opposite effect. Opioid receptor antagonists, particularly δ receptor antagonists, augmented VIP/PACAP/NO release and descending relaxation. Consistent with this pattern, the δ opioid receptor antagonist, naltrindole, augmented descending relaxation in mouse colon. The ability of naltrindole to augment ascending contraction suggests that opioid neurons

also participate in regulating ascending contraction by maintaining a continuous restraint on excitatory cholinergic/tachykinin neurons. The pathways mediating the role of opioid neurons in regulation of ascending contraction have not been fully elucidated.

The sensory pathways mediating the peristaltic reflex in the mouse were similar to those in the rat and human (Grider et al., 1996; Foxx-Orenstein et al., 1996). Minor deformation of the mucosa is sufficient to trigger release of 5-HT from enterochromaffin cells, the second largest store of 5-HT in the body after platelets. In turn, 5-HT stimulates CGRP release from mucosal sensory nerve terminals. The involvement of CGRP in sensory neurotransmission is supported by previous observations showing that exposure of mouse colon to capsaicin or acid pH causes release of CGRP (Roza and Reeh, 2001). In rat colon and human small intestine, blockade of CGRP or 5-HT_4 receptors inhibited VIP and SP release, as well as ascending contraction and descending relaxation (Grider et al., 1996; Foxx-Orenstein et al., 1996). In the present study, the 5-HT_4 receptor antagonist, GR113808A, inhibited CGRP release; this antagonist as well the CGRP antagonist, $hCGRP_{8-37}$, also inhibited ascending contraction and descending relaxation. Thus, in mouse colon as in rat colon and human small intestine, CGRP release and activation of the reflex were mediated exclusively by 5-HT_4 receptors. The involvement of these receptors was corroborated by the ability of selective 5-HT_4 receptor agonists to stimulate CGRP release and initiate the peristaltic reflex. In guinea pig colon, however, stimulation of CGRP release and the peristaltic reflex required activation of both 5-HT_3 and 5-HT_4 receptors (Foxx-Orenstein et al., 1996; Kadowaki, et al., 1996).

The physiological stimulus of peristalsis is most likely the passage of intestinal contents, rather than intraluminal distension. The passage of intestinal contents is mimicked by light mucosal stimulation, which triggers sequential release of 5-HT, CGRP, and motor neurotransmitters. Consistent with this notion, intraluminal 5-HT_4 agonists stimulated propulsive activity in isolated colonic segments (Jin et al., 1999; Nagakura et al., 1997a). Muscle stretch, on the other hand, releases CGRP from extrinsic sensory neurons in the dorsal root ganglion with terminals in the myenteric plexus and does not involve 5-HT release (Grider et al., 1996; Grider and Jin, 1994). Little is known about the intrinsic and extrinsic sensory neurons in the mouse. Although there is a possibility that some of the CGRP detected in the medium was derived from extrinsic neurons, it is not likely since the 5-HT_4 antagonist strongly inhibited CGRP release (about 84%) and both components of the peristaltic reflex. Thus, it is likely that the same arrangement exists in the mouse colon as has been described for rat and guinea pig colon and human intestine.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Excitatory motor neurotransmitters mediating ascending contraction.

Panel A: The peristaltic reflex was elicited by mucosal stroking in the central compartment. Ascending contraction of circular muscle was inhibited by addition of atropine (1 μM) and/or spantide (10 μM) to the orad compartment. Addition of the agents to the caudad compartment had no effect on descending relaxation (data not shown). The results are expressed as percent of maximal contraction (0.68±0.03 g). Values are mean±SE of 4 experiments.

Panel B: Selective release of SP into the orad (solid bar) but not central (open bar) or caudad (hatched bar) compartments. The results are expressed as percent increase above basal level (range of basal values in the three compartments: 38±2 to 42±4 fmol/100 $mg^{-1}.min^{-1}$). Values are means ± SE of 3 experiments.

Figure 2. Inhibitory motor neurotransmitters mediating descending relaxation.

Panel A: The peristaltic reflex was elicited by mucosal stroking in the central compartment. Descending relaxation of circular muscle was inhibited by addition of VIP_{10-28} (10 μM), and/or L-NNA (100 μM) to the caudad compartment. Addition of the agents to the orad compartment had no effect on ascending contraction (data not shown). VIP_{10-28} inhibits also responses mediated by the homologous neurotransmitters, PHI and PACAP. The results are expressed as percent of maximal relaxation (0.46±0.03 g). Values are mean±SE of 4 experiments.

Panel B: Selective release of VIP into the caudad (hatched bar) but not the central (open bar) or orad (solid bar) compartments. The results are expressed as percent

increase above basal level (range of basal values in the three compartments: 41±6 to 49±5 fmol/100 $mg^{-1}.min^{-1}$). Values are means ± SE of 3 experiments.

Figure 3. Regulation of descending relaxation by somatostatin.

Panel A: Selective augmentation of descending relaxation by the SSTR2 receptor agonist DC32-87 and inhibition by the SSTR2 receptor antagonist, PRL-2903 and somatostatin antibody #775 (1:100). Agonists and antagonists were added to the caudad compartment 10 min before, and antibody was added 60 min before the reflex was elicited by stroking. The SSTR-3 antagonist, SST3-ODN-8, had not effect. Addition of agonists, antagonists, or antibody to the orad compartment had no effect (data not shown). Results expressed as percent of control maximal relaxation. Values are mean±SE of 3-4 experiments.

Panel B: Selective release of somatostatin into the caudad compartment (hatched bar). Basal release was decreased in the orad compartment, but did not change in the central compartment. The data is expressed as percent increase above basal level (range of basal values in the three compartments: 0.9±0.1 to 1.2±0.2 fmol/100 $mg^{-1}.min^{-1}$) and are means±SE of 3 values.

Figure 4. Regulation of descending relaxation and ascending contraction by opioid peptides. Augmentation of descending relaxation and ascending contraction by addition of naltrindole (10 μM) to the caudad or orad compartment, respectively. Results are expressed as percent of maximal control response. Values are means±SE of 3 experiments.

Figure 5. Role of CGRP in the regulation of the peristaltic reflex. Inhibition of ascending contraction and descending relaxation by addition of CGRP $_{8-37}$ (10 μM) to the central compartment. Addition of the antagonist to the orad or caudad compartment had no effect (data not shown). Results are expressed as percent of control maximal response. Values are means±SE of 4 experiments.

Figure 6. Pattern of release of 5-HT and CGRP during the peristaltic reflex. Stroking of the mucosa in the central compartment elicited release of 5-HT (Panel A) CGRP (Panel B) into the central (open bar) but not the caudad (hatched bar) or orad (solid bar) compartments. The results are expressed as percent increase above basal level (range of basal values in the three compartments: 21.2±2.4 to 24.6±6.1 pmol/100 $mg^{-1}.min^{-1}$ for 5-HT, and 7.4±6 to 8.2±0.6 fmol/100 $mg^{-1}.min^{-1}$ for CGRP). Values are means±SE of 6 experiments.

Figure 7. Role of 5-HT_4 receptors in mediating CGRP release during the peristaltic reflex. Inhibition of CGRP release elicited by mucosal stroking in the central compartment by addition of the 5-HT_4 receptor antagonist, GR113808A, but not the 5-HT_3 receptor antagonist, LY 278584, to the same compartment. Results expressed as percent increase in CGRP above basal level (basal CGRP release: 7.6±0.4 fmol/100 $mg^{-1}.min^{-1}$). Values are mean±SE of 3 experiments.

Figure 8. Selective activation of 5-HT$_4$ receptors during the peristaltic reflex. Inhibition of the ascending contraction and descending relaxation elicited by mucosal stroking by addition of the 5-HT$_4$ receptor antagonist, GR113808A but not the 5-HT$_3$ receptor antagonist LY 278584 to the central compartment. Addition of either antagonist to the orad or caudad compartment had no effect. Results are expressed as percent of maximal control response. Values are mean ± SE of 3-4 experiments.

Figure 9. Model of the intestinal peristaltic reflex in the mouse. Mucosal stimulation (e.g., passage of digesta) initiates a peristaltic reflex by stimulating 5-HT release from mucosal enterochromaffin cells. In turn, 5-HT activates 5-HT$_4$ receptors located on the terminals of CGRP-containing intrinsic primary afferent neurons. CGRP neurons relay the stimulus to ascending and descending motor neurons via one or more interneurons. In descending pathways, CGRP either directly or via cholinergic interneurons stimulates the release of somatostatin, which in turn inhibits the activity of opioid neurons. The continuous restraint exerted by opioid neurons on inhibitory motor neurons is thus eliminated (disinhibition), leading to release of VIP/PACAP and NO. Somatostatin interneurons also have projections directly to inhibitory motor neurons and the latter are known to express SSTR2 receptors. This suggests that possibility of a second parallel pathway. The interneurons in ascending pathways are likely to be cholinergic neurons that regulate the release ACh and tachykinins (SP and NKA) from excitatory motor neurons. Enkephalin neurons also act as inhibitory neurons in ascending pathways although their exact coupling to interneurons and excitatory motor neurons has not been identified. + and – indicate stimulation and inhibition, respectively

Figure 1

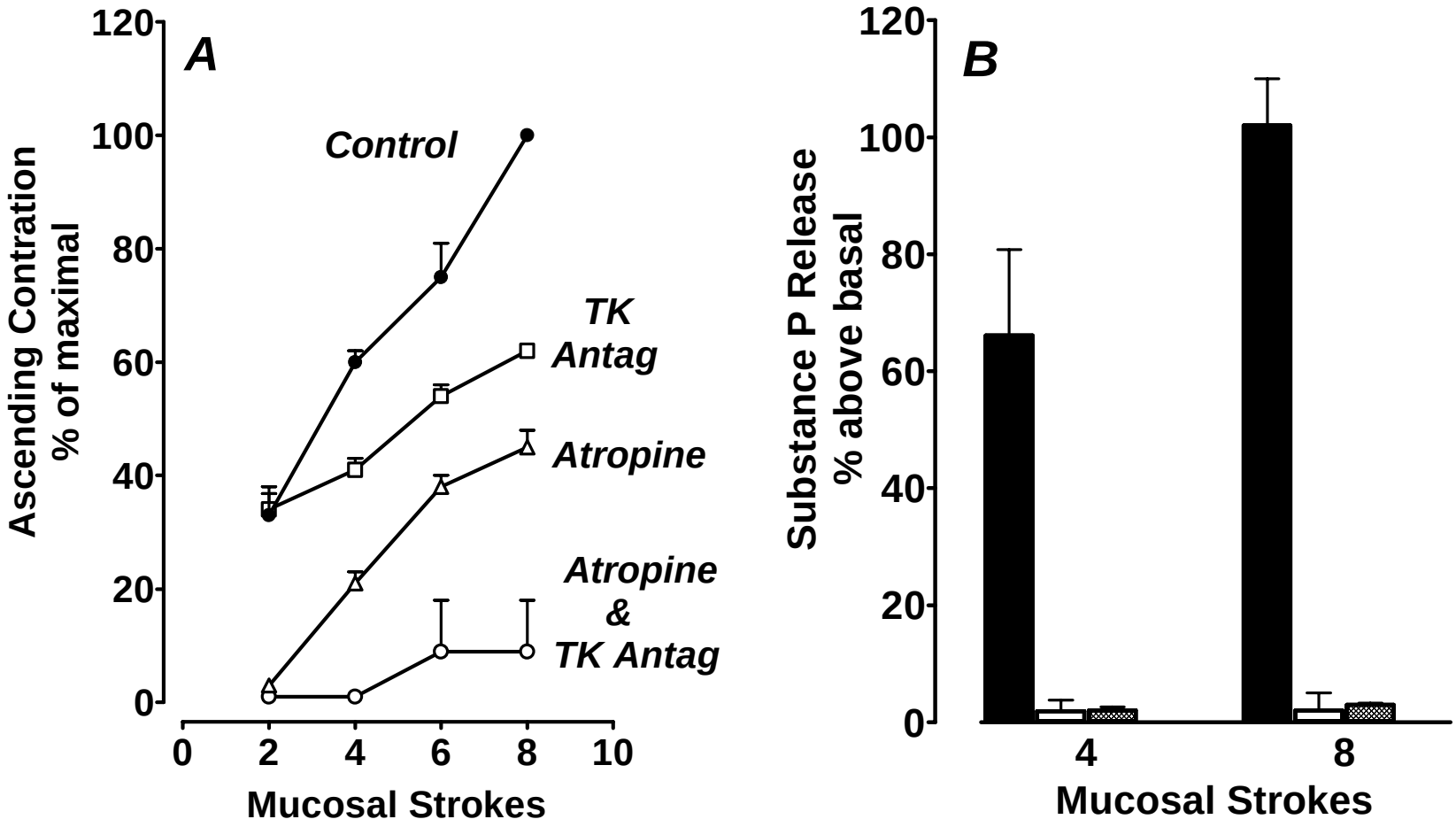
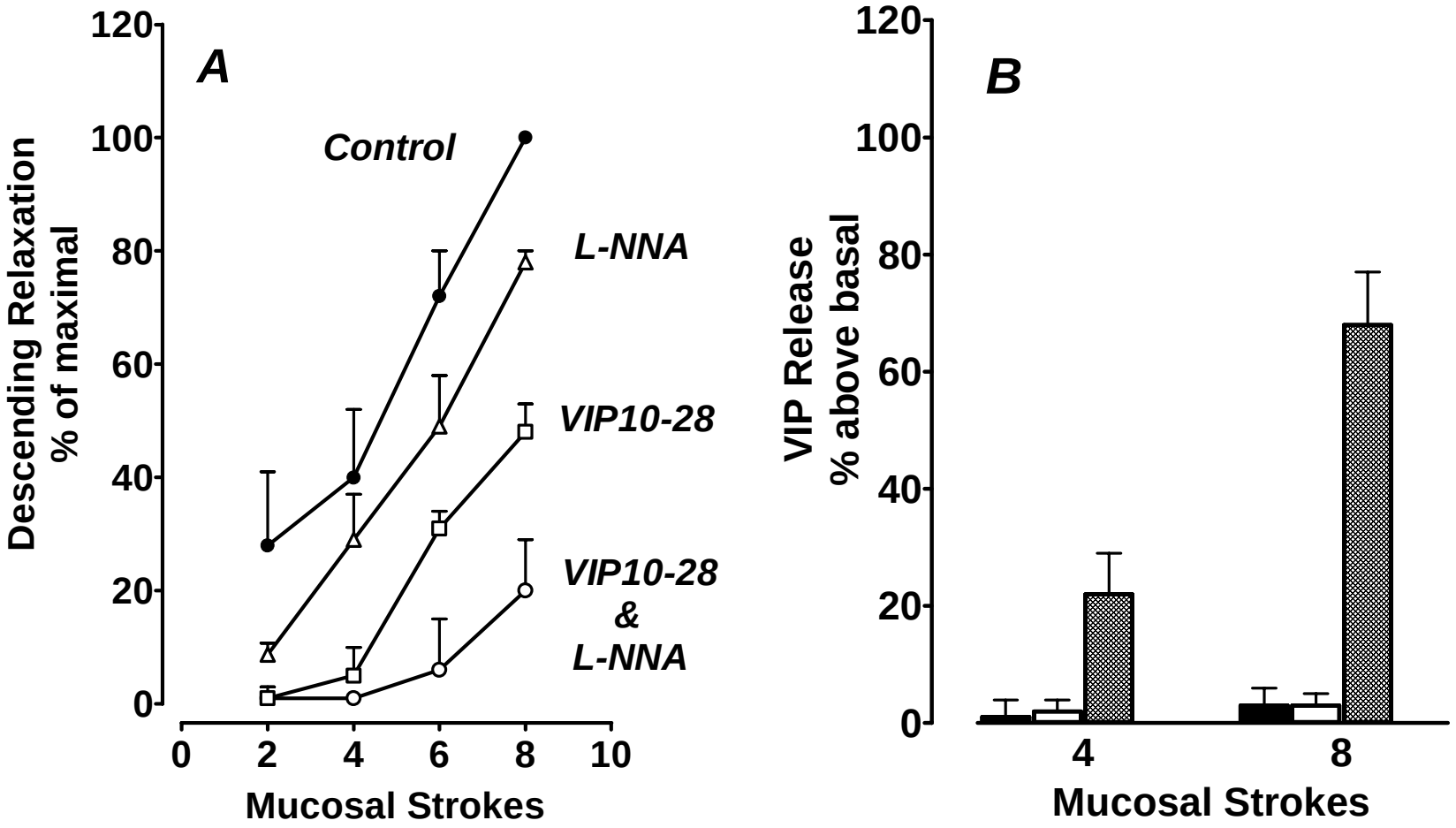


Figure 2



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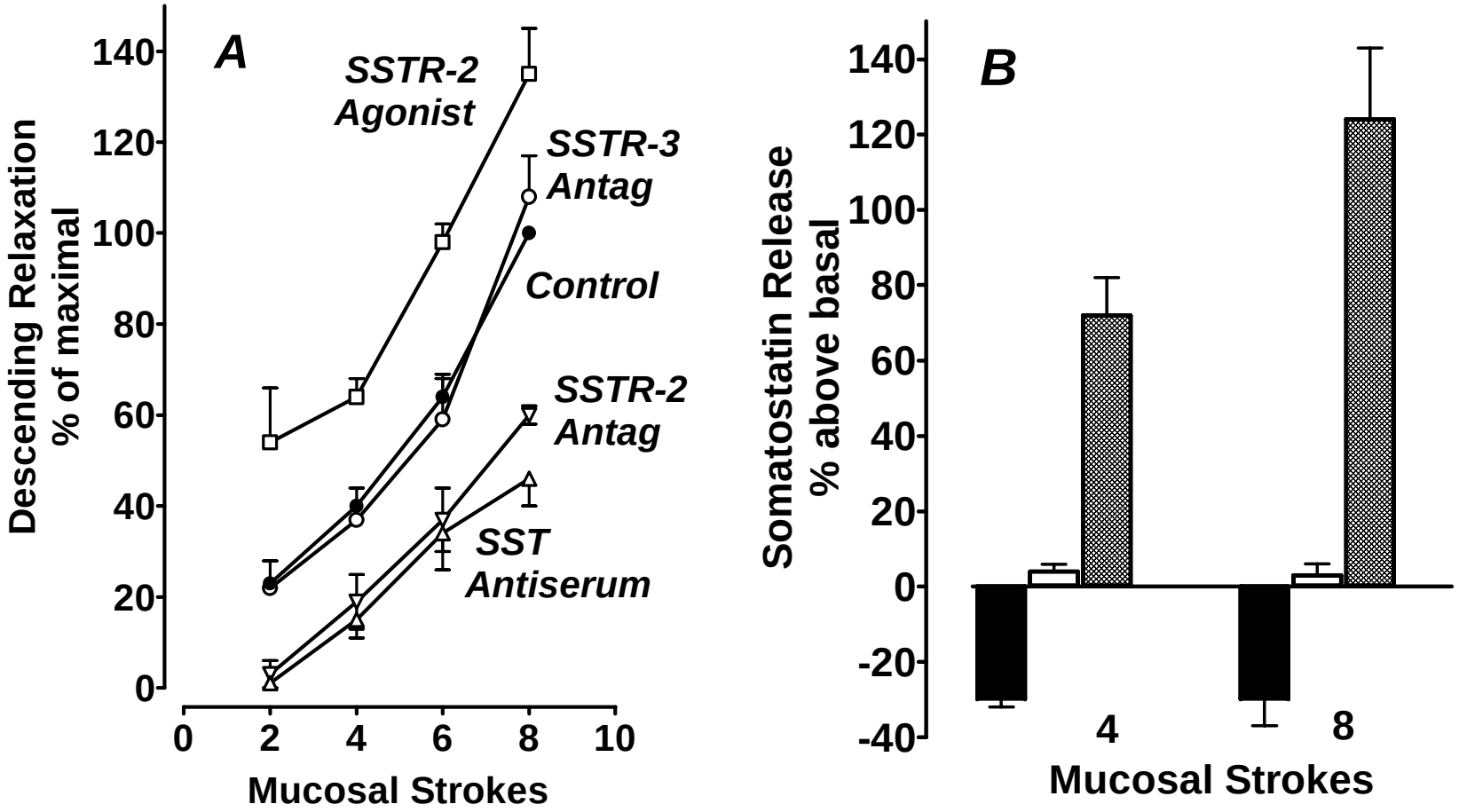

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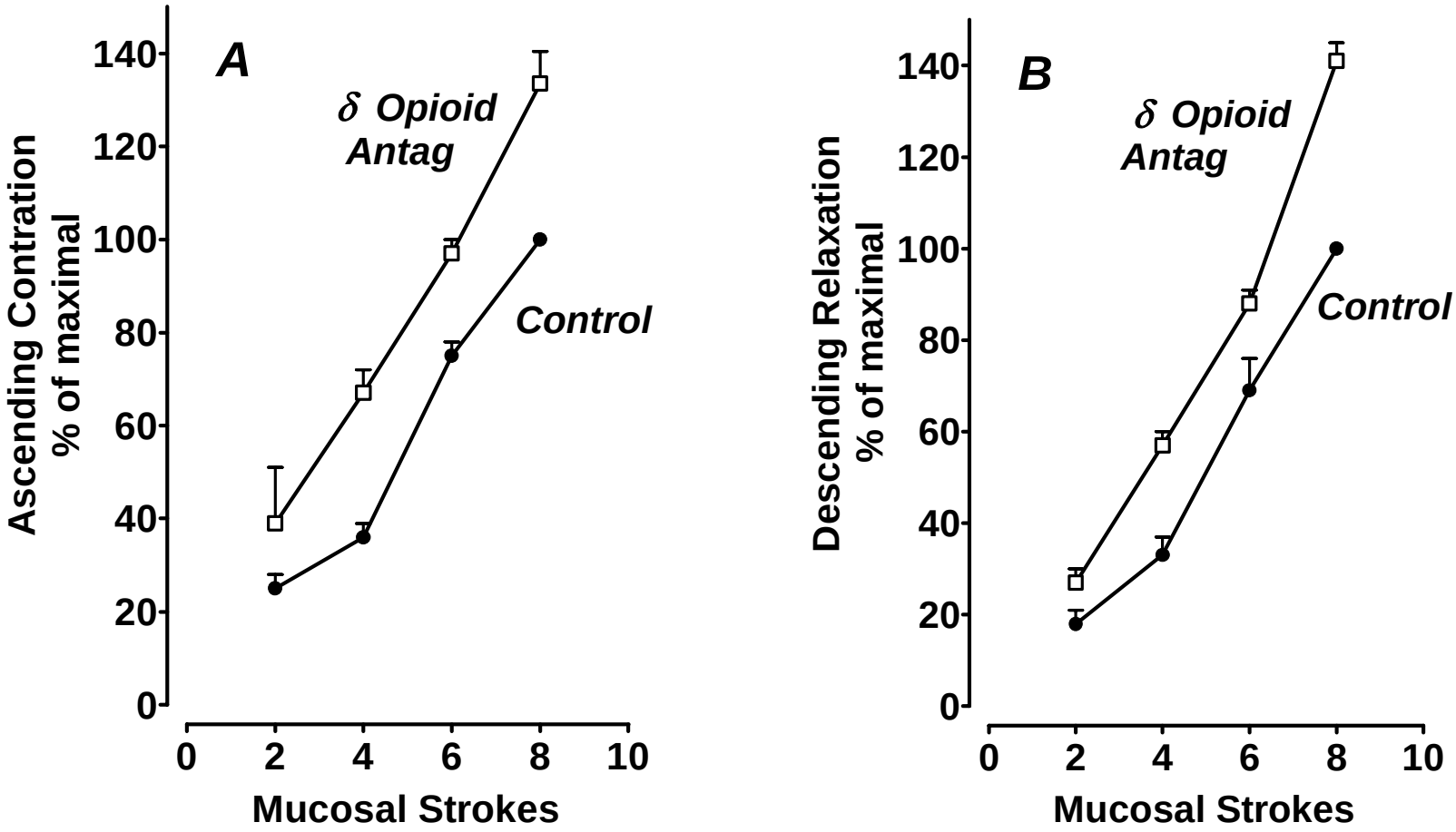

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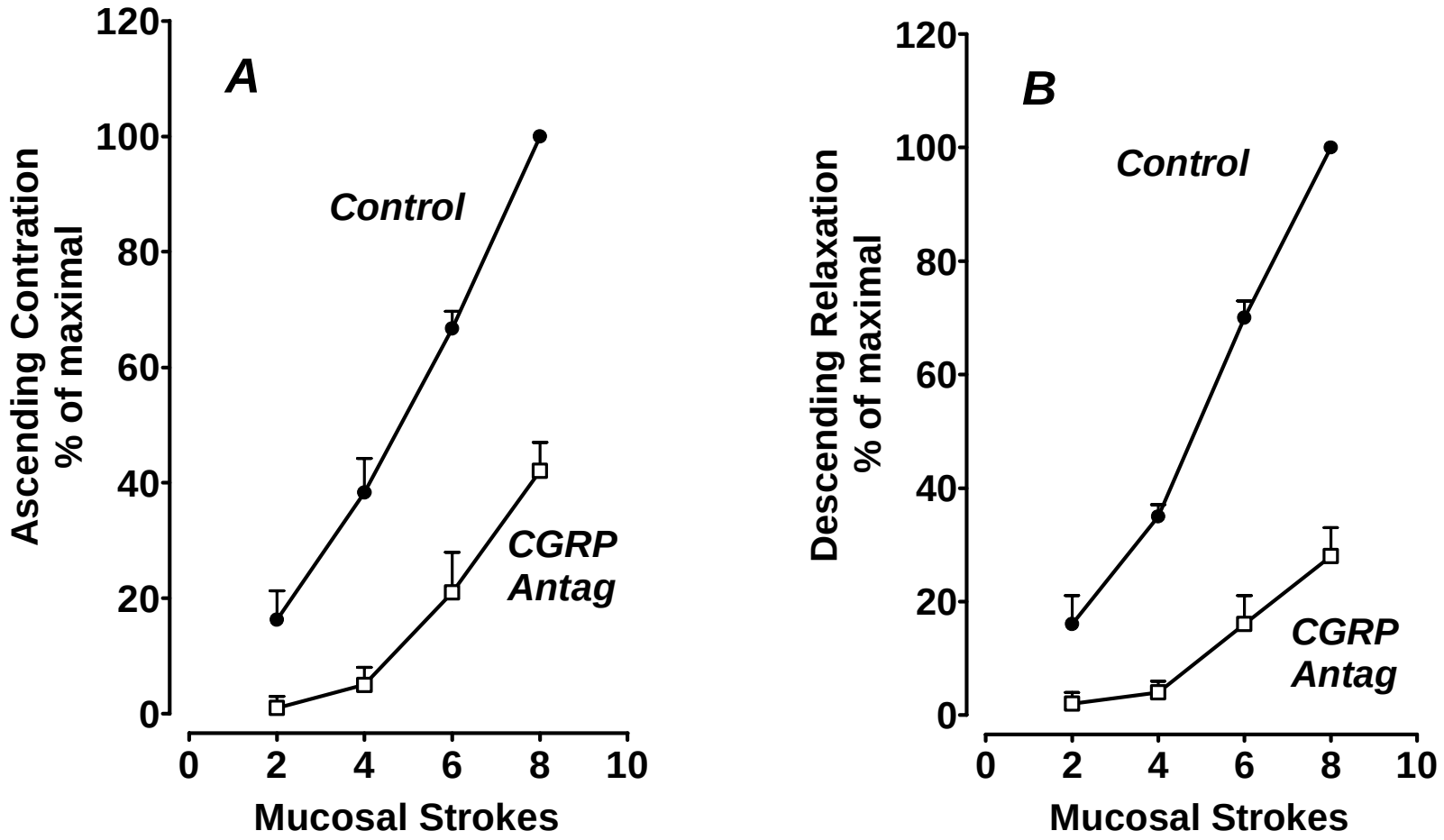

Figure 6

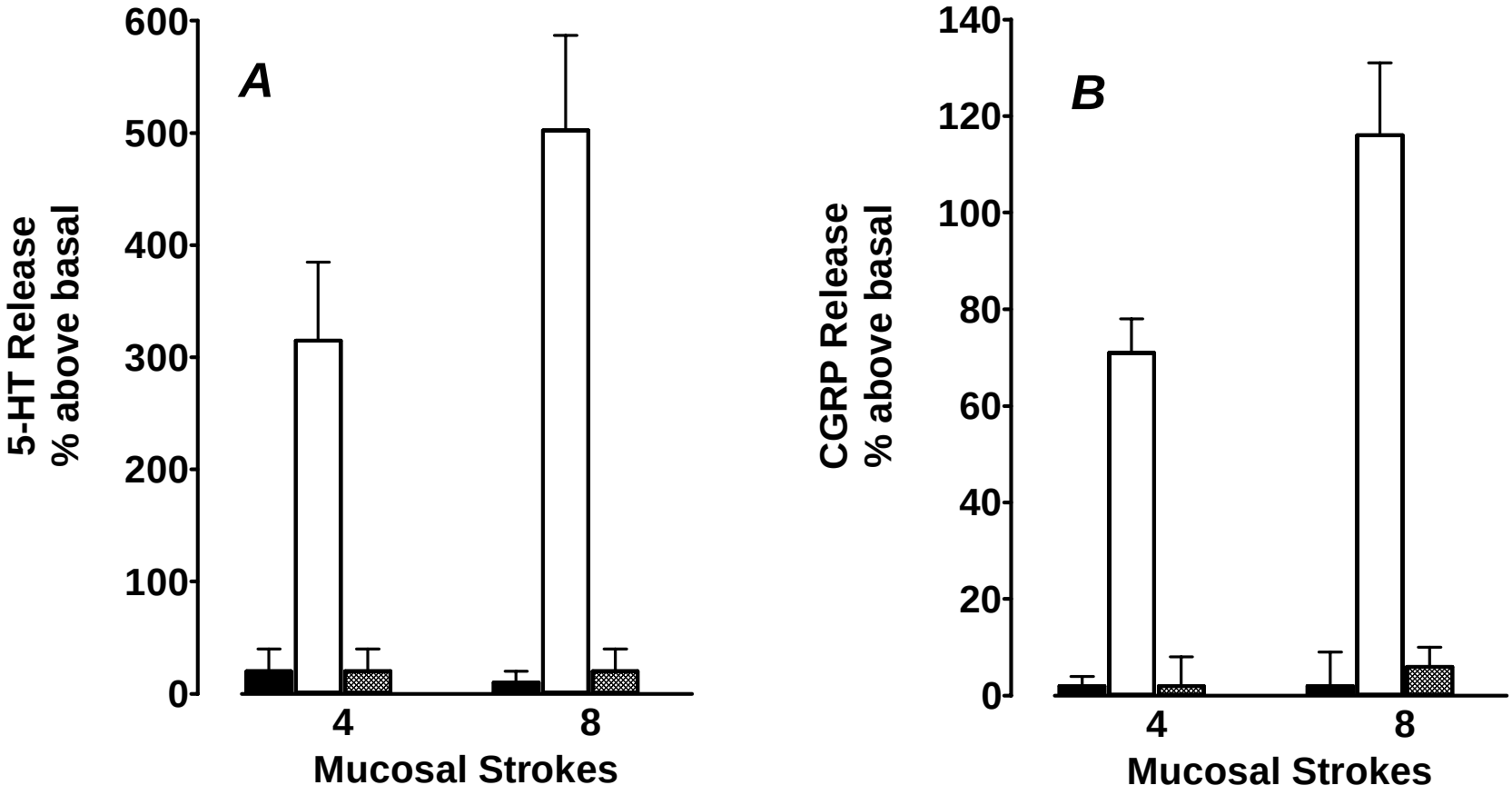

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Figure 7

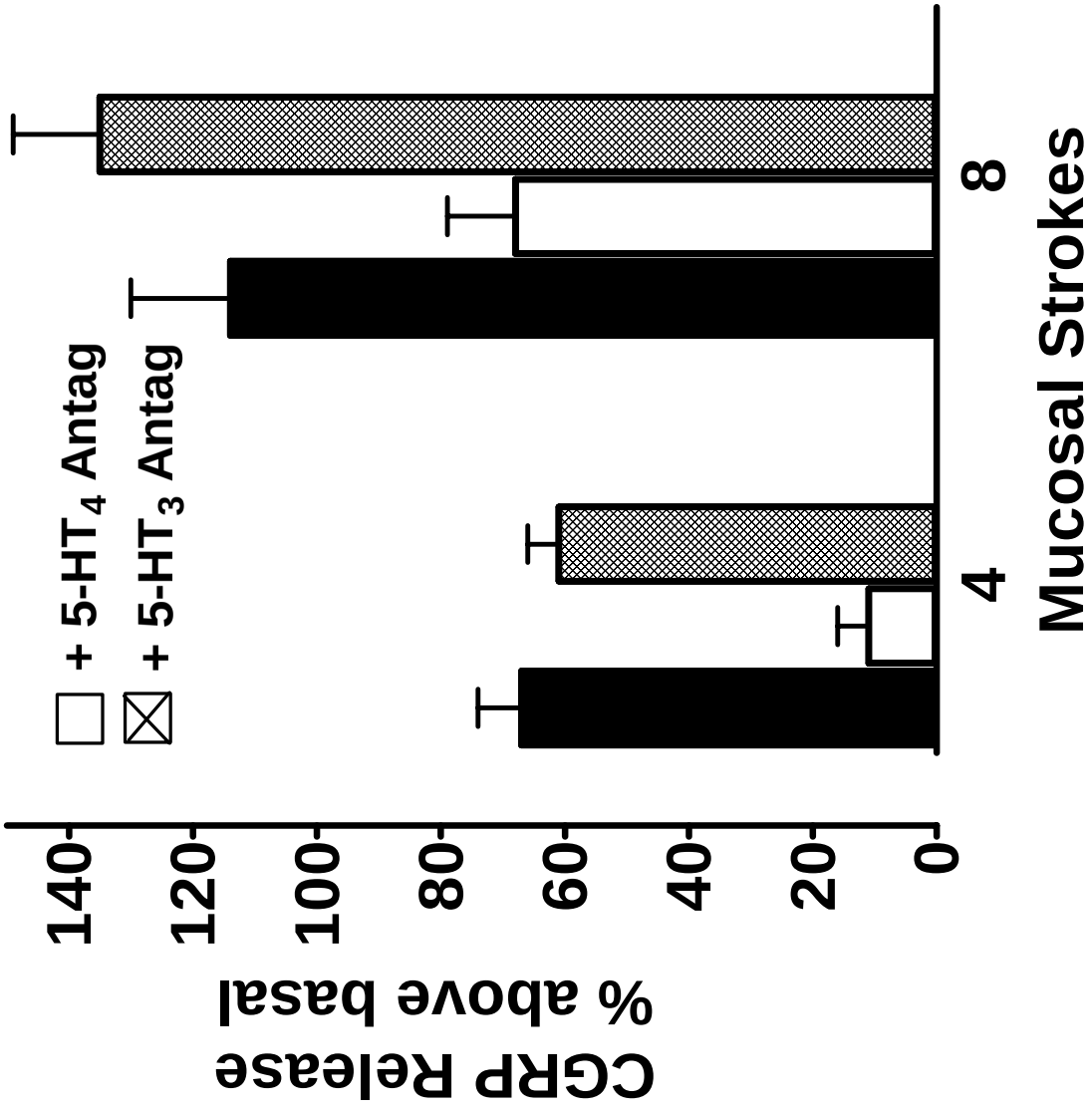

Figure 8

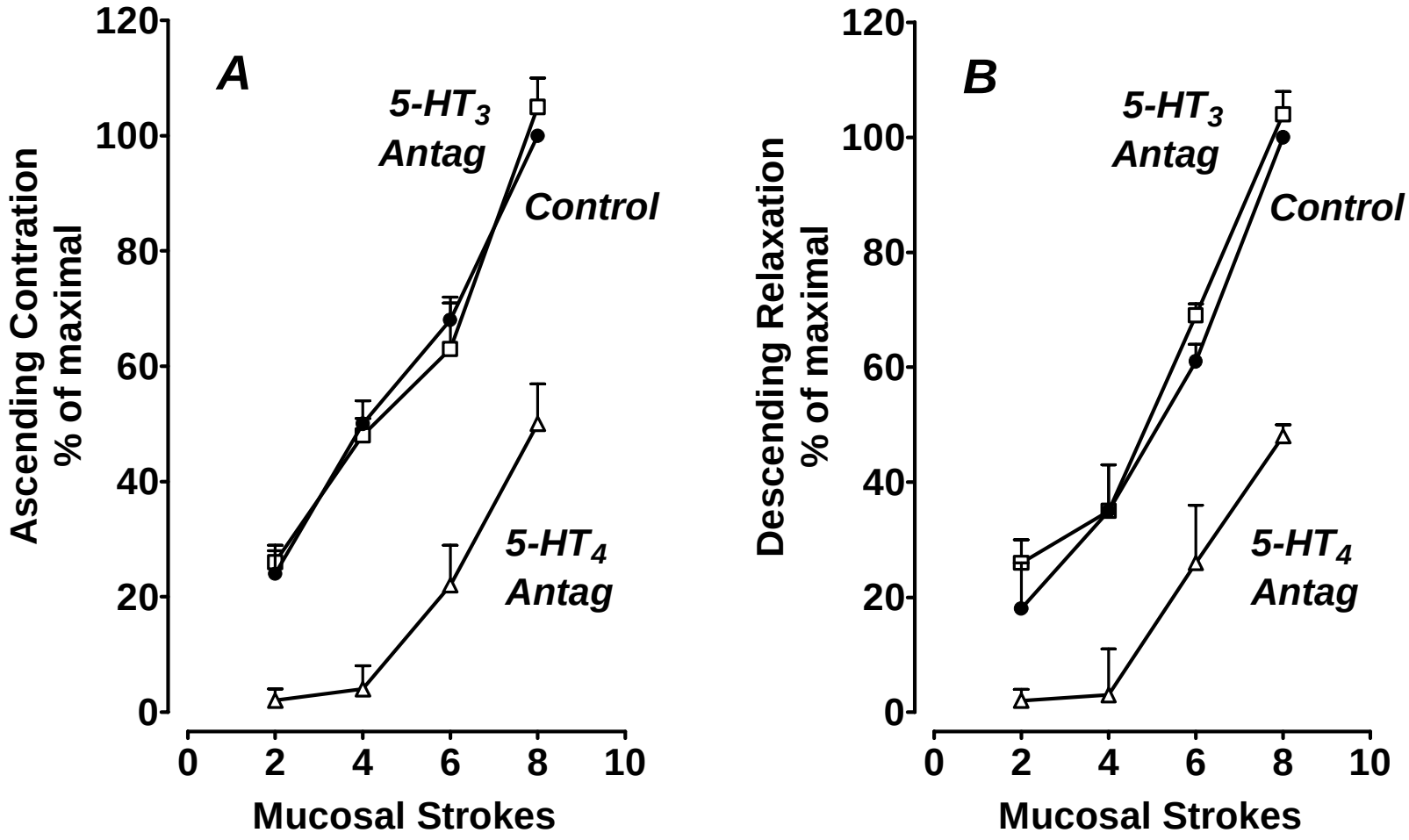

Figure 9