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Smooth Muscle Pharmacology in the Isolated Virgin and Pregnant Rat Uterus and Cervix

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Non-standard Abbreviations:

5-HT	5-hydroxytryptamine
carbachol	2-[(aminocarbonxyl)oxy]-N,N,N-trimethylethanaminium chloride
CGRP	calcitonin gene related peptide
Forskolin	(3R,4aR,5S,6S,6aS,10S,10aR,10bS)-6,10-10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-3-vinyldodecahydro-1H-benzo[f]chroment-5-yl acetate
NO	nitric oxide
PGF2alpha	prostaglandin F2 alpha; (Z)-7-[(1R,2R,3R,5S)-3,5-dihydroxy-2-[(E,3S)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid
PLC	phospholipase C
SNP	sodium nitroprusside

Recommended Section Assignment: Other (this is smooth muscle pharmacology)

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Abstract

Uterine smooth muscle function is established but comparatively little is known about cervical smooth muscle pharmacology. We performed a proof-of-principle experiment that smooth muscle was expressed in the cervix in both virgin and pregnant rats, using the uterus as a comparator. We tested whether all tissues were pharmacologically responsive to contractile and relaxant agonists. Immunohistochemistry revealed the expression of smooth muscle alpha-actin in all tissues. The isolated tissue bath was used to measure isometric contractility of uterine strips and whole cervices from virgin and pregnant (day 11±2) female Sprague-Dawley rats. We tested classical activators of uterine smooth muscle contraction and relaxation in both uterus and cervix. All tissues contracted to the depolarizing agent potassium chloride, prostaglandin F2alpha, muscarinic cholinergic agonist carbachol and 5-hydroxytryptamine. Unlike other tissues, the pregnant cervix did not contract to oxytocin but the oxytocin receptor was present. Both cervix and uterus (virgin and pregnant) had concentration-dependent, near complete relaxation to the adrenergic agonist norepinephrine and adenylate cyclase activator forskolin. The beta adrenergic receptor agonist isoproterenol was less potent in pregnant cervix *vs* virgin by ~10 fold. All tissues, particularly the cervix, responded poorly to the nitric oxide donor sodium nitroprusside, relaxing ~20% maximally. These findings support the importance of smooth muscle in the cervix, the use of the isolated cervix in pharmacological studies, and a similarity between smooth muscle pharmacology of the non-pregnant uterus and cervix. This work highlights the unappreciated smooth muscle function of the cervix *vs* uterus, and cervical changes in pharmacology during pregnancy.

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Introduction

The uterus and cervix depend on passive elements (collagen, extracellular matrix proteins) to carry out their functions in female reproduction, but coordination of active smooth muscle function is also critical, illustrated by pregnancy and labor & delivery. Pregnancy is defined by the quiescence of uterine smooth muscle and the non-pliant, load-bearing cervix. By contrast, in labor & delivery the uterus contracts forcefully to deliver the fetus and placenta, and to do so the cervix must be soft and compliant. In premature labor, this coordinated process that begins too early is one that would be beneficial to stop. In each situation, the function of smooth muscle of the uterus and cervix is important.

While there is considerable knowledge about uterine smooth muscle reactivity to both contractile and relaxant stimuli (Wray 2007; Lopez-Bernal 2007), far less is known about the smooth muscle function of the cervix (Bryman *et al*., 1985). With the recognition that a short cervix may contribute to premature labor, the tensile strength and smooth muscle content of the human cervix have begun to receive attention (Oxlund *et al.,* 2010a, b). The present study represents an initial comparative analysis of limited smooth muscle pharmacology between the uterus and cervix of virgin and pregnant rats. Most importantly, this is a proof-of-principle study that the smooth muscle reactivity of the isolated cervix can be measured and is pharmacologically modifiable.

Some of the seminal papers on cervical smooth muscle function date back to the 1940's (Adler *et al.,* 1944; Rorie and Newton, 1967). Mitchell and Taggart point to

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the paucity of work done on cervical samples (2009), and this is understandable given the difficulty in obtaining human cervical samples on a regular basis. In the present study, we have isolated a strip of the uterine horn (longitudinal muscle) and whole cervix of the same female rat, and investigated the ability of these two separate tissues to respond to a series of pharmacological agonists in a classical isometric contractile assay. We use the rat pregnant between days 9 and 13 of gestation because this time range is a time sufficiently distanced from initiation of pregnancy. We combine this work with histological and immunohistochemical studies to identify smooth muscle and receptor expression.

Using the uterus as a comparator, we chose to use a group of compounds within the cervix based on their importance in: 1) modifying uterine smooth muscle tone; 2) for their role as neurotransmitters within these reproductive tissues, and 3) for their role in modifying smooth muscle function. We discovered that, like the smooth muscle of the uterus, that of the cervix is pharmacologically modifiable, and that responsiveness to pharmacological agonists changes in pregnancy.

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Methods

Animal Use

All animal use protocols were approved by the *Michigan State University* Institutional Animal Use and Care Committee. These experiments have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 8th edition 2011). Virgin female Sprague-Dawley rats (~225 grams, Charles River between 10-12 weeks of age) were used in these experiments. Bedding from the cage of the male rat that the female rat was to be bred with was placed in the cage of the female 3-5 days prior to breeding. Female rats were staged in their estrus cycle, and then paired with a male rat for 5 days in a metabolic cage. This allowed for detection and collection of a vaginal mucus plug. Once this plug was discovered, males were removed from the cage and this was considered Day 1 of the pregnancy. This breeding protocol was approximately 90% successful. Rats (either virgin or Days 9-13 in pregnancy; average 11$\pm$2 days pregnant) were euthanized (80 mg/kg pentobarbital, i.p.) and the ovary, bilateral uterine horns and cervix removed and placed in a dish containing physiological salt solution (PSS) (mM): NaCl, 130; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl_2 \cdot 2H_2O$, 1.6; $NaHCO_3$ 14.9; dextrose, 5.5; and $CaNa_2EDTA$, 0.03 (pH 7.2).

Histology and Immunohistochemistry

Some uterine sections and cervices were cleaned, formalin-fixed, paraffin embedded, processed for sectioning and taken through Masson Trichrome staining within the

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Investigative Pathology lab at *Michigan State University* for identification of collagen (blue), keratin/muscle (red), and cytoplasm (pink). In separate studies, formalin-fixed, paraffin embedded sections of the uterus and cervix were cleared, antigen-unmasked for one minute (citrate-based antigen unmasking solution, Vector, Burlingame, CA, USA), blocked and incubated with a primary antibody against smooth muscle α-actin (clone 1A4, EMD Chemicals, Gibstown, NJ, USA or ab5694, Abcam Cambridge, MA, USA) for identification of smooth muscle or primary antibody against the oxytocin receptor (rabbit, APO692 Acris Antibodies, San Diego, CA, USA). A standard immunohistochemistry protocol was used as written by the manufacturer, Vector (Burlingame, CA, USA), and 3, 3-diaminobenzidine was used to visualize the binding of antibodies to the target of interest (allowing sections to develop for one minute). In parallel sections, the primary antibody was left out of the reaction. Most sections were counterstained with Hematoxylin. In some cases, counterstaining was not performed so as to view antibody-based staining with clarity. Sections were photographed on a Nikon Eclipse inverted microscope and images captured within the program MMI Cell Tools.

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Isolated Tissue Bath Protocol

The largely avascular cervix (color white) was dissected away from the uterine horns. The cervix was placed on two L-shaped stainless steel wires as a circular preparation. Ovaries were removed from the uterine horns, and each horn was dissected. Fat was removed and the horn was dissected open with a longitudinal cut such that the

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placenta and embryos could be visualized. Concepti were gently peeled off the uterus, and longitudinal strips measuring 0.5 cm wide x 2.0 cm long were prepared. Strips were placed in PSS for measurement of isometric contractile force using standard bath procedures. In some experiments, the thoracic aorta was mounted as ring (circular preparations), being used as a control for the efficacy of relaxant agonists. One end of the preparation was attached to a stainless steel rod, and the other was attached to a force transducer (FT03, Grass Instruments, Quincy, MA, USA) and placed under optimum resting tension. In preliminary experiments, length-tension experiments were performed to determine the optimum amount of passive tension applied to the tissues in response to the depolarizing stimulus KCl (100 mM). Optimum passive tension was 1000-1500 mg for the uterus, 1000-1100 mg for the cervix and 4000 mg for aorta. Muscle baths were filled with warmed (37 ^{o}C), aerated (95%O_2/5%CO_2) PSS. Changes in isometric force were recorded on an ADInstruments PowerLab (Model 4) and Quad bridge connected to an eMac. Data were saved electronically and quantified using the program Chart 7.0. After 1-hour equilibration with washes every 15 minutes, tissues were challenged with KCl (100 mM) as an initial contraction, and this response (absolute magnitude in milligrams) was used to normalize tissue response from experiment to experiment. KCl challenge was also repeated at the end of the experiment so as to verify that those tissues that did not contract to an agonist were viable during the course of the experiment. After the initial contraction to KCl, tissues were washed until tone returned to baseline and taken through one of the following protocols.

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Contractile Agonists

Agonists were added to the bath, in a cumulative fashion, in five minute steps. Tissue responses readily plateaued during this time, so this was sufficient to measure agonist-induced changes in contractility. Contractility includes changes in basal tone, increases in oscillatory contraction amplitude and frequency. When a final maximum was achieved, tissues were washed thoroughly over the course of an hour. A second contractile agonist was then investigated in the same manner. Agonists were randomized throughout the experiment and two were used in each tissue. Comparison of agonist response to first and second curves revealed no statistical differences (potency, maximums) and thus these responses were combined in the graphs presented.

Relaxant Agonists

An agonist that caused a stable contraction in all tissues (virgin, pregnant cervix and uterus) was chosen to stimulate tone in these tissues, to which vehicle or a relaxant agonist could be used. Tissues were contracted with a half-maximal concentration of the cholinergic muscarinic agonist carbamylcholine (1-10 μM). Carbamylcholine-induced contraction remained sufficiently stable over the hour necessary to complete a cumulative concentration response curve. A five-minute period of contraction to carbamylcholine was established, and then either vehicle or relaxant agonist was added in a cumulative fashion. Tissue responses readily plateaued over a five-minute period, so this was sufficient to measure agonist-induced relaxation. The range of

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contractions established by carbamylcholine during this five minute period were, for the five different agonists tested: (in integrated units/mg KCl contraction): virgin uterus: 117-142; pregnant uterus: 152-214; virgin cervix: 117-142; and pregnant cervix: 121-161. The magnitude of carbamylcholine-induced contraction established prior to the addition of the different relaxant agonists was not statistically different when compared within group (*e.g.* all agonists in pregnant uterus). When carbamylcholine-induced contraction was compared between a virgin and pregnant tissue for each agonist, all initial contractions were statistically similar save for contraction established prior to CGRP (virgin: 83$\pm$9 vs pregnant 235$\pm$38) in the cervix and NE in the uterus (virgin 117$\pm$17 vs 176$\pm$17) ($p<0.05$). We report these values such that we can interpret whether a different initial contraction would modify the outcome of the relaxant.

For experiments using aorta, tissues were contracted half-maximally with the adrenergic agonist phenylephrine ($2x10^{-8}$ M) prior to addition of a relaxant agonist (SNP, CGRP).

Materials

Sigma Chemical Company (St. Louis MO, USA) as the source of all agonists: acetylcholine chloride, calcitonin gene related peptide, carbamylcholine chloride, forskolin, 5-hydroxytryptamine, isoproterenol, norepinephrine, oxytocin, sodium nitroprusside; PGF2alpha.

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Data Analysis

All data are reported as means±SEM for number of animals indicated in parentheses (N). Adjustments to image data included only brightness and contrast settings, applied uniformly to all images in a figure. Uterine and cervical data from the same rat are graphed separately so as to insure that the response of the cervix, typically less in magnitude than the uterus, was well visualized and could be compared between virgin and pregnant groups. All data are reported as integrated units of uterine/cervical activity over a timed five-minute window. Integrated activity was measured using the integral function of the program Chart (7.0, ADInstruments, Colorado Springs, CO) This calculates the area under the waveform as the sum of the data points multiplied by the sample interval. Data points were taken at 4 per second for 5 minutes; Chart adds these values together and multiplies by the duration of the window (5 minutes). By using integrated activity, we take into account changes in basal tone, oscillatory magnitude and frequency. For contractile experiments, five-minute windows were taken from baseline for each addition, and data are reported as these units of activity normalized to the initial KCl challenge in milligrams. This allows for normalization of tissue size and health from experiment to experiment. For relaxant experiments, a five-minute baseline period (carbamylcholine-induced contraction) just prior to addition of relaxant agonists was measured; this was considered 100% activity. Changes in this integrated activity with addition of relaxant agonists are reported as a percentage of this initial baseline activity. Agonist potencies were calculated using a non-linear regression (curve fit) within GraphPad Prism 5.0 (La Jolla, CA, USA), and are reported as concentrations that caused a half-maximal effect. Maximums are reported as the

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maximal effect achieved. Where a maximal response was not achieved, the actual potency (EC_{50} value) was considered equal or greater than the reported value. Maximums are reported as integrated units/KCl Contraction (for contractile agonists) while maximums for relaxant agonists are reported as the % carbamylcholine-induced contraction remaining. Student's t test was used to compare EC_{50} values of agonist responses in the uterus *vs* cervix, and a $p < 0.05$ was considered significant.

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Results

Histology and Immunohistochemistry

Cross sections of the isolated uterine horn and cervix from virgin and pregnant animals were stained with Masson Trichrome to delineate the presence of cells/keratin (pink/red) and collagen (blue). **Figure 1A** demonstrates that the cervix has significant collagen composition (blue) but does have layers of cells that are irregularly located in the cervical tissue. A midzonal section that lies between the two cervical lumens shows cellularity as well, and the lumen itself demonstrates a robust epithelial cell layer. In pregnancy, this epithelial layer is lost but the cellularity in the stroma of the tissue remains. Staining of smooth muscle alpha-actin demonstrates the presence of smooth muscle in both the virgin (left) and pregnant cervix (right), indicated by the black/brown staining in deep stromal and outer cervical layers (**figure 1B, top**). Staining was absent in sections in which the primary antibody against alpha-actin was not present but tissues counterstained with hematoxylin (**figure 1B, bottom panels**). This was true for two different alpha-actin antibodies used (Calbiochem= antibody 1, Abcam = antibody 2). Antibodies do not recognize other isoforms of actin. Thus, cervices express smooth muscle and continue to do so in pregnancy. **Figure 2** demonstrates that uterine tissue has two cellular layers (pink) consistent with the expression of smooth muscle alpha actin (longitudinal, circular layers of smooth muscle). Smooth muscle alpha actin continues to be expressed in pregnancy. Elongated uterine glands, marked with a star in figure 2A, did not stain for alpha-actin; smaller glands showed some reactivity. Collectively, these data support the idea that all tissues should have the ability to generate smooth muscle tone.

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Response to Contractile Agonists

The uterus and the cervix used in all experiments were from the same animal, providing for the ideal comparison between tissue reactivity. Both the uterus and the cervix are phasic tissues, meaning that tissues showed regular oscillatory activity from baseline. **Figure 3A** shows a representative tracing of spontaneous oscillation during the equilibration period; the tissue had not been exposed to any agonist but was under passive tension. Approximately 50% of both uterine (left) and cervical (right) tissues demonstrated spontaneous oscillations. **Figure 3B** demonstrates that all tissues contracted to cumulative additions of the depolarizing stimulus KCl (6-100 mM) in a concentration-dependent manner. Contraction was lost when KCl was washed out, verifying that all tissues are capable of producing active smooth muscle tone. All tissues contracted to KCl at the beginning and end of the experiment. KCl-induced contraction (in milligrams) did not change during the course of the experiment in the virgin uterus (first challenge: 3700$\pm$251; final challenge: 4539$\pm$1096; $p>0.05$), pregnant uterus (first challenge: 6844$\pm$369, final = 6839$\pm$437, $p>0.05$) virgin cervix (2296$\pm$154, final = 2141$\pm$145, $p>0.05$) or pregnant cervix (first: 1566$\pm$80; final: 1725$\pm$256, $p>0.05$). This finding allows us to use KCl as a normalizer throughout the experiment. However, the magnitude of first contraction to KCl in the pregnant uterus was greater than that of the virgin uterus (6844$\pm$369 *vs* 3700$\pm$251; $p<0.05$), while that of the pregnant cervix was reduced compared to the virgin cervix (1566$\pm$80 vs 2296$\pm$154; $p<0.05$).

Contractile agonists were added in a cumulative fashion, and **table 1** compiles the potencies and efficacies (maximums) of these agonists in the uterus and cervix, virgin

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and pregnant. **Figure 4A (left hand side)** shows a representative tracing of the virgin uterine (top) and virgin cervical (bottom) contraction to cumulative additions of an agonist (oxytocin). PGF2alpha (**figure 5A**; **Table 1)** was a robust contractant in both virgin uterus and cervix. Oxytocin induced contraction in all virgin tissues, and was slightly but not significantly more potent and efficacious in the pregnant *vs* virgin uterus. By contrast, the pregnant cervix lost responsiveness to oxytocin such that no discernible concentration response curve could be identified and potency and maximum values could not be calculated. All tissues contracted to KCl, so these tissues do not lack the ability to contract. The inability to contract to oxytocin is not because of a lack of oxytocin receptors, as the oxytocin receptor could be immunohistochemically detected in both the virgin and pregnant cervices, with and without hematoxylin counterstaining (**figure 6A and B, respectively**).

The uterus and cervix are innervated by the parasympathetic nervous system and the muscarinic cholinergic agonist carbamylcholine (10^{-9} – 10^{-5} M**)** caused a concentration-dependent contraction, one that was particularly robust in the cervix, both virgin and pregnant (**figure 7A, Table 1**). A maximal response to carbamylcholine was not achieved in the uterine strips up to 10 μM. The primary amine 5-hydroxytryptamine (10^{-9} – 10^{-5} M; 5-HT, serotonin) contracted both uterus and cervix (**figure 7B, table 1**). 5-HT was modestly but not significantly more potent in contracting the uterus *vs* the cervix. Overall, potencies of agonists were generally similar between the pregnant *vs* virgin tissues, and the general rank order of potency in both the uterus and cervix was oxytocin> 5-HT = PGF2alpha > carbamylcholine (exception of loss of oxytocin contraction in pregnant cervix). The magnitude of

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contraction in the cervix was generally less than that of the uterus. Collectively, these data confirm for the uterus and supports for cervical smooth muscle the ability to contract to pharmacological agonists.

Response to Relaxant Agonists

Tissues were contracted half-maximally to carbamylcholine (1-10x10^{-6} M). This caused a measurable increase in activity but was not maximal such that additions to the bath had the potential to either decrease or increase tone. **Figure 4B (right hand panel)** shows representative tracings of virgin uterine (top) and virgin cervical (bottom) relaxation to cumulative addition of the adrenergic agonist norepinephrine (NE), and **table 1** compares the potency and efficacy of this and four other relaxant agonists. **Figure 8** demonstrates the effects of the most active vehicle used for relaxant agonists, DMSO. This vehicle was taken through the experiment with the same timing as agonist additions but with vehicle additions (same volume) instead. As a control for vehicle additions, 10-fold dilutions of neat DMSO (considered the final addition in parallel to 10^{-5} M agonist) were used for parallel additions to contracted tissues (final cumulative concentration ~ 0.111% DMSO). Carbachol-induced contraction remained largely stable during these additions but was reduced overall ~25% in all tissues over the course of the experiment.

The sympathetic nervous system innervates both cervix and uterus, and **figure 9** shows the potent and efficacious ability of the adrenergic agonist NE (**figure 9A**) to relax both tissues, as did the β–adrenergic receptor agonist isoproterenol (10^{-10} – 10^{-5}

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M; **table 1; figure 9B**) and the adenylate cyclase activator forskolin (10^{-10} – 10^{-5} M**; table 1, figure 9C**). Isoproterenol was potent in relaxing both the uterus and cervix, so potent in the normal cervix that relaxation was already observed at 10^{-11} M isoproterenol. However, isoproterenol was ~10 fold less potent in the pregnant *vs* virgin cervix, though the contractions established by carbamylcholine were statistically similar in both groups (virgin = 124±24 integrated units/mg KCl contraction; pregnant: 123±24; p> 0.05). These data suggest that while the beta adrenergic receptor function may be reduced, the adenylate cyclase/adrenergic system is largely intact. We examined another smooth muscle relaxant system, NO. The NO donor sodium nitroprusside (SNP, 10^{-10} – 10^{-5} M; **figure 10A**) caused a modest relaxation in the uterus (pregnant and virgin), but this is only ~10-20% maximum relaxation when vehicle-induced effects are taken into account. No discernible relaxation could be detected in carbamylcholine-contracted cervices. By contrast, the same solution of SNP (10 μM) completely relaxed isolated thoracic aorta half-maximally contracted with the adrenergic agonist phenylephrine (not shown). CGRP is a sensory neuropeptide, and it caused a concentration-dependent relaxation in the uterus (to ~40% carbamylcholine contraction remaining; **figure 10B**). This was less clear in the cervix, where in both the virgin and pregnant tissues, high concentrations (10,100 nM) were necessary to observe relaxation. CGRP (100 nM) also caused significantly relaxation (>50%) in the half-maximally phenylephrine contracted rat thoracic aorta. Collectively, relaxant agonists produced similar responses between the uterus and cervix with a potency order of isoproterenol ~CGRP > SNP> NE = forskolin where this order is most strongly validated for the uterus.

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Discussion

Our goal was to investigate the individual contractile and relaxant function of the cervix and uterus in the normal, non-pregnant virgin female rat as a means to establish baseline contractility, using the uterus as a comparator. This represents a proof-of-principle experiment to determine whether the cervix could be routinely studied in this way. We also examined whether the pharmacology of the smooth muscle in these two tissues would be different 1) basally and; 2) during a time in pregnancy that is not close to labor. While the group of compounds studied was not exhaustive, they represent well-known vasoactive substances, neurotransmitters and substances with recognized effects in uterine smooth muscle. In the normal virgin female, the uterine and cervical smooth muscles are largely similar in their qualitative response to contractile and relaxant agonists. In pregnancy, the smooth muscle of both the uterus and cervix continues to contract and relax to stimuli, but the cervical loss of contraction to oxytocin supports the unappreciated fact that changes in smooth muscle responsiveness of these reproductive tissues may be critical to the normal progression of pregnancy.

We confirmed the significant expression of smooth muscle α-actin in the uterus. By contrast, the cervix from the same rat appeared less muscular but did possess smooth muscle, validated by use of two different alpha-actin antibodies (**figure 1**). Estimates of the percentage of smooth muscle in the human uterus are ~35% (Petersen *et al.,* 1991) and in the human cervix have been reported as low as 6-8% (Rorie and Newton, 1967; Oxlund *et al*., 2010a,b) or as high as 45% (Danforth, 1954). This discrepancy needs to be resolved. We speculate that the cervical sampling is different

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in these studies, with samples taken closer to the proper uterus having a higher level of musculature compared to those nearer the vagina. The outer layer cervical layer of smooth muscle has been described as continuous with the outer myometrial layer but cervical electromyographic (EMG) activity suggested that the smooth muscle cells of the outer myometrial and outer cervical layer are differently regulated (bovine tissue; van Engelen *et al*., 2007) and may operate independently. The smooth muscle cells within the deep stromal layer of the cervix are even less well defined, and their function has been described as anything from secretory to contractile (Tiltman, 1998). The few studies that investigate the isolated cervix pharmacologically underscore the paucity of work done in this area (Hollingsworth and Isherwood, 1978; Petersen *et al.,* 1991).

Uterus and Cervix: Similarity in Responses

The contractile and relaxant agonists examined, including neurotransmitters, were similarly potent and qualitatively efficacious in the uterus and cervix (David and Llewellyn-Smith, 2010; Dindyaev and Vinogradov, 2009; Houdeau *et al.*, 2003). Carbamylcholine contracted both tissues from the virgin and pregnant rat, while NE, isoproterenol and the direct adenylate cyclase activator forskolin abolished carbamylcholine-induced contraction. These findings highlight the physiological antagonism exerted by the two autonomic nervous systems, and the similarity of response between uterine and cervical smooth muscle in the normal and pregnant state. Stimulation of the Gs/cAMP pathway facilitated a reduction in reproductive

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smooth muscle activity in both tissues, agreeing with other studies (Price and Lopez-Bernal, 2001; Yuan and Lopez-Bernal, 2007). This differs from a report by Bryman *et al.* (1984) in which NE stimulated uterine smooth muscle activity, and isoproterenol was both stimulatory and inhibitory in cervical strips from early pregnant and nonpregnant women. α_1 adrenergic receptor coupling to PLC is augmented in term myometrium (Dupuis *et al*., 2008) and α_2 adrenergic receptors in the cervix has been described to mediate increases and decreases in cervical resistance (Gal *et al.*, 2009). Terbutaline was reported to increase the cervical resistance of the isolated pregnant rat (Gaspar *et al.,* 2005). The overall variable function of adrenergic receptor warrants future attention as to the specific role of the α and β adrenergic receptor.

Oxytocin, 5-HT, and PGF2alpha caused significant contraction in cervix and uterus. Oxytocin is an inducer of labor (Arthur *et al.,* 2007; Smith and Merrill 2006) as are prostaglandins (Goureau *et al.*, 1992; Griffiths *et al.*, 2006). Responsiveness of the human cervix to prostaglandins is one of the better-characterized responses for cervical smooth muscle. In cervical samples from pregnant and non-pregnant women, different prostaglandins--PGE_2, PGI_2 and 6-keto-PGF1alpha-- inhibited muscle contraction (Bryman *et al.,* 1985). We did not test the relaxant effects of these prostaglandins, but confirm the well-known fact of PGF2alpha as an uterotonic and provide evidence that the cervix (virgin and pregnant) also contracts to PGF2alpha. By contrast, the effects of 5-HT on both uterine and especially cervical smooth muscle are less well known, and our reason for studying 5-HT was its well-known actions as a vasoconstrictor. Interestingly, the ability of 5-HT to contract uterus is species-specific. The rat and human respond with contraction, while that of the pig relaxes (Cordeaux

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et al., 2009). In our hands, 5-HT increased smooth muscle tone in both the uterus and cervix.

Both the isolated uterus and cervix did not relax robustly to the NO donor sodium nitroprusside, though SNP abolished tone in contracted vascular smooth muscle. NO donors cause myometrial relaxation but this is independent of global elevation of cGMP (Buxton 2004). NO has been tested as a treatment of threatened preterm labor (Leszczynska-Gorzelak *et al.*, 2001), suggesting that the uterus of the laboring pregnant rat responds significantly differently to NO donors than the non-pregnant or non-laboring pregnant rat. Nitric oxide synthases have been detected in human cervix of pregnant women at term, and addition of the NO donor nitroglycerin or spermine NONOate at high concentrations (100 nM to 10 □M) reduced contraction (Ekerhovd *et al.*, 2000). A similar report was made for uterine and cervical tissues from the rat (Okawa *et al.,* 2004). Thus, pregnancy likely reshapes these mechanisms such that tissues from the pregnant rat gain responsiveness to NO as labor impends. We studied rats on days 9-13, a time that is still distanced from labor and did not observe robust relaxation to SNP, consistent with report that NO donors were comparable to placebo as treatments for cervical ripening in first trimester abortions (Promsonthi *et al*., 2009). Future studies should investigate the pharmacology of the labor-impending uterus and cervix (Day 21), and we hypothesize that here smooth muscle responsiveness would yet again be markedly changed to support the process of labor. It is possible that different donors of NO would elicit a different outcome, but SNP has been a reliable tool for decades, used by many investigators, and was able to abolish agonist-induced contraction in the vasculature.

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Cervical relaxation to CGRP was also difficult to measure, in that only the highest concentration of CGRP (100 nM) caused a relaxation that was above that caused by vehicle. Uterine relaxation to CGRP was clear and confirmatory (Anouar et al., 1998), but the potencies for CGRP are estimated because the maximum relaxation may not have been achieved by 100 nM, the highest concentration we could attain in the tissue bath. Why the cervix responds so irregularly to CGRP is a question, especially knowing that sensory innervation occurs in the cervix (Ghatei *et al*., 1985) and is a part of ripening (Collins *et al*., 2002).

Uterus and Cervix: Differences in Responses

There were a few notable differences between cervix and uterus. First, the absolute magnitude of contraction achieved in the cervix was less than that of the uterus, likely due to relative lower content of smooth muscle. In pregnancy, contraction to KCl was reduced in the cervix, indicating a potential but not total loss of smooth muscle function. Cervical smooth muscle apoptosis in pregnancy has been suggested (Leppert, 1995). Second, the pregnant cervix lost contractile responsiveness to oxytocin while the uterus did not. This finding was consistent with observations made by Hollingsworth and Isherwood in 1978. Using term pregnant (day 22) strips of rat cervix, they demonstrated a reduced contraction to a given concentration (1 or 10 mu/ml) of oxytocin; a concentration response curve was not performed. Our experiments differ in that we kept the circular nature of the cervix intact, and performed a whole concentration response curve. Given that oxytocin is a major

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stimulant for labor, the loss of cervical response to oxytocin is logical. The mechanism of loss of contraction still needs to be determined, though it is unlikely this is because of OT receptor loss given the significant staining for the OT receptor in cellular sections of the cervix where α-actin is localized. Finally, the loss of sensitivity (reduced potency) to isoproterenol in the pregnant cervix suggests that the beta adrenergic receptor is less effective in transducing the signal stimulated by isoproterenol, either because of loss of receptor or loss of receptor-effector coupling. The lack of change in response to forskolin supports that any change in this adenylate cyclase dependent system is not downstream of the receptor.

Potential Clinical Ramifications

A majority of the focus on the cervix in premature labor has been on the impact of short cervices and cervical ripening, which includes a decrease in collagen, increased water retention, dilated blood vessels and increased plasma extravasation (Ji *et al.*, 2008). The result is a pliable, soft cervix. In premature labor, the cervix may be incompetent, short or dilate (passively) inappropriately (Abdel-Aleem *et al.*, 2010; Berghella *et al.*, 2009; Mella and Berghella 2009; Sinno *et al.*, 2009; Timmons *et al.*, 2010). Our work highlights a largely unstudied function of the cervix, and that is as a contractile tissue. If we can understand the importance of smooth muscle and identify pharmacological differences between the cervix and uterus, we may find therapies that can be used in the treatment of premature labor.

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Limitations and Conclusion

There are limitations to our study. First, we used the longitudinal smooth muscle of the uterus, not the circular smooth muscle. Use of the isometric contractile technique limits studying this tissue in a coordinated fashion. Second, studies suggest regional differences in the upper and lower uterus (Griffiths *et al.*, 2006), and we have used a preparation that extends into both the upper and lower uterus (though excluding the cervical uterus). A third and final limitation is that we used only carbamylcholine as the contractile agonist when investigating the effects of relaxant substances. This is a reasonable choice given the stable contraction elicited by carbamylcholine in uterus and cervix. Use of oxytocin would have been ideal, but we could not use this because oxytocin-induced contraction does not occur in the pregnant cervix. In summary, we present that the longitudinal uterus and circular cervix preparations contracted and relaxed to similar substances, making the important observation that the cervix did contract and relax actively. The uterus had a greater maximum contractile response to the agonists examined compared to the cervix, save for carbamylcholine. Both tissues continued to contract and relax in pregnancy, but oxytocin did not contract the pregnant cervix and isoproterenol sensitivity was reduced in these same tissues. These studies lay a foundation for studying the pharmacology of female reproductive smooth muscle function in normal situations, pregnancy and in labor with the hopes of taking advantage of differences between the uterus and cervix in treatment of premature labor.

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Authorship Contributions

Participated in Research Design, Darios, Seitz and Watts

Conducted experiments: Darios, Seitz and Watts

Performed data analysis: Darios and Watts

Wrote or contributed to the writing of the manuscript: Darios, Seitz and Watts

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Footnotes

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Legends for Figures.

Figure 1. A. Masson Trichrome staining of a cervical cross section (1/2 of whole cervix shown in far left panels) from virgin (left) and pregnant rat (right). Pink = cytoplasm/cell presence, blue = collagen. B. Immunhistochemical staining of smooth muscle cell α-actin in virgin and pregnant uterus. In B, the left panels represent experiments with primary antibody directed against α -actin, and the right panel sections without primary antibody incubation. Bottom row are sections without hematoxylin counterstaining. Representative of four (4) different animals. Arrows point to regions of muscle, L = lumen.

Figure 2. A. Masson Trichrome staining of a cross section of a longitudinal uterus preparation from virgin (left) and pregnant rat (right). Pink = cytoplasm/cell presence, blue = collagen. B. Immunhistochemical staining of smooth muscle cell α-actin in virgin and pregnant uterus. In B, the left panels represent experiments with primary antibody directed against α -actin, and the right panel sections without primary antibody incubation. Representative of four (4) different animals. Arrows point to regions of muscle. * = uterine gland.

Figure 3. A. Representative tracings of virgin uterine (left) and cervical (right) spontaneous oscillations as isolated preparations. B. Contraction of virgin and

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pregnant uterus (left) and cervix (right) to cumulative additions of KCl. Points represent means±SEM for number of animals in parentheses.

Figure 4. Representative tracings of the contraction of virgin tissues to oxytocin (left) and relaxation to NE (right) demonstrating how cumulative concentration response curves were carried out. Representative of over 100 experiments.

Figure 5. Concentration dependent contraction of the uterus (top) and cervix (bottom) to PGF2α (A) and oxytocin (B). Points represent means±SEM for number of animals in parentheses. * = significantly different from comparable responses of virgin tissue.

Figure 6. Oxytocin receptor localization in the virgin (top) and pregnant (bottom) cervix with (left) and without (right) hematoxylin counterstain. Representative of four different animals. Arrows point to regions of interest. L = lumen.

Figure 7. Concentration dependent contraction of the uterus (top) and cervix (bottom) to carbamylcholine (A) and 5-HT (B). Points represent means±SEM for number of animals in parentheses.

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Figure 8. Effect of vehicle additions (DMSO) on carbamylcholine-induced contraction in the virgin and pregnant uterus (top) and cervix (bottom). Points are the percentage of contraction remaining over a 5 minute period after additions, and represent means±SEM for the number of animals in parentheses.

Figure 9. Effect of NE (A), isoproterenol (B) and forskolin (C) on half-maximal carbamylcholine-induced contraction in isolated rat uterine strip (top) and cervix (bottom). Points represent means±SEM for number of animals in parentheses.

Figure 10. Effect of sodium nitroprusside (SNP; A) and calcitonin gene related peptide CGRP; B) on half-maximal carbamylcholine-induced contraction in isolated rat uterine strip (top) and cervix (bottom). Points represent means±SEM for number of animals in parentheses.

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Table 1. Pharmacological parameters for agonists in stimulating contraction and relaxation in the isolated virgin and pregnant uterus and cervix.

Contractants	Maximum Contraction (Integrated area/mg KCl contraction)				-log EC_{50} [M]			
	Uterus		Cervix		Uterus		Cervix	
	Virgin	*Pregnant*	*Virgin*	*Pregnant*	*Virgin*	*Pregnant*	*Virgin*	*Pregnant*
$PGF_{2\alpha}$	200.5±13.7	169.9±14.5	29.4± 3.8	33.6± 5.3	6.73±0.19	6.68±0.28	7.29±0.42	7.24±4.39
Oxytocin	229.0±15.6	301.5±10.7	70.0±14.8	NC	8.81±0.26	9.03±0.17	9.83±1.22	NC
Carbachol**	262.2±10.9	255.7±89.3	162.1±19.7	233.9±29.7	5.62±0.085	5.47±0.45	6.09±0.14	6.14±0.12
5-HT	118.1± 9.5	145.7± 7.5	49.4± 6.3	33.7± 4.4	7.09±0.25	7.33±0.17	6.64±0.30	7.09±0.35

Relaxants	Maximum Relaxation (% Carbachol contraction remaining)				-log EC_{50} [M]			
	Uterus		Cervix		Uterus		Cervix	
	Virgin	*Pregnant*	*Virgin*	*Pregnant*	*Virgin*	*Pregnant*	*Virgin*	*Pregnant*
NE	21.2± 8.4	2.5± 1.2	10.8±6.9	38.6±11.8	6.50±0.23	6.45±0.11	5.98±0.56	6.06±0.46
Iso	1.3± 1.0	0.3± 0.3	10.4±6.1	20.8± 5.5	9.51±0.15	9.37±0.11	9.95±0.37	8.51±0.58*
Forskolin	1.2± 0.8	3.4± 3.3	12.8±2.5	17.2± 6.8	6.26±0.22	5.62±0.18	5.73±0.21	6.31±0.31
SNP	66.8±14.7	60.4±12.1	NC	NC	9.01±0.84	9.23±0.99	NC	NC
CGRP**	36.9±10.6	37.6±11.1	30.4±8.9	60.4±10.4	9.35±0.53	9.10±0.55	8.11±0.49	7.64±0.27

NC = not calculable. 5-HT = 5-hydroxytryptamine, carbachol = carbamylcholine, $PGF_{2\alpha}$ = prostaglandin 2 alpha, Iso = isoproterenol, SNP = sodium nitroprusside, NE = norepinephrine, CGRP = calcitonin gene related peptide.

* = significantly different from virgin value; ** = EC_{50} are estimates as maximums may not have been obtained.

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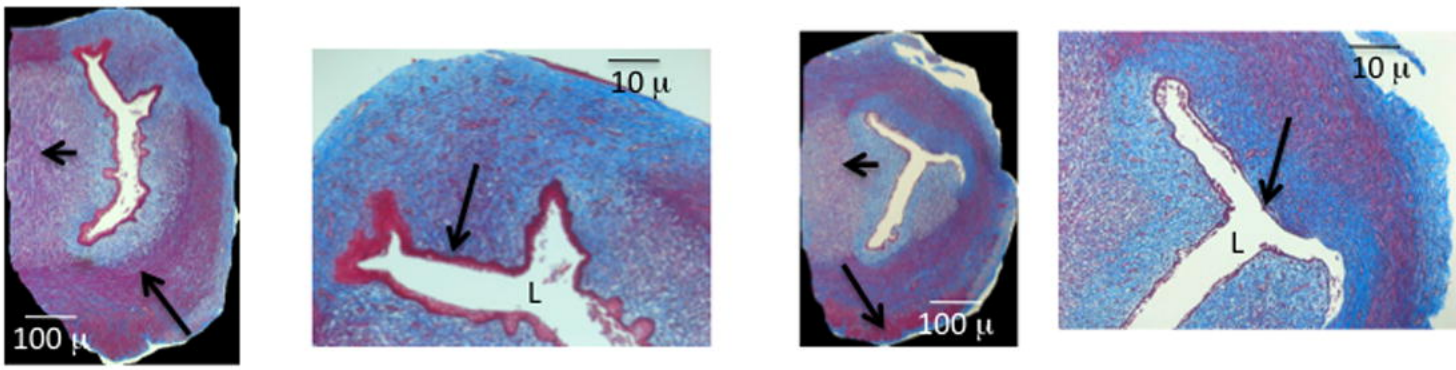
A.

Trichrome

Virgin

Pregnant



B.

Smooth Muscle α-actin

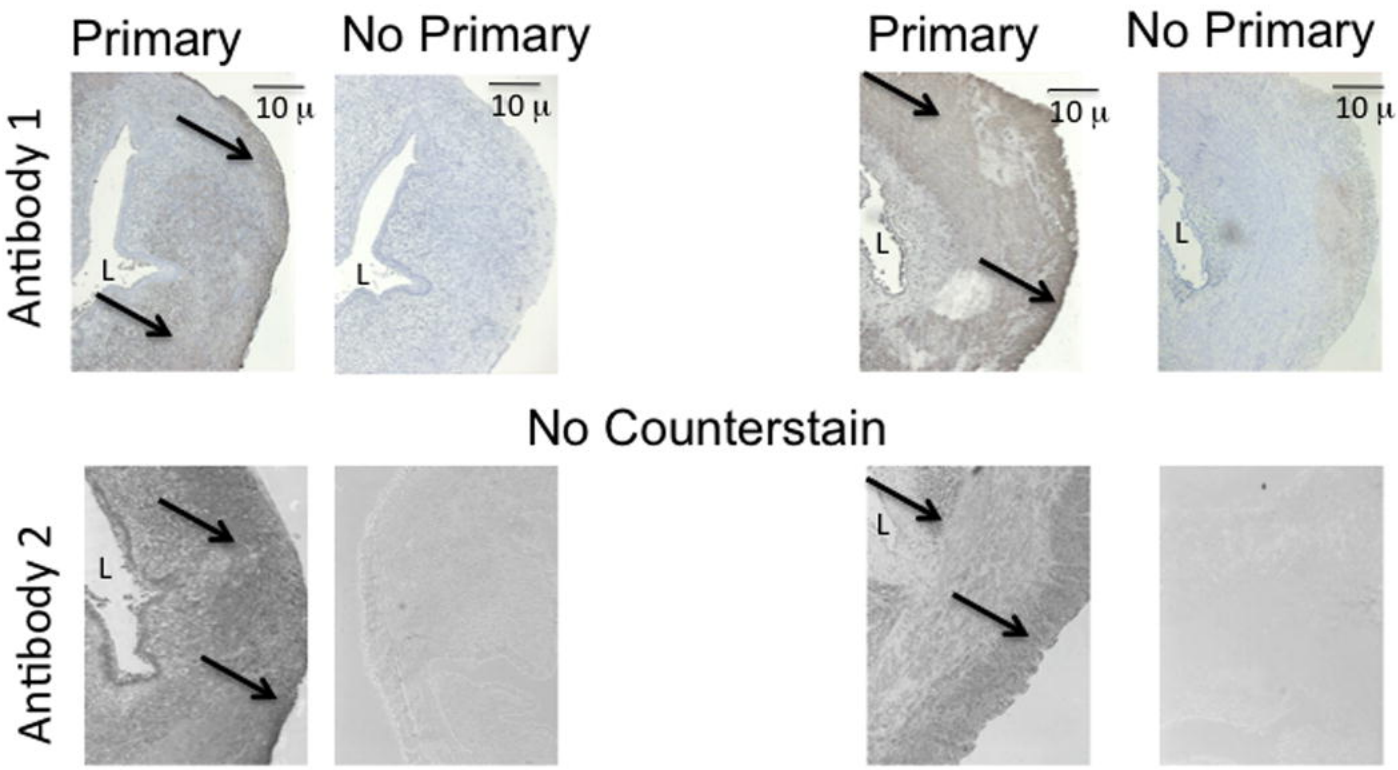
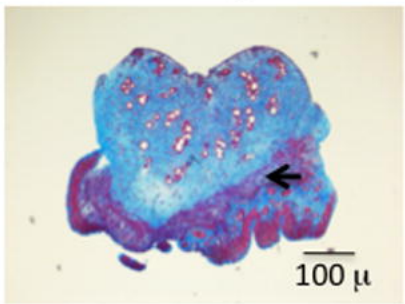


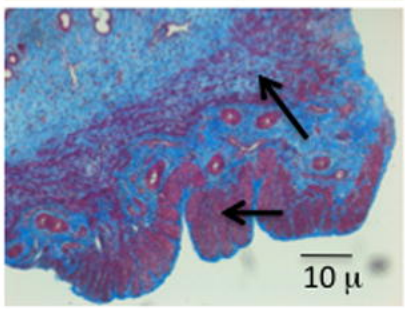
Figure 1

A.

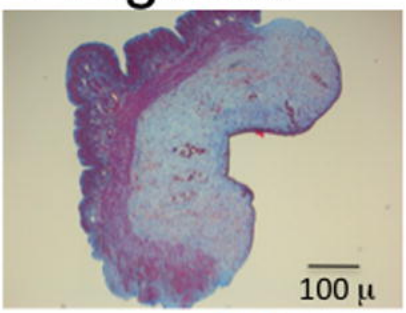
Trichrome

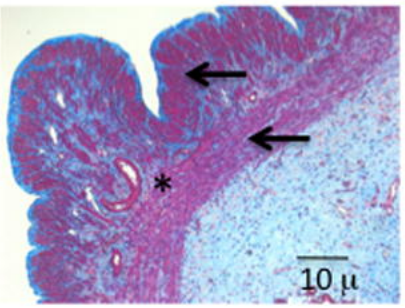
Virgin





Pregnant

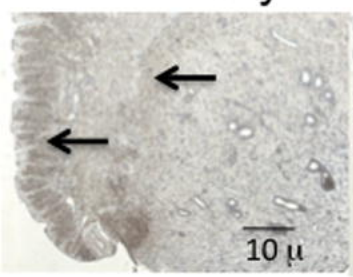




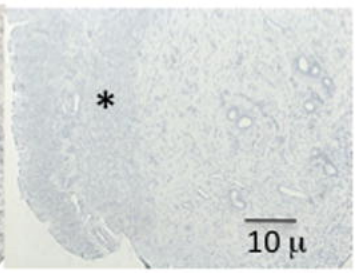
B.

Smooth Muscle α-actin

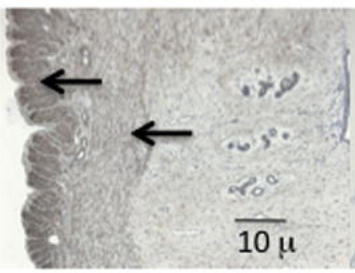
Primary



No Primary



Primary



No Primary

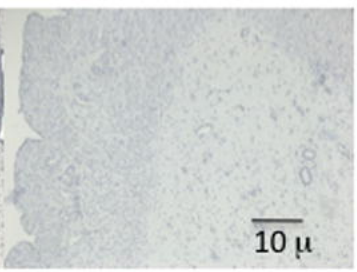
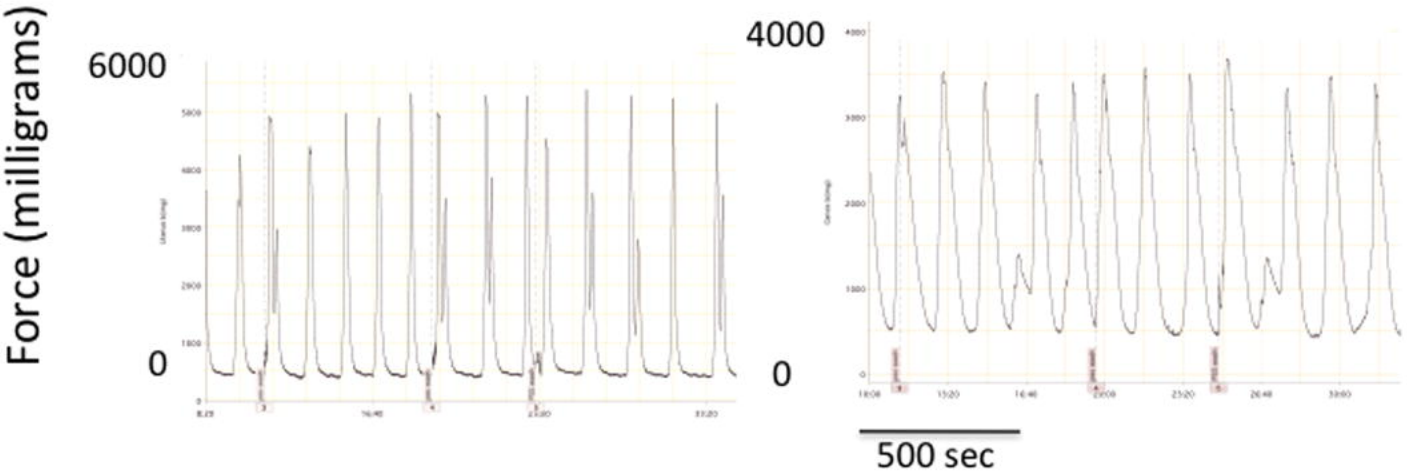


Figure 2

A.


Force (milligrams)
6000
0
4000
0
500 sec

B.

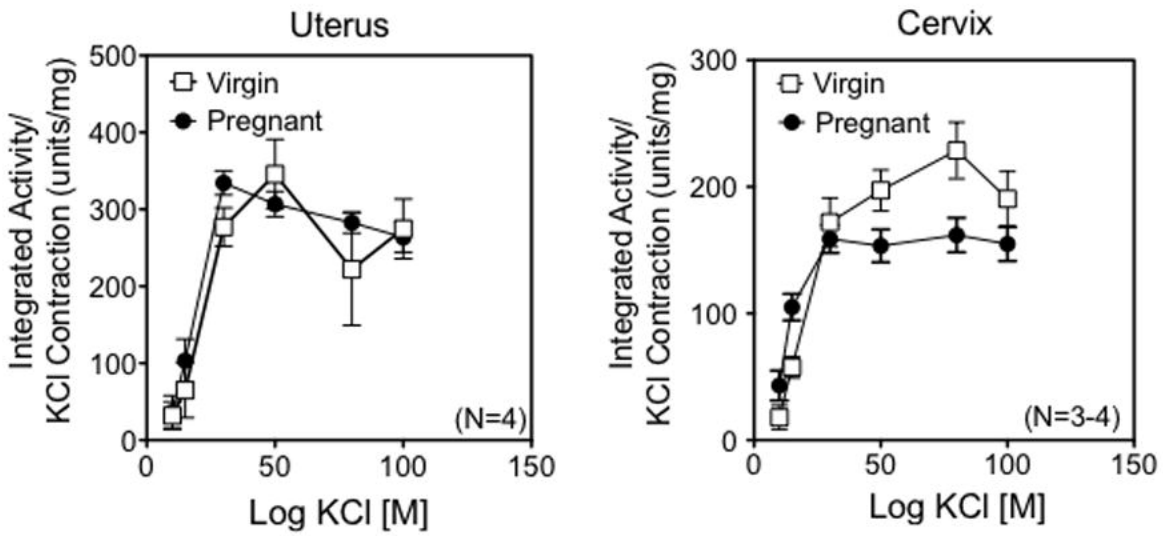

Uterus
Integrated Activity/
KCl Contraction (units/mg)
500
400
300
200
100
0
Virgin
Pregnant
(N=4)
0
50
100
150
Log KCl [M]
Cervix
Integrated Activity/
KCl Contraction (units/mg)
300
200
100
0
Virgin
Pregnant
(N=3-4)
0
50
100
150
Log KCl [M]

Figure 3

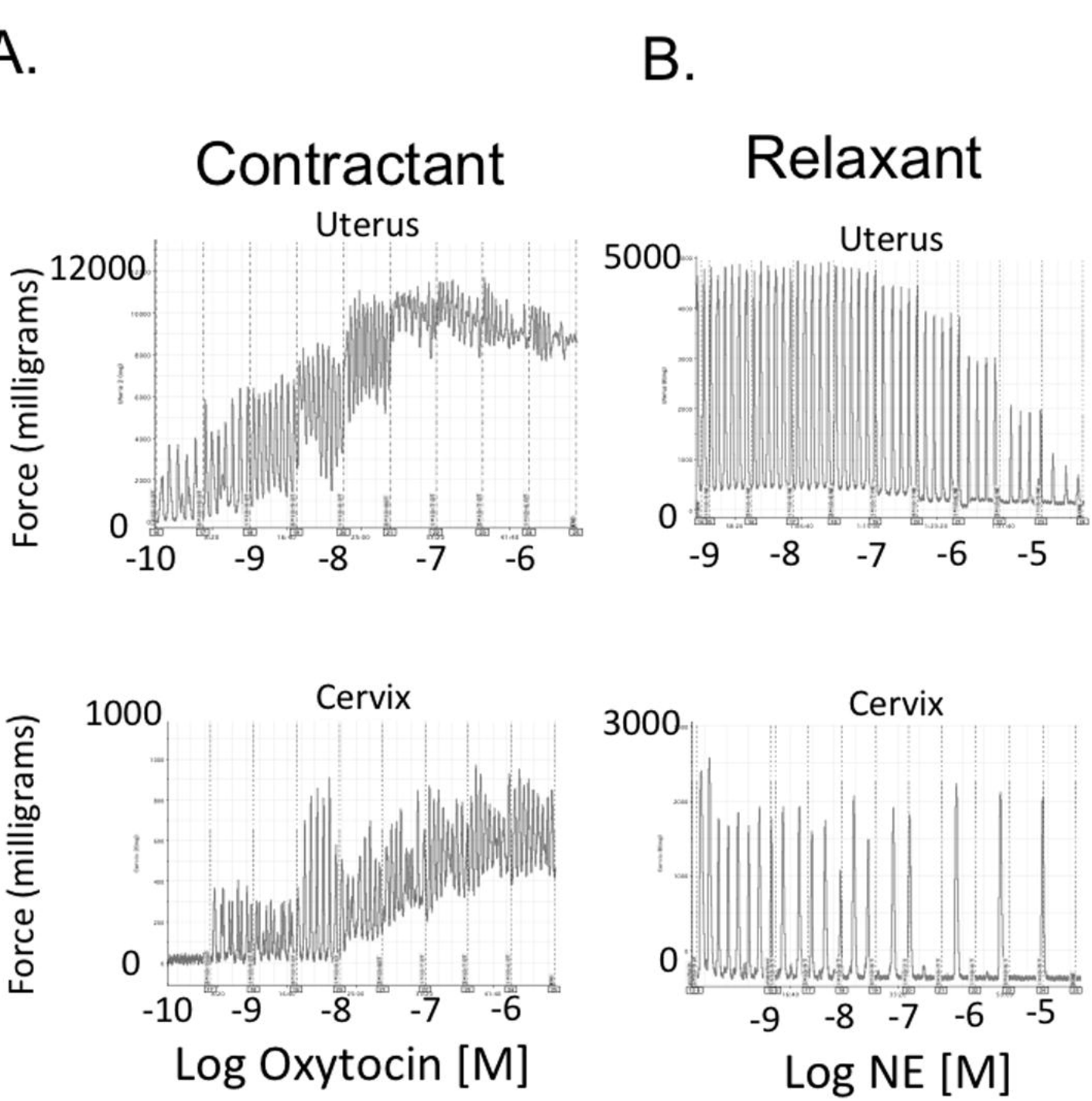

A.
B.
Contractant
Relaxant
Uterus
Uterus
12000
0
5000
0
Force (milligrams)
-10
-9
-8
-7
-6
-9
-8
-7
-6
-5
Cervix
Cervix
1000
0
3000
0
Force (milligrams)
-10
-9
-8
-7
-6
-9
-8
-7
-6
-5
Log Oxytocin [M]
Log NE [M]

Figure 4

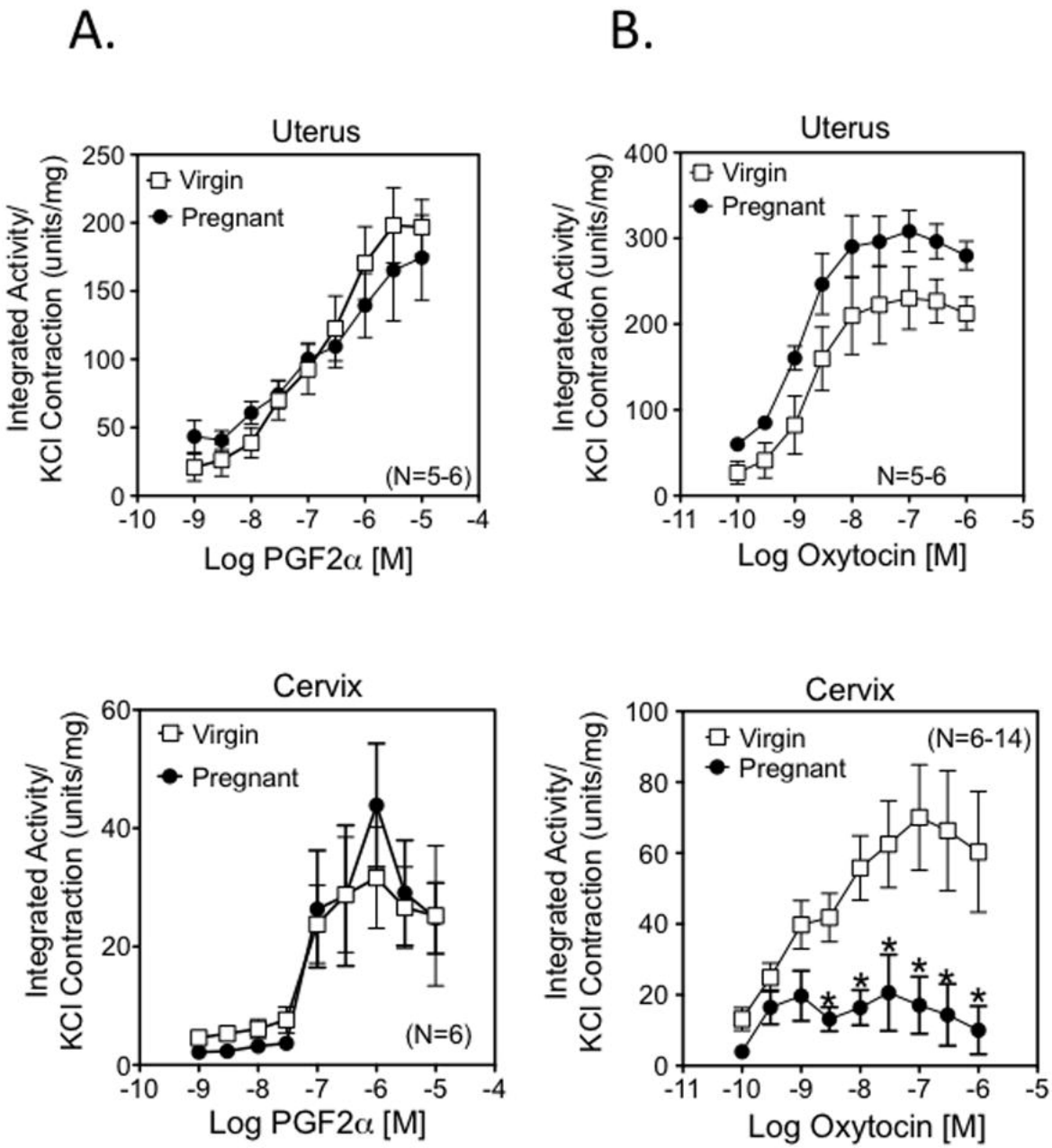

A.
B.
Uterus
Integrated Activity/
KCl Contraction (units/mg)
Virgin
Pregnant
(N=5-6)
Log PGF2α [M]
Uterus
Virgin
Pregnant
N=5-6
Log Oxytocin [M]
Cervix
Virgin
Pregnant
(N=6)
Log PGF2α [M]
Cervix
Virgin
Pregnant
(N=6-14)
Log Oxytocin [M]

Figure 5

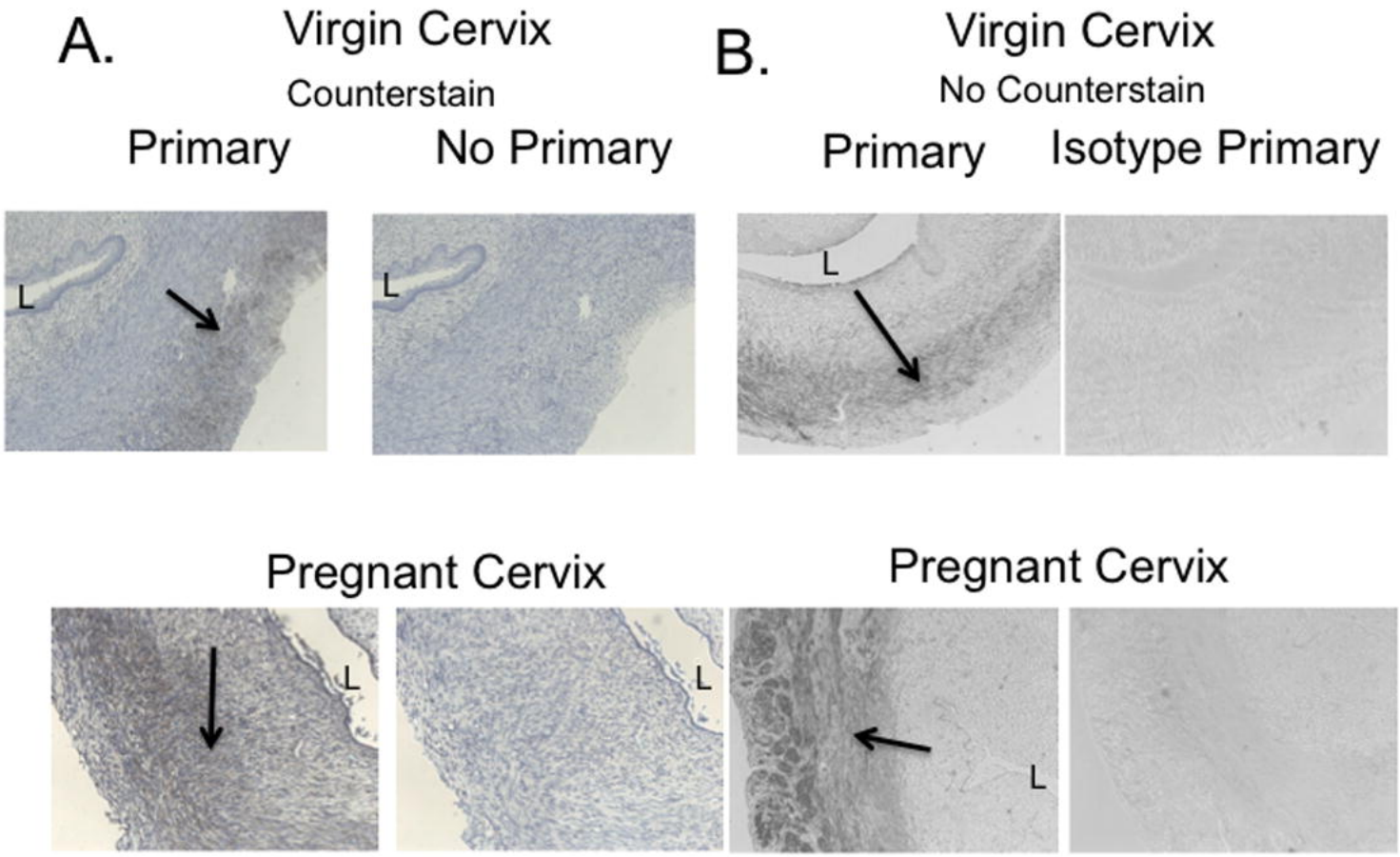

A.
Virgin Cervix
Counterstain
Primary
No Primary
B.
Virgin Cervix
No Counterstain
Primary
Isotype Primary
L
L
L
L
Pregnant Cervix
Pregnant Cervix
L
L
L

Figure 6

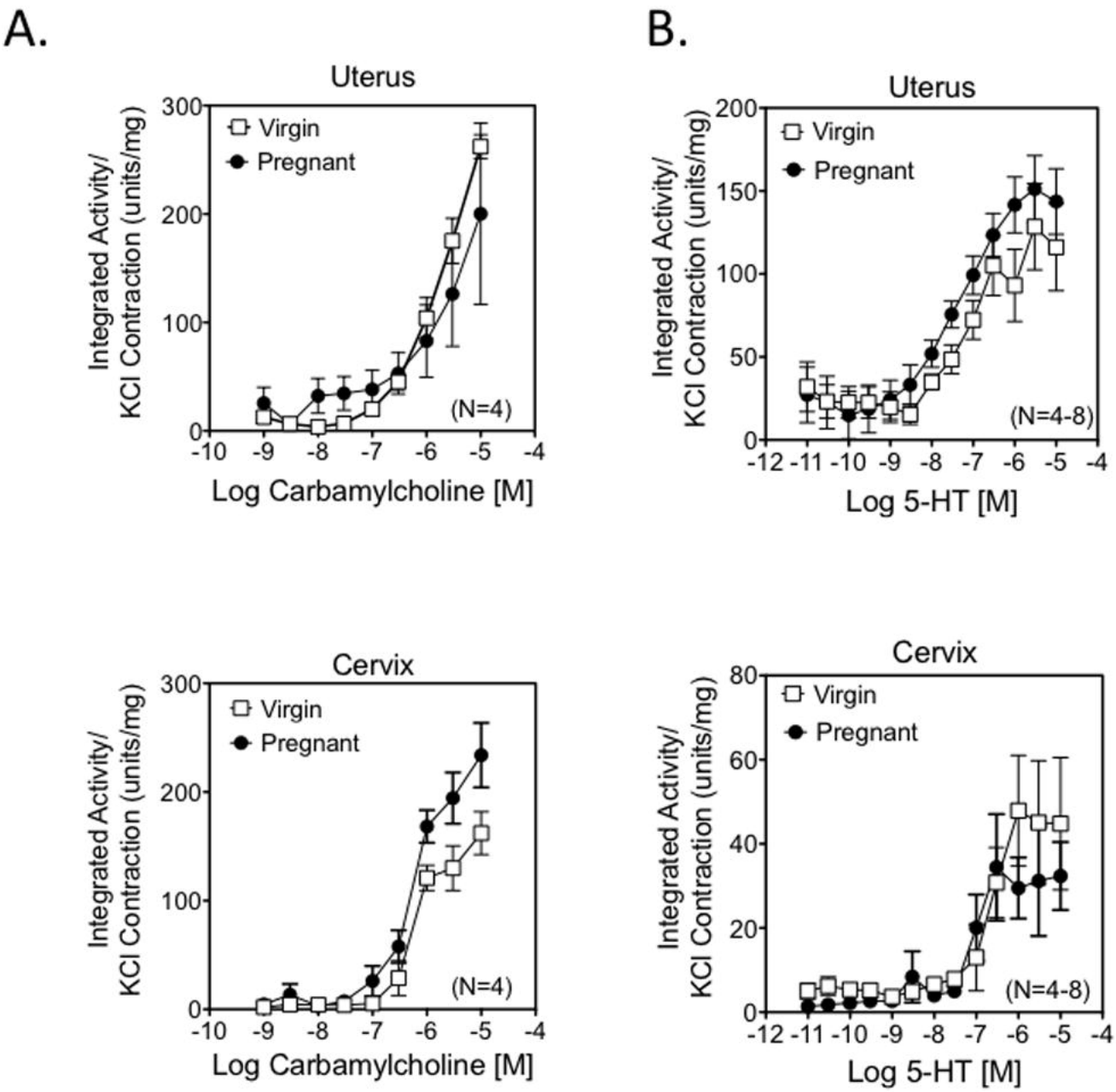

A.
Uterus
Integrated Activity/
KCl Contraction (units/mg)
Virgin
Pregnant
(N=4)
Log Carbamylcholine [M]
B.
Uterus
Integrated Activity/
KCl Contraction (units/mg)
Virgin
Pregnant
(N=4-8)
Log 5-HT [M]
Cervix
Integrated Activity/
KCl Contraction (units/mg)
Virgin
Pregnant
(N=4)
Log Carbamylcholine [M]
Cervix
Integrated Activity/
KCl Contraction (units/mg)
Virgin
Pregnant
(N=4-8)
Log 5-HT [M]

Figure 7

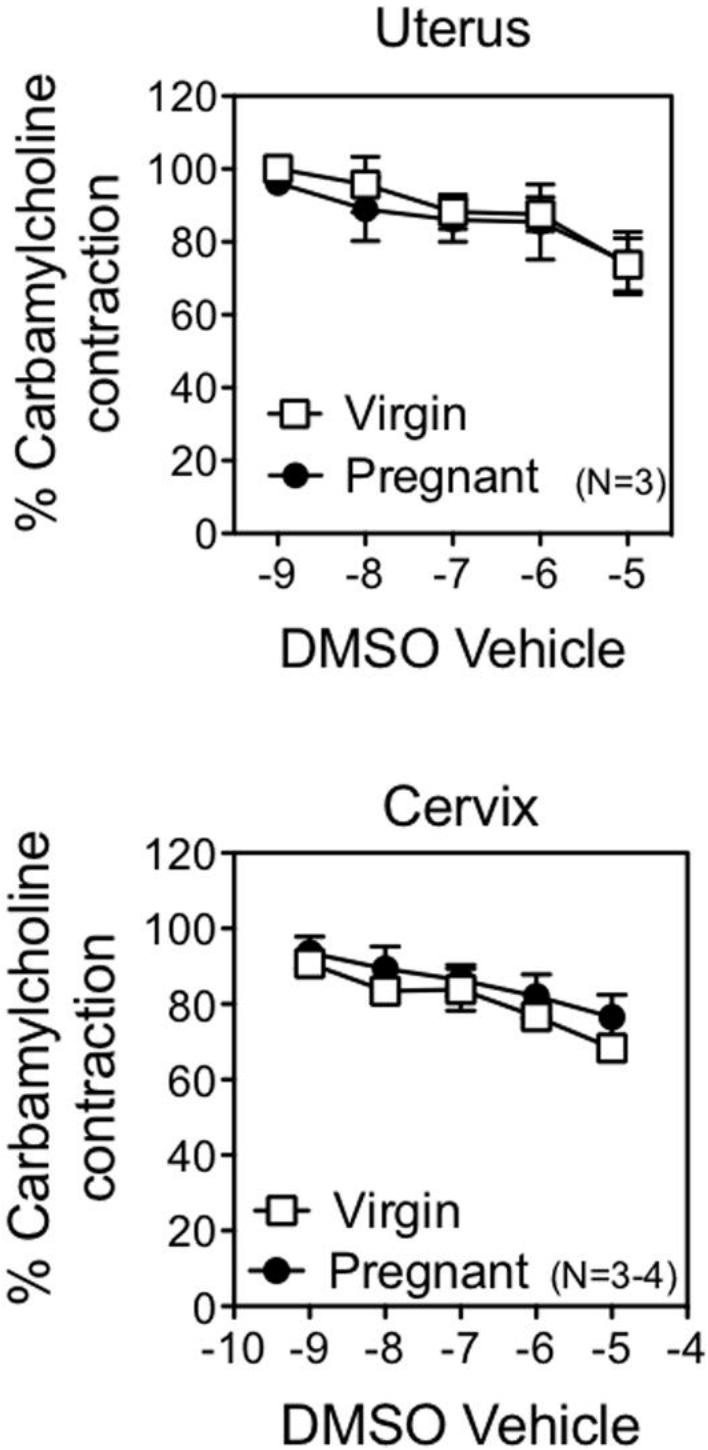

Uterus
% Carbamylcholine contraction
120
100
80
60
40
20
0
Virgin
Pregnant
(N=3)
-9
-8
-7
-6
-5
DMSO Vehicle
Cervix
% Carbamylcholine contraction
120
100
80
60
40
20
0
Virgin
Pregnant
(N=3-4)
-10
-9
-8
-7
-6
-5
-4
DMSO Vehicle

Figure 8

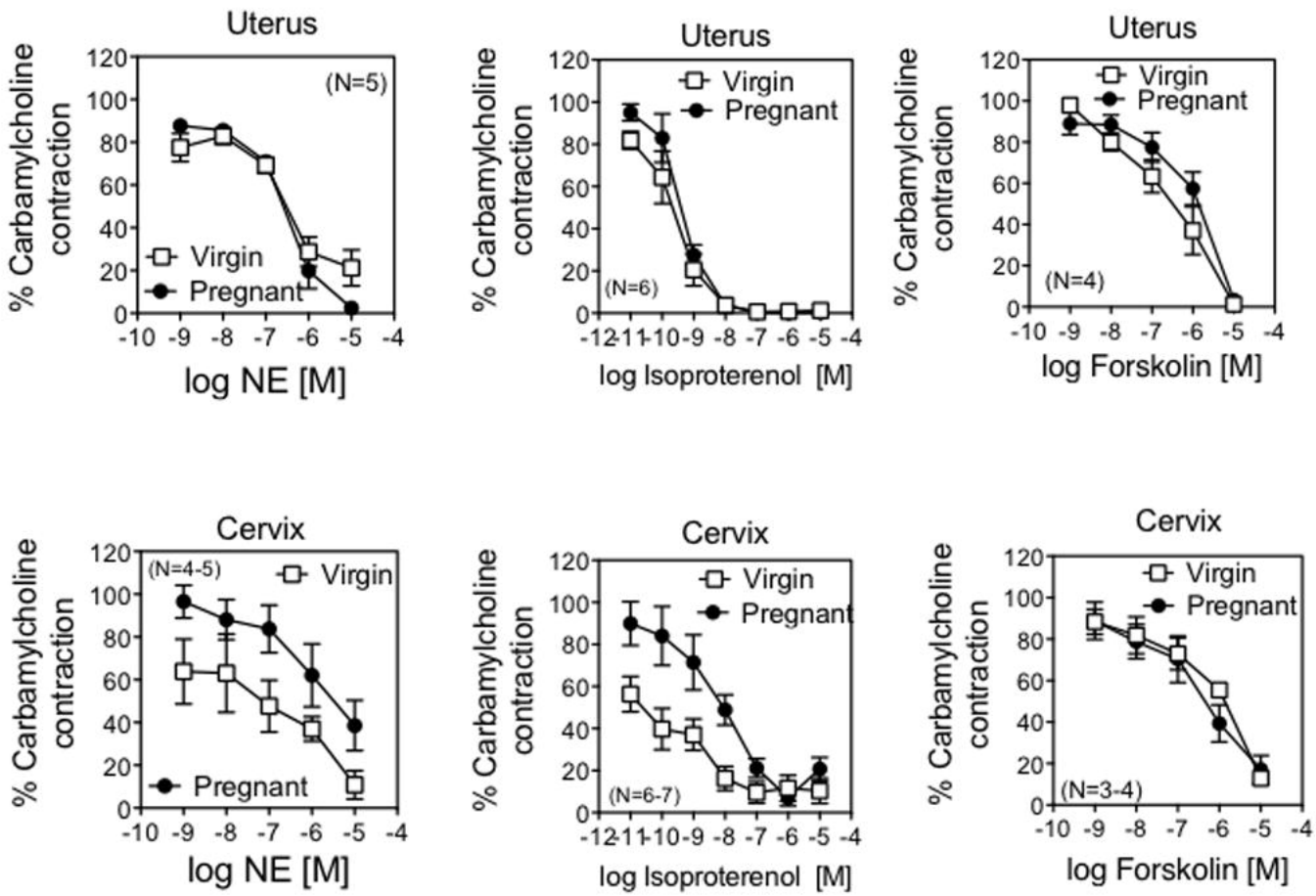
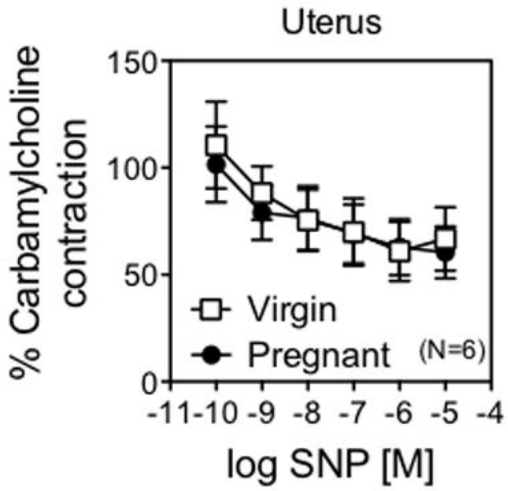
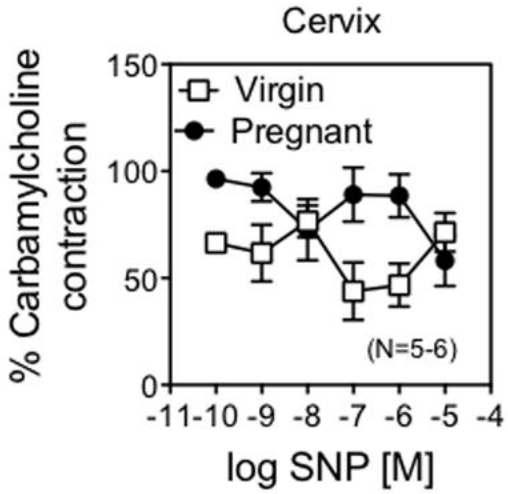

A.
B.
C.
Uterus
% Carbamylcholine contraction
(N=5)
Virgin
Pregnant
log NE [M]
(N=6)
log Isoproterenol [M]
(N=4)
log Forskolin [M]
Cervix
(N=4-5)
(N=6-7)
(N=3-4)

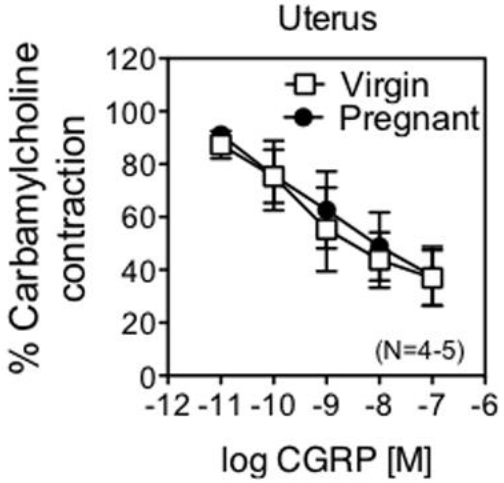
Figure 9

A.


Uterus
% Carbamylcholine contraction
150
100
50
0
Virgin
Pregnant
(N=6)
-11 -10 -9 -8 -7 -6 -5 -4
log SNP [M]


Cervix
% Carbamylcholine contraction
150
100
50
0
Virgin
Pregnant
(N=5-6)
-11 -10 -9 -8 -7 -6 -5 -4
log SNP [M]

B.


Uterus
% Carbamylcholine contraction
120
100
80
60
40
20
0
Virgin
Pregnant
(N=4-5)
-12 -11 -10 -9 -8 -7 -6
log CGRP [M]

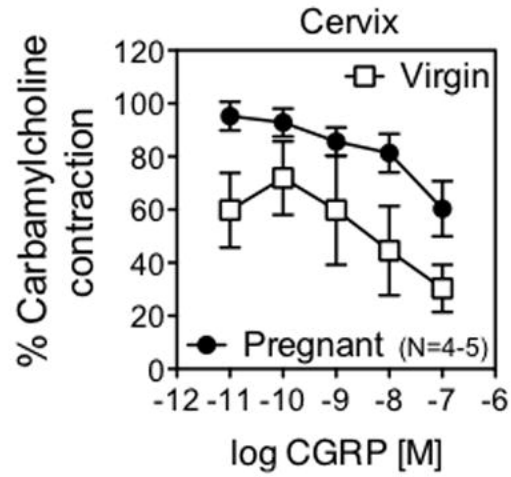

Cervix
% Carbamylcholine contraction
120
100
80
60
40
20
0
Virgin
Pregnant (N=4-5)
-12 -11 -10 -9 -8 -7 -6
log CGRP [M]

Figure 10