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Pharmacologic specificity of nicotinic receptor mediated relaxation of muscarinic receptor pre-contracted human gastric clasp and sling muscle fibers within the gastroesophageal junction

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Abbreviations: BIC, bicuculline, CGRP, calcitonin gene related polypeptide; CO, carbon monoxide; EFS, electric field stimulation, GABA, gamma amino butyric acid; GEJ, gastroesophageal junction; GERD, gastroesophageal reflux disease; HPZ, high-pressure zone; LEC, Lower esophageal circular muscle; LES, lower esophageal sphincter; L-NAME, L-nitro arginine methyl ester; NANC, non-adrenergic, non-cholinergic; NIC, nicotine; NO, nitric oxide; NOS, nitric oxide synthase; PACAP, pituitary adenylate cyclase-activating peptide; PROP, propranolol; SNAP, S-Nitroso-N-acetyl-DL-penicillamine, STRY, strychnine; TLESR, transient lower esophageal sphincter relaxation; TTX, tetrodotoxin; SR95531, 2-(3-Carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide (Gabazine); VIP, vasoactive intestinal polypeptide.

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Abstract:

Relaxation of gastric clasp and sling muscle fibers is involved the transient lower esophageal sphincter relaxations underlying the pathophysiology of gastroesophageal reflux disease (GERD). These fibers do not contribute tone to the high pressure zone in GERD patients, indicating their role in pathophysiology. This study identifies some mediators of the nicotine induced relaxation of muscarinic receptor pre-contracted gastric clasp and sling fibers. Muscle strips from organ donors pre-contracted with bethanechol were relaxed with nicotine then rechallenged after washing and adding inhibitors: tetrodotoxin (TTX), the nitric oxide synthase inhibitor L-NAME , the β adrenoceptor antagonist propranolol, the glycine receptor antagonists strychnine or ginkgolide B and the $GABA_A$ receptor antagonists bicuculline or SR95531. TTX only inhibited clasp fiber relaxations. L-NAME and propranolol inhibited and ginkgolide B was ineffective in both. SR95531 was ineffective in clasp fibers and partially effective in sling fibers. Strychnine and bicuculline prevented relaxations with low potency indicating actions not on glycine or $GABA_A$ receptors but more consistent with nicotinic receptor blockade. Bethanechol pre-contracted fibers were relaxed by the nitric oxide donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) and by the β adrenergic agonist isoproterenol (clasp fibers only) but not by the glycine receptor agonists taurine or glycine or the $GABA_A$ agonist muscimol. These data indicate that nicotinic receptor activation mediates relaxation via release of nitric oxide in clasp and sling fibers, norepinephrine acting on β adrenoceptors in clasp fibers and GABA acting on $GABA_A$ receptors in sling fibers. Agents that selectively prevent these relaxations may be useful in the treatment of GERD.

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Introduction

A complex anatomy, physiology and mechanics mediates functions of the gastro-oesophageal sphincter that are necessary to regulate esophageal emptying into the stomach and to permit air venting, while protecting against unwanted reflux of gastric content. The relative tonic contributions to the protective function of the gastroesophageal sphincter and the relative loss of tone during transient lower esophageal sphincteric relaxations (TLESR) underlie sphincteric function (Dent et al., 1980; Boeckxstaens, 2005).

The circular muscle fibers at the distal end of the esophagus have been traditionally considered to be the lower esophageal sphincter (Kahrilas, 1997; Cheng et al., 2005; Mittal and Goyal, 2006). However, the first barrier to gastric reflux is actually the clasp and sling muscle fiber complex of the proximal stomach. Several investigators also use this same term “lower esophageal sphincter” (LES) to indicate this clasp and sling muscle fiber complex (Gonzalez et al., 2004; Tian et al., 2004). The intrinsic inner circular muscles of the distal 2-3 centimeters of the esophagus (the lower esophageal circular muscles, LEC) together with the gastric sling/clasp fiber muscle complex constitute the internal, smooth muscle mechanism of the high-pressure zone. The crural diaphragm, positioned between and overlapping these two anatomically distinct smooth muscle structures constitutes the external, skeletal muscle mechanism. The sling/clasp muscle complex has been described as a thickened wall of smooth muscle at the gastroesophageal junction (GEJ) in cadaver studies (Liebermann-Meffert et al., 1979; Stein et al., 1991; Stein et al., 1995). Sling muscle fibers surround the junction of the esophagus on the greater curvature side of the gastric cardia. Clasp muscle fibers, found on the lesser curvature junction of the stomach, attach to the sling muscle. It has been proposed that the perfect match between the manometric pressure and the gastric

sling/clasp complex indicates that the gastric sling/clasp muscle fiber group is actually the anatomic correlate of the manometric lower esophageal sphincter (Stein et al., 1995). However, because these muscles are anatomically part of the stomach and not the esophagus, a more appropriate name for the sling/clasp smooth muscle fiber complex is the "upper gastric sphincter". Since the lower esophageal circular smooth muscle component, the crural diaphragm and the gastric sling/clasp muscle fiber complex are anatomically contiguous in normal individuals, intraluminal pressure is due to contractions of a combination of all of these muscles.

Many of the published studies on relaxation of gastrointestinal smooth muscle focused on either electric field stimulation (EFS) induced relaxations or inhibition of stretch induced active tone. It has been known for at least 50 years that the pressure of the human gastroesophageal high pressure zone (HPZ) is reduced by administration of the muscarinic receptor antagonist atropine (Bettarello et al., 1960; Skinner and Camp, 1968; Opie et al., 1987). The pressure generated specifically by the human clasp and sling muscle fiber complex as well as the lower esophageal circular muscle in-vivo is reduced by atropine (Miller et al., 2009) . These findings suggest that the in-vivo tone of the HPZ is maintained by the neuronal release of acetylcholine acting on smooth muscle muscarinic receptors. In-vitro stretch induced tone in muscle strips from human GEJ is active and calcium sensitive (Kovac et al., 2005). However, this stretch induced tone not decreased by atropine (Preiksaitis and Diamant, 1997; Gonzalez et al., 2004). Therefore, the in-vitro stretch induced tone develops by a different mechanism than the in-vivo, atropine sensitive tone that is responsible for generating in-vivo pressure in the gastroesophageal HPZ. Thus relaxation of muscarinic

receptor pre- contracted clasp and sling muscle may be the in-vitro physiologic equivalent of in-vivo TLESR and deglutitive inhibition.

A number of investigators have demonstrated that nicotine induces relaxation of stretch induced tone. To the best of our knowledge no similar studies have been performed on bethanechol pre-contracted clasp and sling muscle strips. Nicotine causes relaxation of stretch induced tone indirectly through various secondary mediators and signal transduction pathways. For these reasons, we chose to study nicotine induced relaxations of bethanechol pre-contracted clasp and sling muscle fibers. This paradigm allows use of specific agonists and antagonists to explore the secondary messengers that mediate nicotine induced relaxation of clasp and sling muscle fibers. These fibers relax during transient lower esophageal sphincter relaxations that underlie the pathophysiology of gastroesophageal reflux disease (GERD).

Methods:

All drugs and chemicals were obtained from Sigma Chemical Company (St. Louis, MO). A total of 14 specimens of human stomach with attached esophagus were obtained from subjects with no known history of GERD. The mean age of the subjects was 46.6 ±5.9 years, 11 of which were men and 3 were women, 9 of the subjects were Caucasian, 2 were Hispanic, 1 was African American, 1 was Asian and 1 was Indian. The average height and weight was 174 cm and 86.4 Kg respectively. We obtained these specimens from brain dead patients maintained on life support who consented to organ transplant donation. Their next of kin consented to donation of non-transplantable organs for research. These human organ specimens were obtained from organ procurement agencies (National Disease Research Institute and the International Institute for the Advancement of Medicine). The specimens were harvested within 30 minutes after cross clamping the aorta. The stomach contents were gently rinsed out with saline. The esophageal and pyloric openings were ligated and the entire specimen was transported to our laboratory on ice by overnight courier immersed in either University of Wisconsin (Beltzer's Viaspan) organ transport media (UW) or HTK which is composed (in mM) of: NaCl – 15, KCl – 9, Potassium hydrogen 2-ketoglutarate – 1, $MgCl_2$ – 4, histidine NaCl – 18, tryptophan – 2, mannitol – 30, $CaCl_2 \bullet 2\ H_2O$– 0.015.

Preparation of smooth muscle strips: The specimens were dissected in a cold room (0-5C). The greater and lesser omentums were removed. The outermost longitudinal fibers descending from the esophagus across the stomach were removed by sharp dissection. This exposed the sling muscle fibers as a U shaped group of fibers approximately 8 mm wide enveloping the esophagus around the greater curvature of the stomach and the semicircular clasp muscle fibers around the lesser curvature opposite to the cardiac notch. The clasp

muscle fibers are oriented perpendicular to the sling muscle fibers and connect between the open ends of the U shaped sling muscle fibers. Beginning at the cardiac notch, the sling muscle fiber complex was separated from the underlying submucosa by sharp dissection and this tissue plane was followed completely around the lesser curvature thus separating the clasp muscle fibers from underlying submucosa. The clasp muscle fibers were removed from the sling fibers by sharp dissection and cut into 18-24 smooth muscle strips approximately 2 X 2 X 8 mm with the long axis parallel to the direction of the muscle fibers. Similar muscle strips (32-48) were cut from the sling muscle fibers. The LEC fibers (32-48) were obtained from the thickened area of the esophagus approximately 1-2 cm proximal to the stomach. At the conclusion of the experiment each muscle strip length between the suspending clips was measured with a caliber, cut off from the suspending clips and the tissue participating in the contractile response was weighed. The average length and weight of the muscle strips was 7.9±0.2 mm and 7.6±0.3 mg for the clasp fibers, 8.9±0.2 mm, 8.8±0.4 mg for the sling fibers and 8.0±0.1 mm and 13.7±0.5 mg for the LEC fibers. These dissections were performed to isolate the clasp, sling and LEC fibers and avoid the confounding effects of the overlying longitudinal smooth muscle or underlying mucosal or submucosal layers.

Contraction and relaxation responses of smooth muscle strips: Responses of the clasp muscle fibers were studied first followed by sling fibers then LEC fibers. The specimen remained on ice immersed in the organ transport medium in the interim. These smooth muscle strips were suspended in 10 ml muscle baths in Tyrode's solution continuously bubbled with 95% O_2/5% CO_2 and maintained at 37C. The length of the muscle strips were adjusted over 15 minutes to produce 1 gram of basal tension and then strips were allowed to accommodate to the muscle bath for an additional 15 minutes. The strips were then washed

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by 3 exchanges of fresh bathing solution and allowed an additional 15 minutes of accommodation prior to investigation of their contractile response. All studies were completed within 36 hours after receiving the tissues.

Strips were exposed to a near maximally effective concentration of bethanechol (30 μM) then, after reaching a plateau in tension, nicotine was added. After a 60 minute repeated washing (3 bath exchanges every 15 min) and re-equilibration period, strips were exposed to potential inhibitors of relaxations for 30 minutes then rechallenged with bethanechol followed by nicotine. The following compounds were tested for their ability to inhibit relaxation: the nitric oxide synthase (NOS) inhibitor L-NAME (10, 30 and 100 μM), the β adrenergic receptor antagonist propranolol (1, 3 and 10 μM), the glycine receptor antagonists strychnine (3, 10 and 30 μM) and ginkgolide B (10 μM), the $GABA_A$ receptor antagonists bicuculline (methiodide salt, 10,30 and 100 μM) and SR95531 (30 μM) and the sodium channel blocker tetrodotoxin (1 μM). Agonists of these receptor systems were also added to separate strips after inducing a plateau of tension with 30 μM bethanechol. This included the nitric oxide donor SNAP (1, 10 and 100 μM), the beta adrenoceptor agonist isoproterenol (1, 10 and 100 μM), the $GABA_A$ receptor agonist muscimol (10, 10,100 and 1,000 μM), and the glycine agonists taurine and glycine (0.1, 1 and 10 mM).

Immunohistochemistry: After the above experiments muscle strips were flash frozen on dry ice, cryosectioned into 12 micrometer longitudinal sections, mounted onto charged slides (Fisher Plus slides), and then fixed for 30 minutes in 4% paraformaldehyde in PO_4 buffer (pH 7.4). After drying overnight, adjacent sections, on slides, were washed in phosphate buffered saline (PBS), incubated in 3% H_2O_2 in methanol (4^oC) for 30 min, washed, incubated

in 10% goat serum with 4% dried milk in PBS and 0.3% triton-X-100 for 30 min, and then overnight at 24^{o}C with either a NeuN antibody (a neuronal nuclear marker; Millipore, Billerica, MA; catalog no. MMB337; 1:200 dilution in PBS) or anti-PGP9.5 (a pan neuronal marker; Abcam, Cambridge, MA, catalog no. ab8189, 1:50 dilution in 10% goat serum in PBS). After washing, sections were incubated for 2 hrs at 24^{o}C with goat anti-mouse peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. HRP was visualized as a black immunoreactive stain using diaminobenzidene (DAB) with cobalt (Sigma-Aldrich, St. Louis, MO). These sections were counterstained lightly with eosin. One cm x 1 cm blocks of tissue (full thickness) were collected from an esophagus and stomach specimen that was fixed for 48 hours in 4% paraformaldehyde in PO_4 buffer as a whole organ from an additional donor with no known history of GERD, equilibrated in 30% sucrose in PO_4 buffer for 3 days, cryosectioned into full thickness 12 micrometer slices, mounted onto charged slides, and stained with hematoxylin and eosin.

Data and statistical analysis: IC_{50} values for the antagonists and EC_{50} for agonists were determined by linear regression of the dose-effect data (Tallarida, 2000). For each condition, a minimum of 6 separate muscle strips obtained form 2-4 different donors were used. Statistical analysis of the contractility data was performed using analysis of variance (ANOVA) followed by Newman-Keuls post-hoc tests. In the event that homogeneity of variance tests (Hartley's F-max, Cochran's C or Bartlett's Chi-Square) showed statistically significant differences in the variances between groups, the data was converted to ranks prior to performing the ANOVA. Differences were considered statistically significant at $p<0.05$.

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Results:

Figure 1A ,B shows hematoxylin and eosin stained sections from the esophagus and stomach specimen that was fixed as a whole organ. Figure 1A is a cross section of the esophagus at approximately 2 cm proximal, to the gastroesophageal junction. Figure 1B is a cross section of the stomach at approximately 1 cm distal to the GEJ on the lesser curvature. LEC, clasp and sling regions are indicated in the inner circular muscle layer. After the in-vitro contractility assays, smooth muscle strips from these same 3 regions were fixed, sectioned and probed immunohistochemically for neuronal cell bodies (NeuN+) and axons (PGP9.5+). As shown in Figure 1 C, D, F, G, I and J, small NeuN+ neuronal neurons are located between individual smooth muscle fibers located within the inner circular layer of each region. Each set of neuronal cell bodies were associated with Pgp9.5+ neuronal axons (Figure 1 E, H and K).

All muscle strips developed decreased tone below the 1 gram of basal tone during the second 15 minute accommodation period (0.71±0.03 for clasp, 0.51±0.02 g for sling and 0.88±0.03 g for LEC fibers). At the end of the 15 minute period after the wash, immediately before bethanechol addition the basal tone was 0.54±0.02 g for clasp, 0.52±0.02 g for sling and 0.69±0.03 g for LEC fibers. This basal tension after the second 15 minute period and the tension immediately prior to the first exposure to bethanechol was statistically significantly higher in LEC fibers than clasp or sling fibers ($p<0.01$). The effect of exposure to nicotine was determined without pre-contracting the strips to bethanechol in a separate group of 8 muscle strips from each of the 3 different muscle fibers. 1 mM nicotine caused relaxation of clasp fibers (-0.22±0.07 g), contraction of sling fibers (2.02±0.06 g) and relaxation of LEC fibers (-0.17±0.06 g). The response of the sling fibers was statistically significantly greater than the clasp and LEC responses ($p<0.01$).

Nicotine induced relaxations of bethanechol pre-contracted muscle strips: Bethanechol induces contraction of clasp and sling fibers with similar potency: EC_{50} = 8.4±1.8 μM for clasp, 11±1.3 μM for sling and 7.1±1.2 μM for LEC fibers (figure 2). Based on these findings 30 μM bethanechol was used to pre-contract clasp, sling and LEC fibers to near maximal tension. Cumulative addition of nicotine (10 μM-1 mM) induces relaxation of these pre-contracted muscle strips (figure 3, open symbols). In clasp fibers, the maximal relaxation occurred at 100 μM nicotine and in LEC fibers maximal relaxation occurred at 30 μM. Higher concentrations induced relaxations, however, these were not as great as the effect of 100 μM, possibly due to receptor desensitization. In sling fibers, the maximal relaxation occurred at 1 mM nicotine. In separate strips from different donors, a single dose of 1 mM nicotine induced relaxations that were not statistically significantly different from the maximal relaxation obtained during the cumulative addition of nicotine (figure 3, closed symbols). Based on these results, a single concentration of 1 mM nicotine was used for determination of IC_{50} values for the antagonists. Representative traces from these experiments in clasp muscle fibers are shown in figure 4. As can be seen in the traces at the left in figure 4, a relaxation is induced in response to 1 mM nicotine added after the clasp strips contracted to 30 μM bethanechol. The traces on the right in figure 4 show representative traces of the blockade of these nicotine induced relaxations by the different antagonists as detailed below. Similar representative traces for sling and LEC fibers can be found in supplemental figures 1 and 2 respectively.

Inhibition of nicotine induced relaxations: Our initial approach was to determine which inhibitors either blocked the maximal nicotine induced relaxation or shifted the nicotine concentration effect curve to the right. Thus, muscle strips were pre-contracted with 30 μM bethanechol and induced to relax with 3 cumulative additions of increasing concentrations of

nicotine (0.1, 0.3 and 1.0 mM nicotine). Following extensive washing and addition of either vehicle or antagonist for 30 min, the muscle strips were stimulated to contract with 30 µM bethanechol and then relax to the 3 concentrations of nicotine. Five of the inhibitors tested: bicuculline (100 µM), L-NAME (100 µM), propranolol (10 µM), strychnine (300 µM) and tetrodotoxin (TTX, 1 µM), significantly attenuated the maximal nicotine induced relaxation in clasp fibers. Similar findings were found in sling and LEC fibers, except that TTX did not attenuate the relaxation in sling or LEC fibers. The mean ± sem of the effect of these inhibitors on the contraction induced by bethanechol and on maximal relaxation response induced by nicotine for both clasp, sling and LEC fibers are shown in the bar graphs of figure 5. Detailed concentration response curves for nicotine with each antagonist are shown in supplemental figures 3-5 for clasp, sling and LEC fibers respectively. None of the antagonists significantly affected the bethanechol induced contraction in clasp, sling or LEC fibers. In a separate group of strips, a single concentration (100 µM) of the ganglionic nicotinic receptor antagonist hexamethonium was tested in the 3 different tissues for its ability to block 1 mM nicotine induced relaxation of 30 µM bethanechol pre-contracted strips. Hexamethonium had no effect on bethanechol induced contractions but caused a statistically significant inhibition of the nicotine induced relaxation ($p<0.05$ for clasp, $p<0.01$ for sling and LEC fibers, data not shown).

In a second series of experiments performed on different specimens from the initial studies described above, various concentrations of the antagonists (L-NAME, propranolol, bicuculline, and strychnine) were used to determine IC_{50} values. In these experiments, a single maximally effective concentration of 1 mM nicotine was used to induce relaxations of the muscle strips pre-contracted with 30 µM bethanechol. Shown in figure 6, is the effect of the individual concentrations of propranolol on both the contractile response to bethanechol and the

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relaxation response to nicotine for clasp, sling and LEC fibers. Similar data was obtained for bicuculline, L-NAME and strychnine and can be found in supplemental figures 6-8 for clasp, sling and LEC fibers respectively. The relaxations were normalized to the pre-antagonist relaxations from the same muscle strip in order to determine average percent effect for each dose of antagonist (Figure 7). The dose-effect data for each were analyzed by linear regression of effect on log dose from which the IC_{50} was determined as described by Tallarida (Tallarida, 2000) and shown in Table 1. The IC_{50} values for L-NAME, bicuculline and strychnine were not statistically significantly different between clasp, sling and LEC fibers. The IC_{50} value for propranolol was significantly lower in sling and LEC than in clasp fibers, demonstrating that propranolol is more potent in inhibiting nicotine induced relaxations in sling and LEC fibers than in clasp fibers.

The potencies of bicuculline and strychnine in clasp, sling and LEC fibers (Table 1) were much lower than their reported potencies at $GABA_A$ (Maggi et al., 1984; Huang and Johnston, 1990) and glycine receptors (Lynch and Lynch, 2004) respectively, and within their reported potency for antagonism of nicotinic receptors (Zhang and Feltz, 1991; Kuijpers et al., 1994; Albuquerque et al., 1998; Demuro et al., 2001). Therefore, other antagonists with no reported activity at nicotinic receptors were used: the $GABA_A$ antagonist SR95531 (Tonini et al., 1989; Zhang and Feltz, 1991) and the glycine receptor antagonist ginkgolide B (Kondratskaya et al., 2002; Kondratskaya et al., 2004) in 6-8 separate clasp and sling muscle strips from 2 different donors. Ginkgolide B (10 μM) had no effect on nicotine induced relaxations in clasp or sling fibers and SR95531 (30 μM) was also ineffective in clasp fibers but reduced nicotine induced relaxations by 35±8% (n=11 strips from 2 donors) in sling fibers.

Agonist induced relaxations of bethanechol pre-contracted muscle strips: In order to determine whether agonists of these receptor systems could mimic the nicotine induced relaxations, strips were contracted with 30 µM bethanechol then exposed to increasing concentrations of isoproterenol, SNAP, muscimol, taurine and glycine. Neither taurine nor glycine had any relaxatory effect on plateau tension at concentrations up to 10 mM (data not shown). Figure 8 shows concentration effect curves for muscimol, isoproterenol and SNAP along with nicotine for comparison and time control strips that indicate the slow reduction in tension over the same time period in 30 µM bethanechol contracted strips that were not exposed to any additional agents. Muscimol in both clasp and sling fibers and isoproterenol in sling fibers did not induce a greater degree of relaxation than in the time control strips and therefore did not mimic the nicotine induced relaxations. SNAP in both clasp and sling fibers and isoproterenol in clasp fibers induced relaxations that were similar in extent to the nicotine induced relaxations.

Discussion:

We previously demonstrated that the in-vivo resting tone of the clasp and sling muscle fibers is mediated in large part by neuronal release of acetylcholine acting on muscarinic receptors (Brasseur et al., 2007). We also demonstrated that the clasp/sling muscle fiber complex does not contribute to the pressure generated by the HPZ in patients with GERD as they do in normal volunteers (Miller et al., 2009). In addition, clasp fibers from organ donors with histological evidence of reflux (Barrett's esophagitis) have a reduced contractile response compared to donors without GERD (Miller et al., 2010). These finding suggest that reflux may be the result of an abnormal clasp/sling muscle fiber complex which could be due to either an underactive contractile mechanism or an overactive relaxation mechanism. The aim of the current study is to understand the mechanism of sling and clasp muscle fiber relaxation in response to activation of nicotinic receptors in the setting of contraction through activation of muscarinic receptors.

We previously reported that the bethanechol induced maximal contraction is greater in the sling muscle fibers than in all other fibers studied in these subjects (Braverman et al., 2009). This result is in general agreement with previous studies showing that human gastric sling muscle fibers contract significantly greater to acetylcholine than human clasp muscle fibers (Tian et al., 2004). Over the span of the last three decades, the formation and regulation of the HPZ by the gastric sling and clasp muscle fibers has been studied in vitro. Differences have been reported between human clasp and sling stomach muscle fibers in the sensitivity and maximal responses to acetylcholine, dopamine, phenylephrine, and isoproterenol (Tian et al., 2004).

Most of the previously published studies pertaining to the relaxation of gastrointestinal smooth muscle focused on either EFS induced relaxations or inhibition of stretch induced active tone. EFS is known to induce effects on smooth muscle strips indirectly, by inducing release of neurotransmitters from nerve endings. In the current study, TTX prevented a large portion of the nicotine mediated relaxation of the clasp fibers. This indicates that a portion of the nicotinic mediated relaxation of the clasp fibers is neuronally mediated and likely results from nicotine induced stimulation of action potentials. Therefore these nicotinic receptors must be located at sites along the nerve more proximal than the neuromuscular junction, for example on the enteric ganglion cells or axons rather than at the nerve terminals. Because TTX was ineffective in preventing nicotine induced relaxations in sling and LEC fibers, this indicates that nicotine induced stimulation of action potentials are not required for relaxations and thus these nicotinic receptors are likely to be located more distally and closer to the neuromuscular junction.

With regard to the location of enteric neuronal cell bodies in the clasp, sling and LEC regions, our immunohistochemical investigation showed that there were several small neuronal cell bodies located within the inner circular layer itself. The location of these small neurons match those described in the opossum lower gastro esophageal high pressure zone (Sengupta et al., 1987). In that study, small groupings of “atypical” thionin-Sudan black stained (i.e. Nissl stained) neurons were buried within the circular muscle, in addition to groupings of neurons buried within the longitudinal muscle layer and between the longitudinal and circular layers. The latter two areas were not studied here because our focus was on the possible location of neurons within the muscle strips that were examined physiologically. Our findings in the LEC region also match those by Kim et al (Kim et al., 2008), who observed PGP9.5 neuronal

structures buried within muscular layers of esophageal wall of patients with esophageal myopathies. Since our study and Kim's are the first to examine the location of neuronal structures in the GEJ of humans using specific neuronal markers, perhaps the location of these neurons are in fact not "atypical" but the norm.

Neuronal nitric oxide (NO) appears to be a major neurotransmitter mediating nerve evoked relaxations in human gastric clasp muscle fiber strips (Gonzalez et al., 2004). β-adrenergic and D_2 dopamine receptor agonists (Tian et al., 2004) as well as vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP) and adenosine have also been reported to relax human clasp muscle fibers (Gonzalez et al., 2004). The non-adrenergic, non-cholinergic (NANC) neurotransmitters NO, VIP, PACAP, ATP, calcitonin gene related polypeptide (CGRP) and carbon monoxide (CO) all have inhibitory effects in the gastrointestinal tract. Immunohistochemistry of the LES myenteric neurons shows the presence of NOS, the peptides VIP, PACAP and CGRP and the constitutive enzyme heme oxygenase type 2 that is involved in the synthesis of CO (De Man et al., 1991; Murray et al., 1991; Tottrup et al., 1991; Oliveira et al., 1992; Tottrup et al., 1993; Kortezova et al., 1996; Yuan et al., 1998; Farre et al., 2006; Farre et al., 2007).

The role of NO in relaxation of LES smooth muscle has been extensively studied in vitro. Stimulation of the intrinsic nerves by EFS induces an "on" contraction instead of an "on" relaxation in circular esophagogastric junction muscle strips from achalasic patients suggesting that nitrergic neurotransmission is severely impaired (Tottrup et al., 1990). In many different species, NO, NO donors and EFS can induce relaxation and hyperpolarization of smooth muscle strips from the LES by a cyclic GMP (cGMP) pathway. Experiments using cGMP inhibitors and direct measurements of cGMP confirmed that activation of nitrergic myenteric

neuron induces smooth muscle relaxation via the guanylate cyclase-cGMP pathway (Torphy et al., 1986; Barnette et al., 1989; Rattan and Moummi, 1989; Conklin and Du, 1992; Jun et al., 2003; Farre et al., 2006). A current hypothesis proposes that NO inhibits calcium-activated chloride current via intracellular increase of cGMP, leading to inhibitory junction potentials and relaxation (Zhang et al., 2003). Nitric oxide is considered the main inhibitory neurotransmitter at the LES.

Nicotine relaxes carbachol pre-contracted human esophageal muscle strips which is mostly blocked by L-N-nitro arginine and completely blocked by hexamethonium (Gonzalez et al., 2004). Studies using porcine clasp fibers demonstrate that nicotine indirectly relaxes smooth muscle because relaxation is inhibited by hexamethonium and TTX (Farre et al., 2006). Nicotine has been reported to stimulate two independent pathways. One is NO mediated and the other is sensitive to the ATP-type calcium-activated K^+ channel blocker apamin and mediated mostly by P2Y with a minor P2X purinergic receptor contribution (Farre et al., 2006). In the current study we demonstrated that clasp, sling and LEC muscle strips, pre-contracted with bethanechol, showed relaxation with administration of nicotine and that the relaxation was blocked to variable degrees using TTX (only in clasp fibers), the NOS inhibitor L-NAME and the β adrenergic receptor antagonist propranolol. The glycine receptor antagonist strychnine and the $GABA_A$ antagonist bicuculline inhibited relaxations with potencies consistent with nicotinic receptor blockade. Because the glycine receptor antagonist ginkgolide B had no effect on nicotine induced relaxations and the glycine agonists taurine and glycine did not induce relaxation we can conclude that the nicotine induced relaxations are not mediated by activation of glycine receptors in clasp or sling fibers. The more selective $GABA_A$ antagonist SR95531 did not affect nicotine induced relaxations in clasp fibers but reduced sling

fiber relaxations by approximately one third. Thus nicotine induced relaxations are not mediated by activation of $GABA_A$ receptors in clasp fibers but at least a portion of the relaxation in sling fibers is mediated by $GABA_A$ receptors.

The fact that TTX blocks the nicotine mediated relaxation in clasp fibers suggests that nicotine causes the relaxation early on in the pathway at a neuromuscular junctional site. Historically, NO has been thought to be the final mediator of the relaxation. However, NOS inhibition did not completely block the relaxation, suggesting that other non-nitrergic neurotransmitters are also involved. The nitric oxide donor SNAP caused the same degree of relaxation in both clasp and sling fibers as nicotine. The β adrenoceptor agonist isoproterenol relaxed clasp fibers nearly as much as nicotine but did not relax sling fibers whereas the $GABA_A$ agonist muscimol did not relax either clasp or sling fibers. It is not entirely clear how the β adrenoceptor antagonist propranolol can completely prevent the nicotine induced relaxations in both clasp and sling fibers whereas the β adrenoceptor agonist isoproterenol only relaxes the clasp and not sling fibers. Likewise it is unclear how the $GABA_A$ antagonist SR95531 can inhibit nicotine induced relaxations in sling fibers by 35% whereas the relaxation effect of the $GABA_A$ agonist muscimol was not evident in sling fibers. One possible explanation for these findings is that a threshold concentration of each of the neurotransmitters (norepinephrine and GABA) is needed in order to induce a relaxation. Therefore, any one of the individual antagonists could prevent the relaxations. A second explanation is that the neural pathways involved in relaxation are connected in series rather than in parallel. Therefore blocking one neurotransmitter blocks the action of the other neurotransmitter and effectively blocks the relaxation.

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Based on the findings of this study, we can make the conclusions that, in the face of muscarinic receptor stimulation, activation of nicotinic receptors on the enteric neurons in GEJ smooth muscle causes release of multiple substances that relax the clasp ,sling and LEC smooth muscles including NO in clasp, sling and LEC fibers, norepinephrine acting on β adrenoceptors in clasp fibers and GABA acting on $GABA_A$ receptors in sling fibers. Because selective relaxation of these particular smooth muscle fibers of the GEJ are early events in the TLESRs that mediate GERD, identification of agents that selectively prevent these relaxations may be useful therapeutic agents to treat GERD by preventing TLESRs.

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Authorship Contribution

Participated in research design: Braverman, Vegesna, Miller, Ruggieri

Conducted experiments: Braverman, Vegesna, Barbe, Tiwana, Hussain, Ruggieri

Contributed new reagents or analytic tools: Barbe

Performed data analysis: Braverman, Vegesna, Ruggieri

Wrote or contributed to the writing of the manuscript: Braverman, Vegesna, Miller, Ruggieri

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Footnotes:

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Legends for figures:

Figure 1. Photomicrographs of histochemistry and immunohistochemistry. Panels A and B are composite low power images of hematoxylin and eosin stained sections obtained from an esophagus and stomach specimen that was fixed as a whole organ. Scale bar indicates 1,000 µm. Panel A is a cross section of the entire esophagus approximately 2 cm proximal, cephalic or orad from the gastro esophageal junction defined by the cardiac notch. Panel B is a cross section taken approximately 1 cm distal to the GEJ on the lesser curvature of the stomach. Panels C-K are images obtained from smooth muscle strips that were fixed, sectioned and subjected to immunohistochemical staining after performing the in-vitro contractility assays. C-E are clasp muscle fibers, F-H are sling muscle fibers and I-K are LEC muscle fibers. Panels C, D, F, G, I and J are stained for the neuronal nuclear marker NeuN. Panels E, H, and K are stained for the pan neuronal marker Pgp9.5. Panels E, H and K are from sections adjacent to panels D, G and J respectively.

Figure 2. Bethanechol concentration response curves for human clasp, sling and LEC muscle fiber contractile response. Contraction is shown in grams (A) and as a percent of the maximal contraction (B). * denotes statistically significant difference in the maximal contraction between clasp and sling fibers and between sling and LEC fibers. n=14 muscle strips from 4 specimens for clasp, n=37 muscle strips from 8 specimens for sling and n=30 muscle strips from 6 specimens for LEC.

Figure 3. Nicotine concentration response curves for relaxation of bethanecol (30 µM) pre-contracted human clasp and sling fibers. The data is shown as percent relaxation from the maximal tension after bethanechol addition. The number of muscle strips for the clasp fiber cumulative concentration response curve n=36 from 4 specimens and n=88 from 8 specimens

for clasp fiber non-cumulative 1 mM nicotine response. For the sling muscle fibers n=98 strips from 5 specimens for the cumulative concentration response curve and n=114 strips from 5 specimens for non-cumulative 1 mM nicotine response. For the LEC muscle fibers n=45 strips from 2 specimens for the cumulative concentration response curve and n=86 strips from 4 specimens for non-cumulative 1 mM nicotine response.

Figure 4. Representative traces of clasp fiber contractile responses to bethanechol, the relaxation response to nicotine and the inhibitory effects of bicuculline, L-NAME, propranolol and strychnine on the nicotine induced relaxations. Similar results were obtained with sling fibers as shown in supplemental figure 1.

Figure 5. Mean ± SEM of the maximal contraction to 30 µM bethanechol (A, B, C) and the maximal relaxation response (D, E, F) to nicotine (NIC, 0.1, 0.3, and 1 mM) before (open bars) and after (shaded bars) 30 minute exposure to vehicle (H_2O, Veh), 100 µM bicuculline (BIC), 100 µM L-NAME, 10 µM propranolol (Prop), 10 µM strychnine (Stry) and 1 µM tetrodotoxin (TTX). For clasp and sling fibers, all bars represent mean ± SEM from at least 8 muscle strips from 4 different specimens except for TTX which was derived from at least 6 muscle strips from 3 different specimens. For LEC fibers, n=at least 6 muscle strips from 4 specimens except for Veh (2 specimens) and TTX (1 specimen). Statistical difference between pre and post responses is indicated by * ($p<0.05$) and ** ($p<0.01$).

Figure 6. Mean ± SEM of the contraction response to bethanechol and relaxation responses to nicotine before (open bars) and after (shaded bars) 30 minute exposure to vehicle (H_2O, VEH) or propranolol (1, 3 and 10 µM) for clasp fibers (A) ,for sling fibers (B, 0.1, 0.3, 1, 3, and 10 µM) and for LEC fibers (C, 1, 3 and 10 µM). Statistical difference between pre and post responses is indicated by * ($p<0.05$) and ** ($p<0.01$). n=4-15 clasp muscle strips from 2-4

specimens, n=4-30 sling muscle strips from 2-4 specimens and n=3-12 LEC muscle strips from 2-4 specimens.

Figure 7. Concentration response curves for bicuculline (BIC), L-NAME, propranolol (PROP) and strychnine (STRY) inhibition of 1 mM nicotine induced relaxations of 30 µM bethanechol pre-contracted clasp (A), sling (B) and LEC (C) muscle fibers. The data is shown as the percent (mean ± SEM) of the relaxation response to nicotine after the addition of antagonist to the relaxation response to nicotine before the addition of antagonist calculated for each muscle strip. These curves were derived from between 4-15 clasp muscle strips from 2-4 specimens, 4-30 sling muscle strips from 2-4 specimens and 3-12 LEC muscle strips from 2-4 specimens for each concentration of antagonist.

Figure 8. Agonist concentration response curves for relaxation of 30 µM bethanechol pre-contracted human clasp (A) and sling (B) muscle fibers. The data shown is the mean ± sem grams relaxation in response to cumulative addition of agonist. The data for nicotine is the same as in figure 2, for comparison purposes. Clasp fibers; for time control, n=4 strips from 1 specimen; for muscimol, n=9 strips from 2 specimens; for isoproterenol n=15 strips from 3 specimens; for SNAP, n=16 strips from 3 specimens. Sling fibers; for time control, n=5 strips from 2 specimen; for muscimol, n=13 strips from 3 specimens; for isoproterenol n=25 strips from 4 specimens; for SNAP, n=19 strips from 4 specimens.

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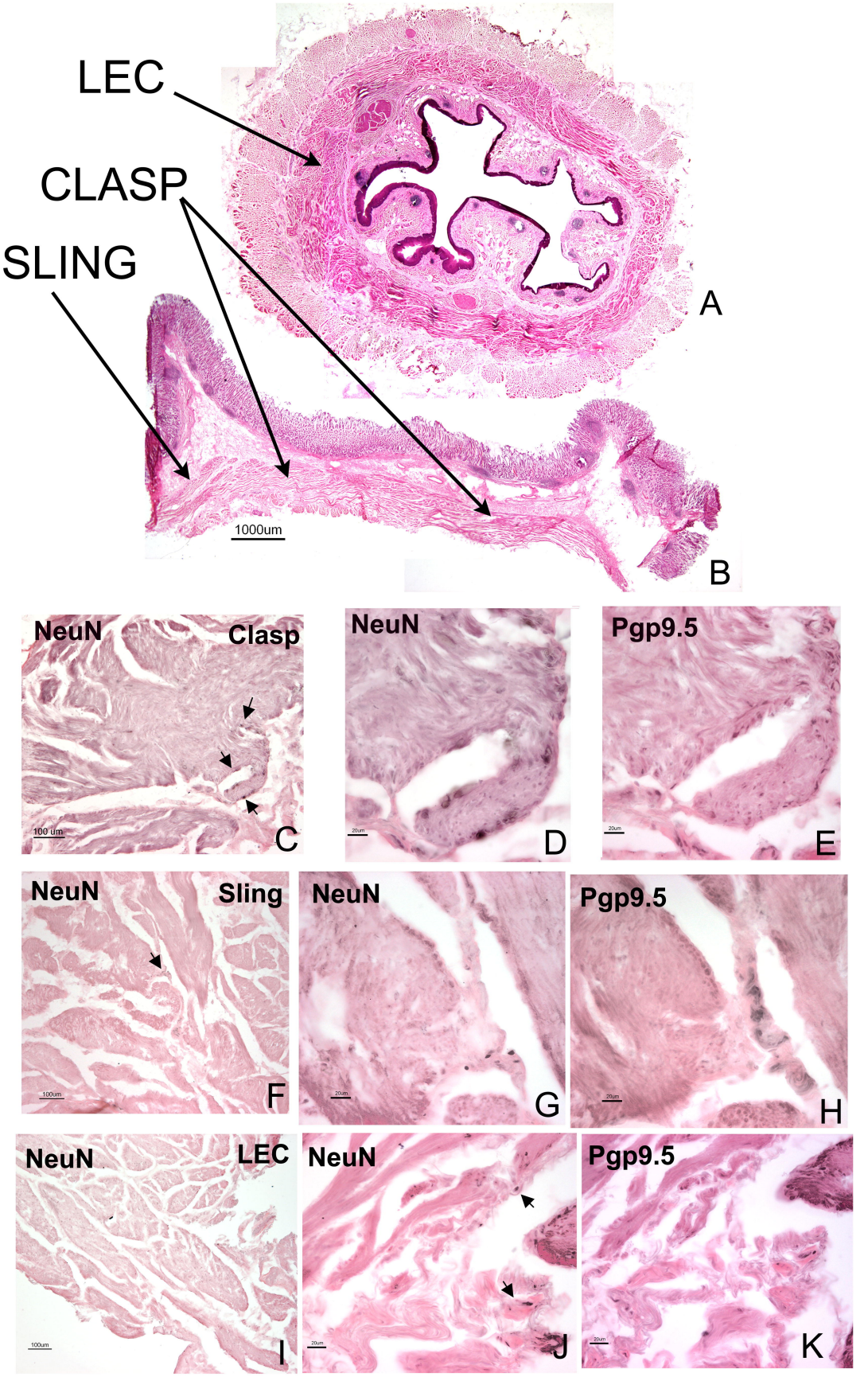
Table 1. Potencies of antagonists for inhibition of nicotine induced relaxation. Values are derived from the data displayed in figure 7.

Antagonist	Clasp IC_{50} (μM)	Sling IC_{50} (μM)	LEC IC_{50} (μM)
Bicuculline	38.5±3.4	47.6±7.4	85±9.1
L-NAME	24.7±6.0	7.9±1.2	102±94
Propranolol	6.1±2.1	1.27±0.47*	1.83±1.1
Strychnine	9.0±1.1	26.2±17	44.9±28


LEC
CLASP
SLING
A
1000um
B
NeuN
Clasp
100 um
C
NeuN
D
Pgp9.5
E
NeuN
Sling
100um
F
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G
Pgp9.5
H
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LEC
100um
I
NeuN
J
Pgp9.5
K

Figure 1

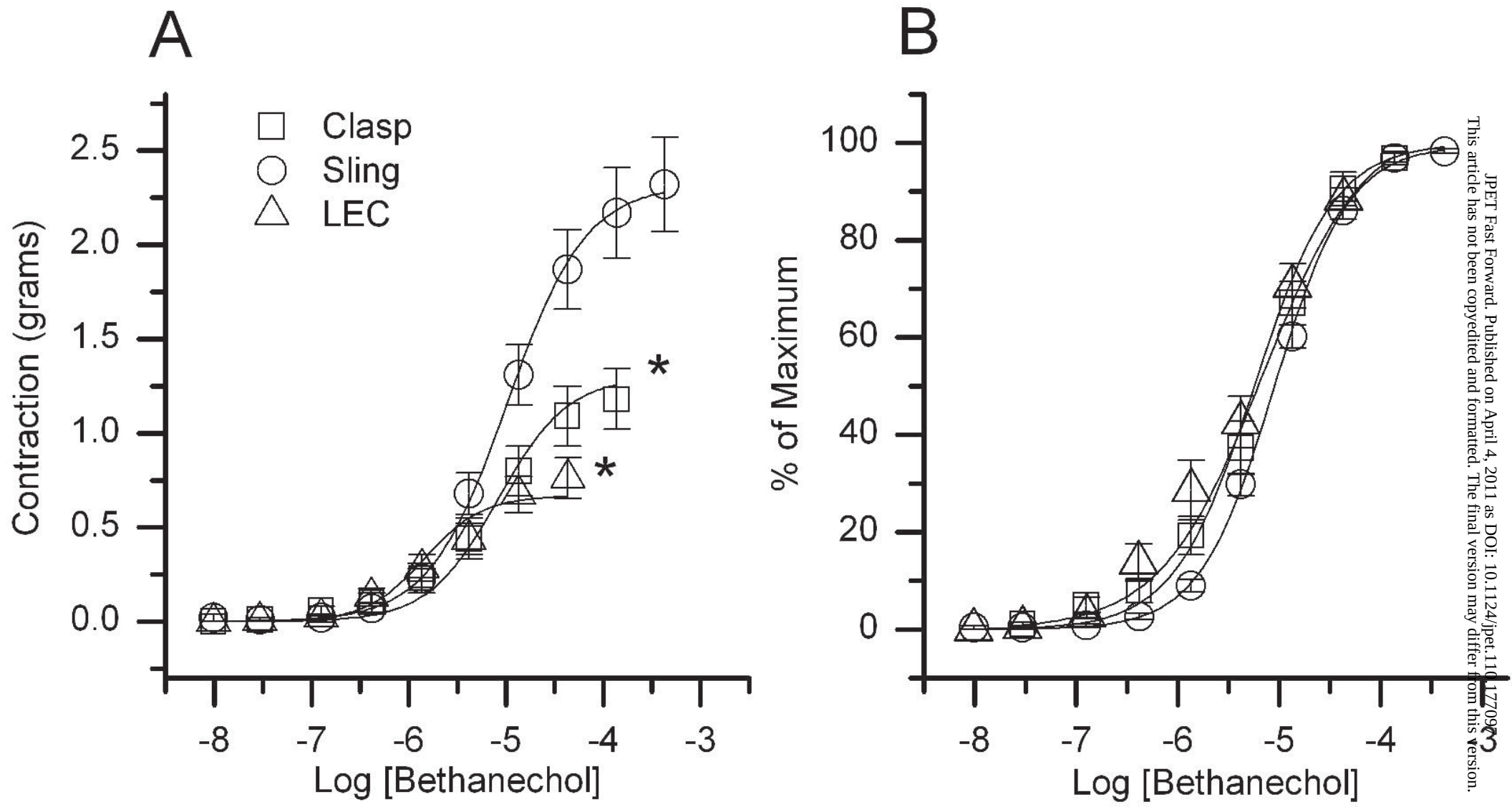

Figure 2

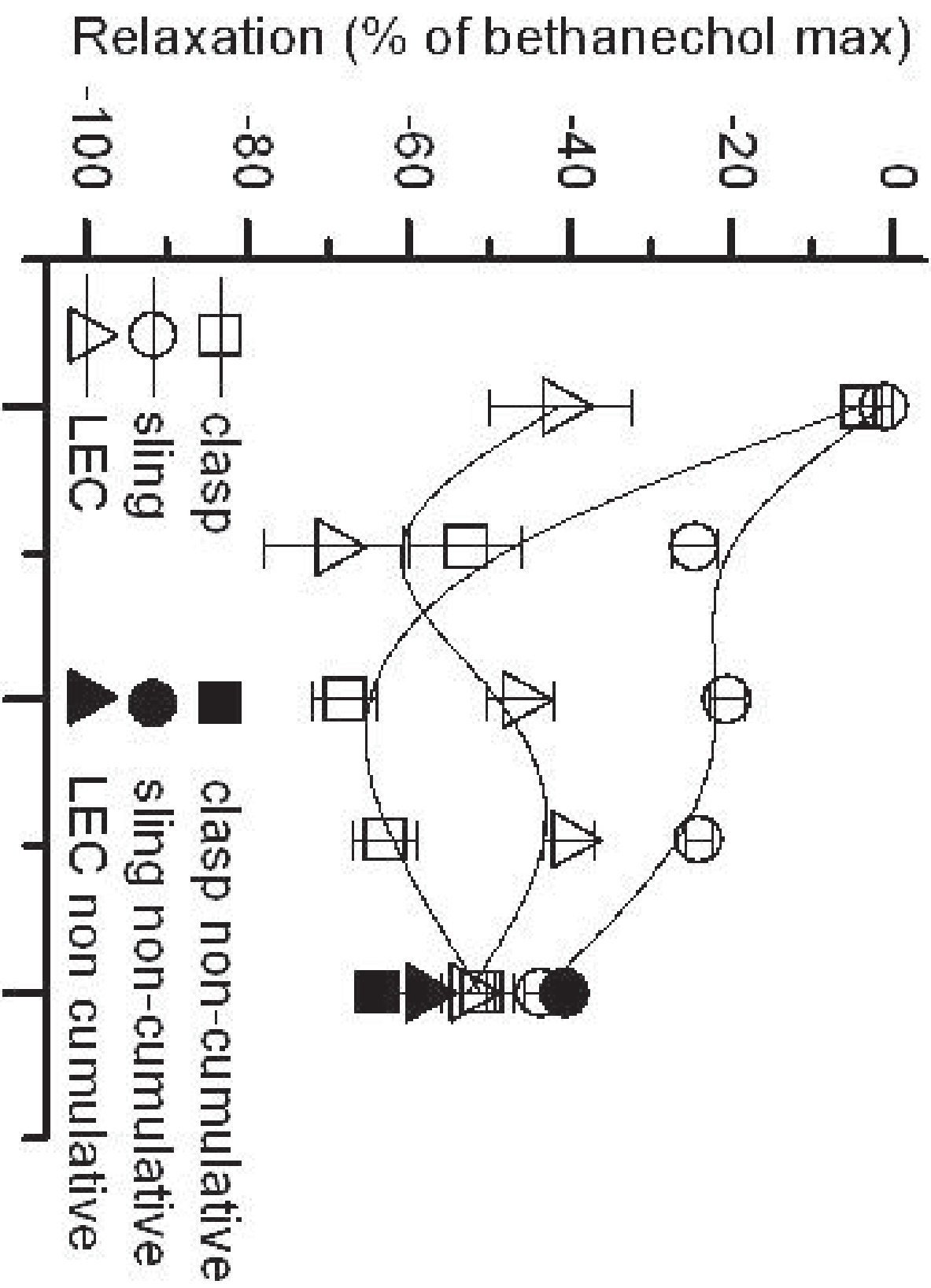

Figure 3

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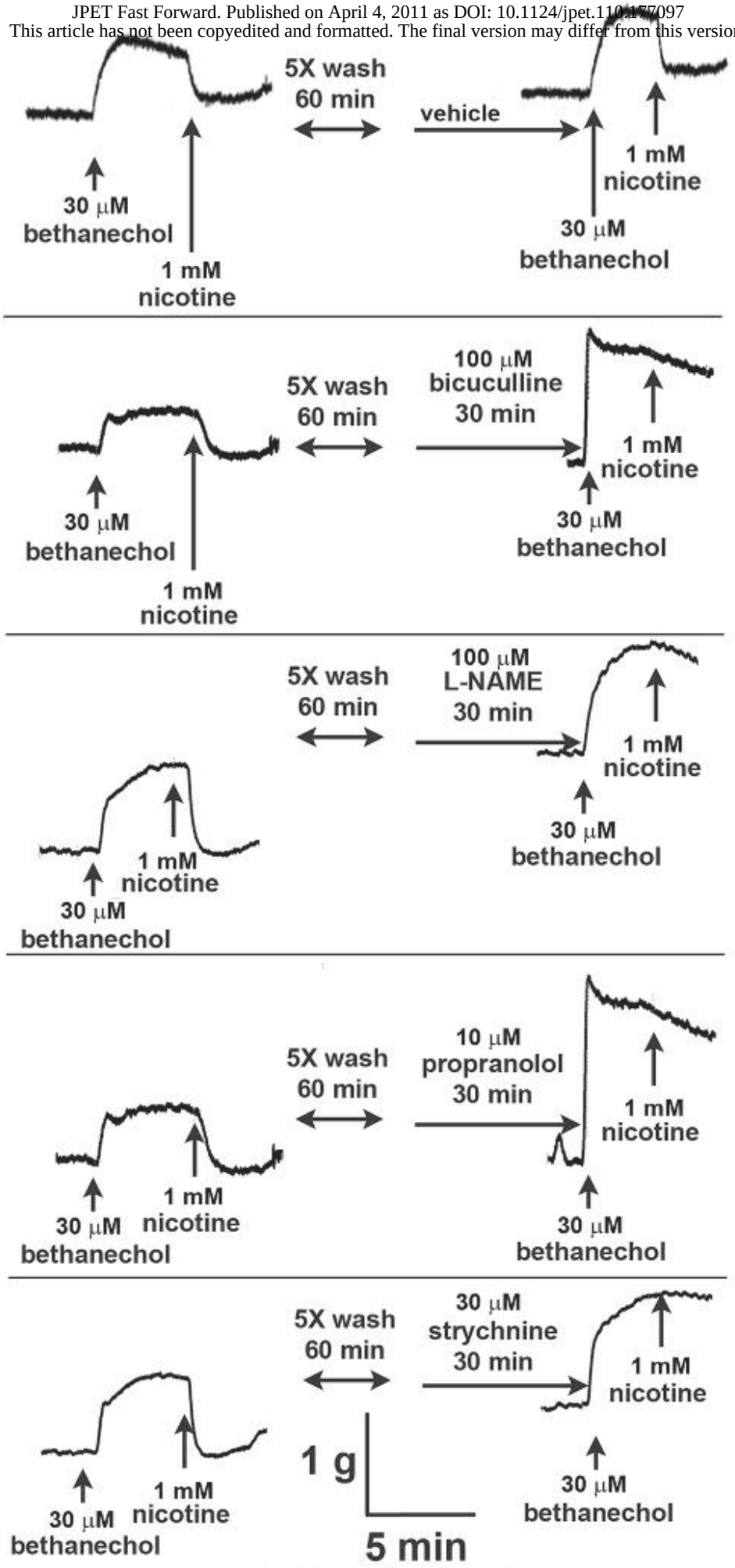

Figure 4

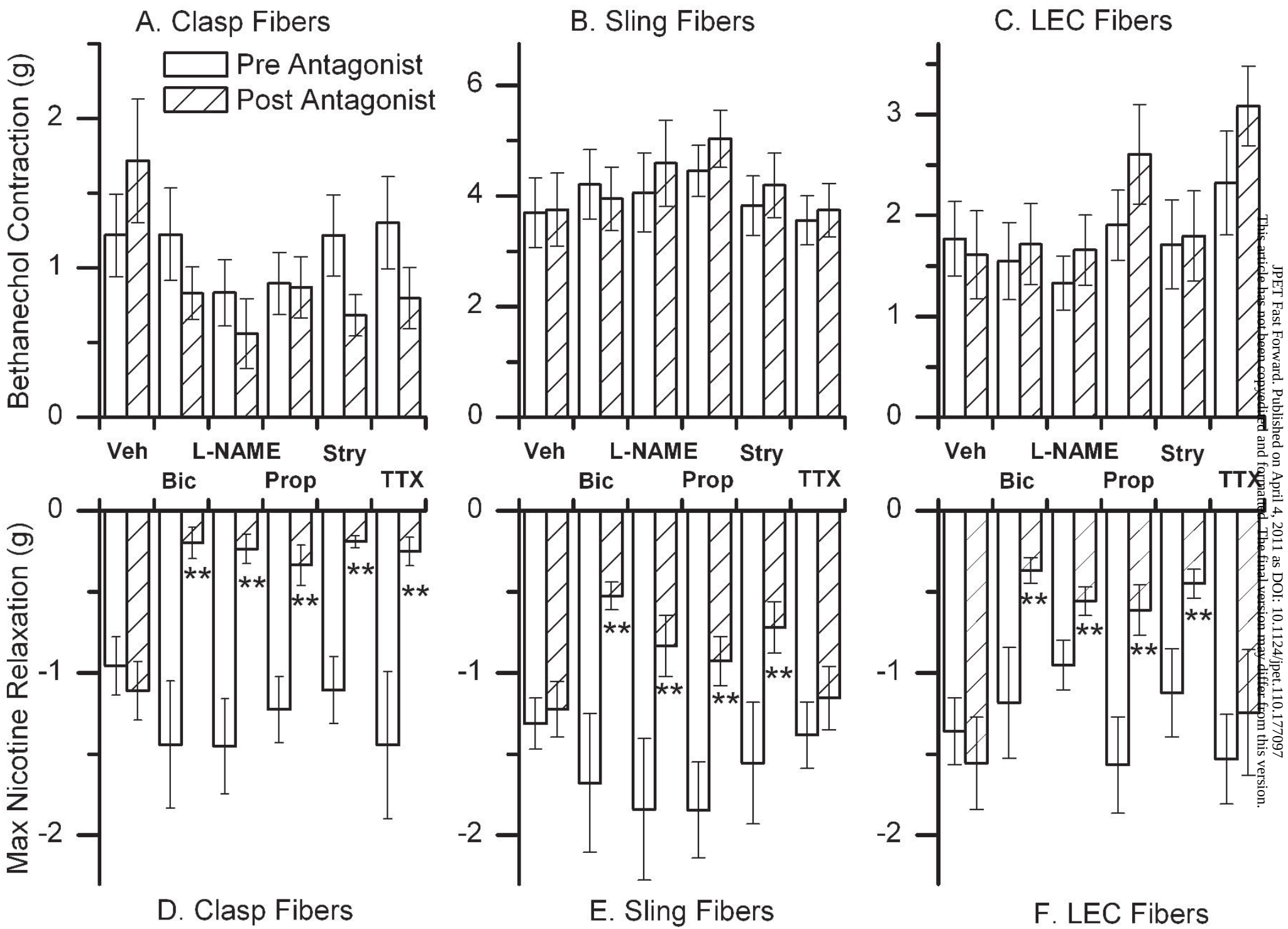

Figure 5

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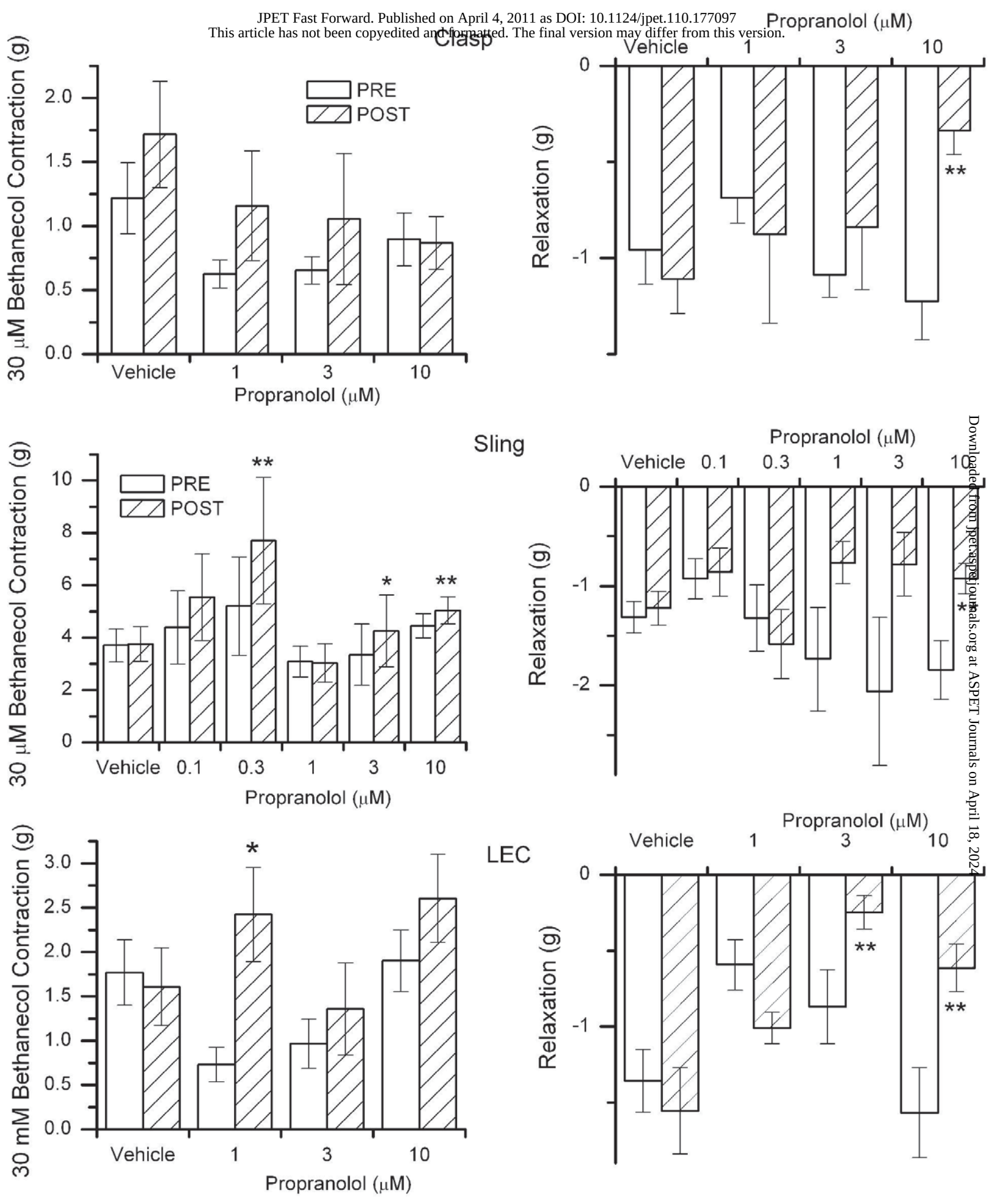


Figure 6

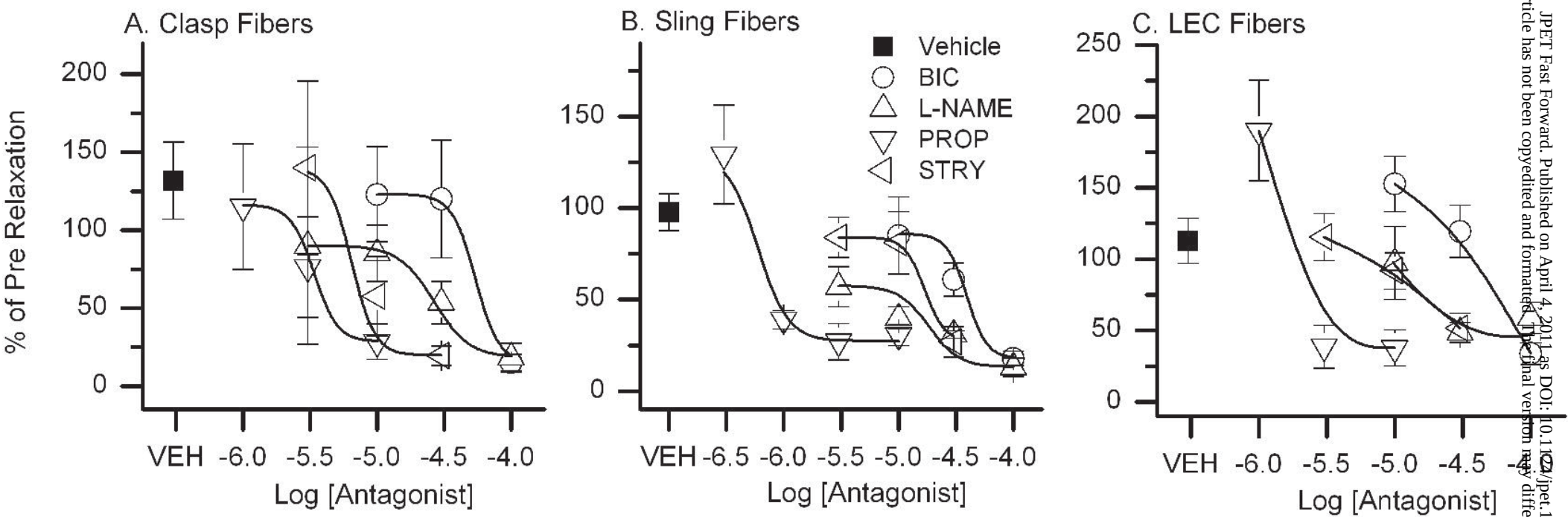
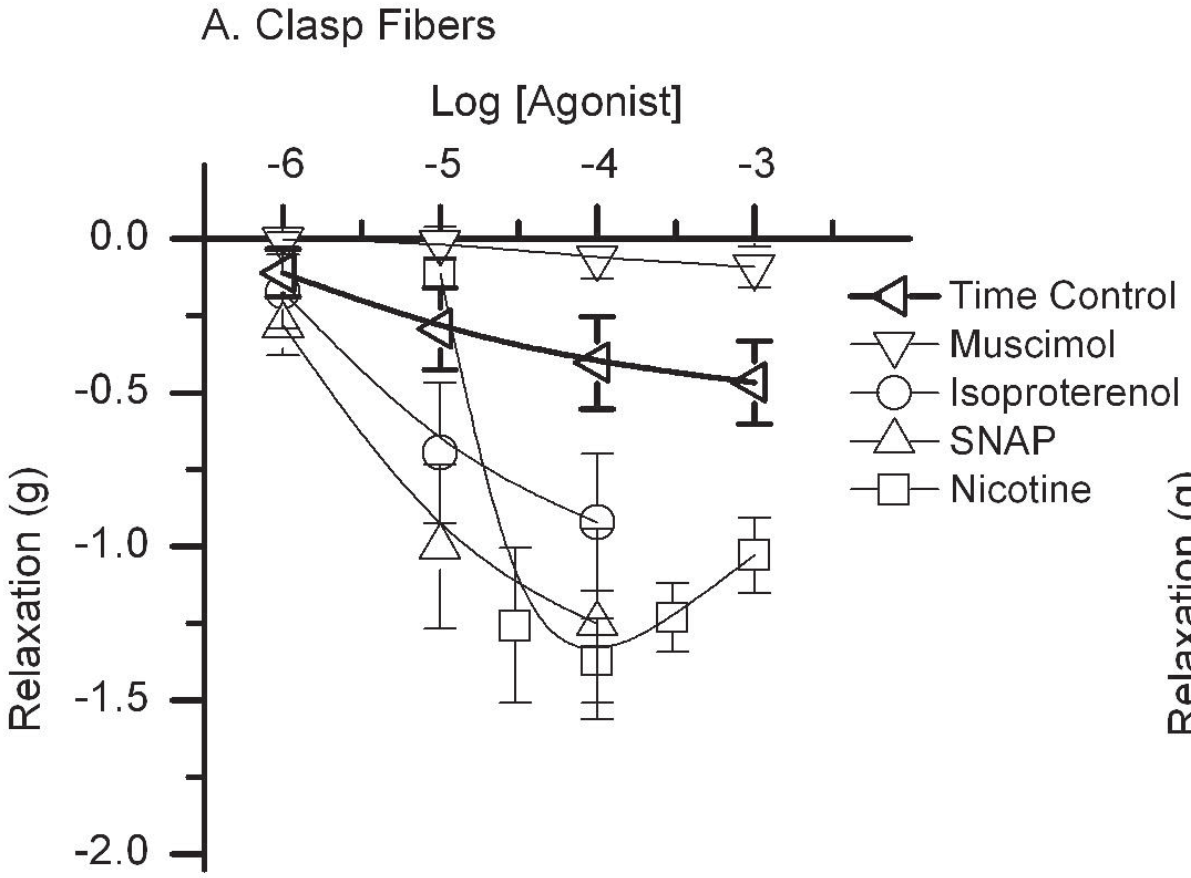


Figure 7



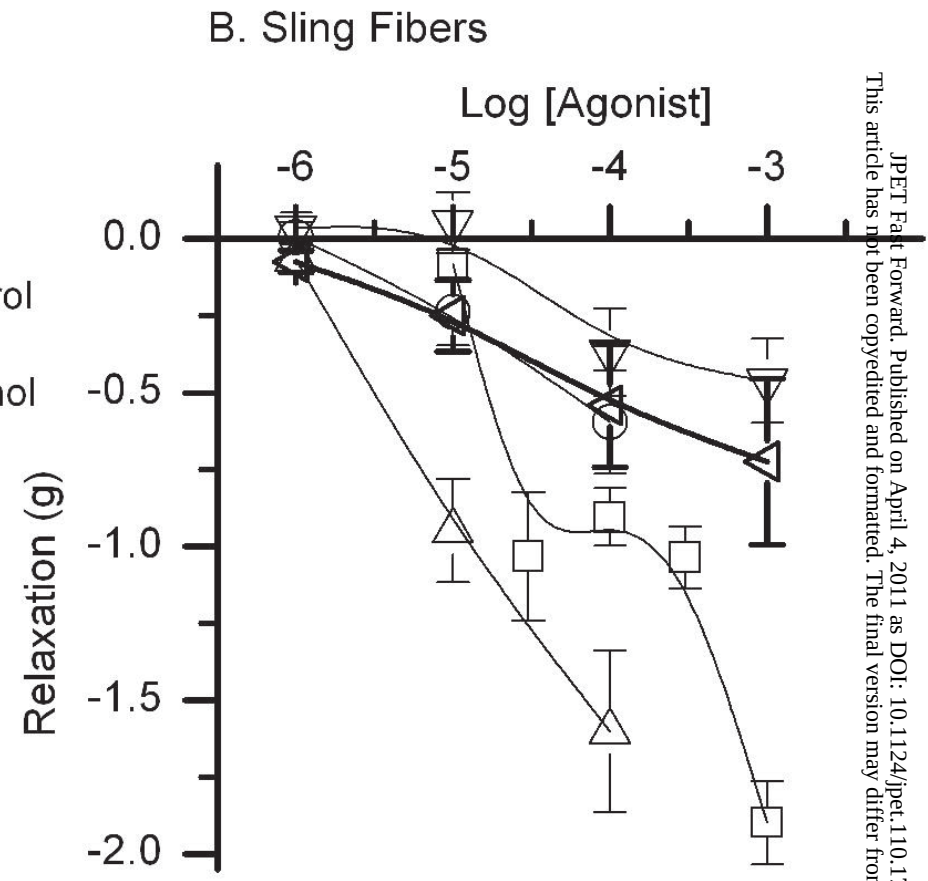


Figure 8