Uterine Cervical Afferents in Thoracolumbar Dorsal Root Ganglia Express Transient Receptor Potential Vanilloid Type 1 Channel and Calcitonin Gene–related Peptide, but Not P2X3 Receptor and Somatostatin

Chuanyao Tong, M.D.,* Dawn Conklin, B.A.,† Brittany B. Clyne, M.D.,‡ Jennifer D. Stanislaus, B.A.,§ James C. Eisenach, M.D.¶

**Background:** Little is known regarding the phenotype of afferents that innervate the uterine cervix. Chronic estrogen sensitizes uterine cervical afferents to mechanical distension, but whether this reflects changes in afferent neurotransmitter or excitatory ion channel expression is unknown. The authors used immunocytochemistry to characterize uterine cervical afferents and the effects of estrogen on them.

**Methods:** Fluorogold was injected into the uterine cervix of intact rats (n = 7) and those with ovariectomy alone (n = 9) or with estrogen supplementation (n = 8). Bilateral dorsal root ganglia at T12–L2 were removed and immunostained for transient receptor potential vanilloid type 1 (TRPV1), P2X3 receptor, calcitonin gene–related peptide, and somatostatin. The proportion of fluorogold-traced dorsal root ganglion neurons expressing each of these markers was compared with untraced neurons.

**Results:** Most fluorogold-traced cells were found at L1 (>55%) and were of small diameter (24 μm). TRPV1 expression was similar between traced and untraced cells, except the estrogen treatment increased TRPV1 expression in traced cells. Calcitonin gene–related peptide expression was greater in traced than in untraced cells, with no effect of experimental treatment. No traced cells expressed the P2X3 receptor or somatostatin, although each of these was present in untraced cells.

**Conclusion:** Uterine cervical afferents in the hypogastric nerve express TRPV1, an important nociceptive channel, which may play a role in estrogen-induced sensitization of cervical afferents. High expression of calcitonin gene–related peptide suggests a sensory and efferent role for this peptide. In contrast to other viscera, these afferents do not express somatostatin or P2X3 receptor, indicating a unique phenotype of these C fibers.

PAIN during labor, as well as some gynecologic pain originating from distension of the uterine cervix and lower uterine segment, has received little neurophysiologic study. Acute dilatation of the uterine cervix produces stimulus dependent pain mostly referred to lower thoracic dermatomes in humans.1 In rats, the uterine cervix receives innervation from hypogastric and pelvic nerves, the former primarily entering the spinal cord at T12–L2 and the latter at L6–S1.2,3 Although neuropeptide content and ion channel and receptor expression have been studied in L6–S1 cervical afferents,4,5 no such characterization has been done for T12–L2 afferents.

We have recently examined afferent, spinal cord, and nocifensor reflex responses to uterine cervical distension in rats. Uterine cervical distension produces a stimulus-dependent increase in hypogastric afferent nerve firing, increased expression of cFos in the thoracolumbar spinal cord (T12–L2), and reflex abdominal muscle activity.6,7 Although thoracolumbar uterine cervical afferents are polymodal, responding to mechanical and thermal stimuli as well as bradykinin,8 little is known regarding the neurotransmitters or ion channels they express.

The current study focuses on expression of two excitatory receptors present in nociceptors. Transient receptor potential vanilloid type 1 (TRPV1) responds to heat, protons, and vanilloid compounds, including capsaicin, and, in colonic visceral afferents, to distension.9–11 The P2X3 receptor, a member of the P2X purinoreceptor family, is activated by adenosine triphosphate.12 TRPV1 and P2X3 receptors are commonly expressed in peripheral somatic and visceral C-fiber afferents, and their cell bodies in the dorsal root ganglia (DRGs).5,10–19 Activation of TRPV1 and P2X3 receptors are important for acute nociception from distension in other visceral structures, and their expression or responsiveness is up-regulated by chronic inflammation.9,15,16

C-fiber nociceptors also express several peptide neurotransmitters. Calcitonin gene–related peptide (CGRP) is an excitatory neuropeptide found primarily in C fibers of somatic and visceral afferents and plays roles both as an excitatory neurotransmitter in the spinal cord and as a potent vasodilator and immune cell attractant in the periphery.4,20–25 Somatostatin, an inhibitory neuropeptide, is also found in sensory neurons and may inhibit nociceptive neurotransmission in the periphery as well as in the spinal cord.20–27 One purpose of the current study is to define the expression of these channels and neurotransmitters in afferents that innervate the uterine cervix via the hypogastric nerve.

Estrogen has been implicated in enhancing pain by decreasing pain threshold and increasing response to suprathreshold stimuli, both in humans and in ani-

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*A Associate Professor; † Laboratory Technician; ‡ Obstetric Anesthesia Fellow; § Medical Student; ¶ FM James Ill Professor.

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Address reprint requests to Dr. Tong: Department of Anesthesiology and Pain Mechanisms Laboratory, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157. ctong@wfubmc.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.
Many chronic pain syndromes are more common in women than in men, including irritable bowel syndrome, chronic pelvic pain syndrome, and interstitial cystitis, and estrogen has been speculated as a potential cause of this sexual dimorphism.29–31 Estrogen supplementation to ovariectomized rats decreases withdrawal threshold and increases response to noxious stimuli administered to the colon or the vagina.32–35 In addition, estrogen supplementation increases spontaneous activity of uterine cervical afferents and enhances their response to mechanical stimuli.36 The mechanisms of estrogen’s effects are not clear but may involve increased expression of nociceptive neurotransmitters or ion channels. A second purpose of this study is to determine whether chronic estrogen supplementation alters the expression of TRPV1 and P2X3 receptors, CGRP, or somatostatin.

Materials and Methods

The study was approved by the Animal Care and Use Committee of Wake Forest University School of Medicine (Winston-Salem, North Carolina), and experiments followed the animal research ethical guidelines of the International Association for the Study of Pain. Female Sprague-Dawley rats were housed two per cage in our central animal facility, with free access to food and tap water ad libitum and with a 12 h:12 h light–dark cycle.

Surgery and Uterine Cervical Sensory Neuron Labeling

Anesthesia was induced with 5% and maintained with 2% halothane in oxygen with spontaneous ventilation. A laparotomy was performed via a midline incision in the lower abdomen. A bilateral ovariectomy was performed, and then a pellet containing either estrogen or placebo was implanted subcutaneously. Intact animals served as a control group; estrous stage was not determined at the end of surgery. 0.5% bupivacaine was infiltrated in the incision for postoperative pain control. Postoperatively, animals showing any signs of infection or hemotoma or impairment of water and food intake resulting in significant weight loss were killed and not included in the study.

Tissue Preparation

Twenty-one days after surgery, animals were anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal) and perfused transcardially with 250 ml phosphate-buffered solution, 0.01 M, containing 1% sodium nitrite and then with 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered solution. Bilateral DRGs at the T12–L2 levels were removed and postfixed in the same fixative for 4 h and then transferred into 30% sucrose in phosphate-buffered solution for cryoprotection for 48 h. A pair of DRGs from each level was mounted in Tissue-tek O.C.T. (Sakura Finetechnical Co., Torrance, CA) embedding medium and cryostat sectioned at 16 μm. Sections were thaw-mounted onto poly-l-lysine–coated slides (Fisher Scientific, Pittsburgh, PA) and stored in a −80°C freezer until use.

Immunostaining

After thawing, slides were washed with 0.001 M phosphate-buffered solution–triton (pH 7.4) four times and blocked with 1.5% normal donkey serum for 1 h. Slides were then incubated with one of the following specific antibodies: TRPV1 (guinea pig, 1:2,500; Neuromics, Northfield, MN), P2X3 (guinea pig, 1:1,000; Neuromics), CGRP (rabbit, 1:1,000; Bachem, King of Prussia, PA), or somatostatin (rabbit, 1:500; Santa Cruz, Santa Cruz, CA) overnight at 4°C in a humidified chamber. After washing with phosphate-buffered solution–triton (2 × 5 min), the slides were incubated with donkey species-specific tetramethyl rhodamine–labeled secondary antibodies (Jackson, West Grove, PA) diluted 1:200 in 1.5% normal donkey serum for 1 h. The slides were then coverslipped with Vectashield Mounting Medium (Vector, Burlingame, CA). Two control experiments were performed to validate the specific staining for each antibody: (1) incubation with specific blocking peptide (obtained from the same source company, except somatostatin, which was not performed) and (2) omitting the primary antibody.

Image Analysis

Only sections with fluorogold-labeled cells were selected for quantification. Every third section was used to avoid double counting, and effort was made to obtain three sections from each spinal level. Only cells with intact structure and observable nucleus were counted.

Images were captured with Q-imager under epifluorescence microscopy (Nikon E600; Tokyo, Japan) and with proper filters (UV-2A for fluorogold and G-2E/C for tetramethyl rhodamine). Images were later analyzed using...
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Considered statistically significant.

A total of 26 rats were studied (ovariectomy = 9, estrogen supplement = 8, intact = 7, bilateral hypogastric neurectomy = 2), and 727 fluorogold-labeled cells were identified. Injection of fluorogold into the dorsal surface of the uterine cervix and lower uterine segment labeled neurons bilaterally in DRGs at T12–L2 spinal levels. This labeling was via retrograde transport through the hypogastric nerve, because fluorogold-positive neurons were not found in animals that received bilateral hypogastric neurectomy at the time of tracer injection. Fluorogold-labeled cells appeared solid white to light blue in color with fluorescent staining in the cytoplasm (fig. 1), and those cells with calibrated density above 180 were counted as positively stained. Overall, the population of fluorogold-labeled neurons was 1–2% in studied DRG sections. In staining for TRPV1 and P2X3 receptors, a total of 412 fluorogold-positive cells were identified, and 315 fluorogold-positive cells were identified for the staining of CGRP and somatostatin. Most fluorogold-positive cells were found at L1, followed by L2 and T13, with few fluorogold-labeled cells at T12 (fig. 2). The average diameter of fluorogold-labeled neurons was 24 ± 6.1 μm (average ± SD), which was significantly larger than neurons stained with TRPV1 (20 ± 6.0 μm), P2X3 (22 ± 5.6 μm), CGRP (20 ± 5.2 μm), or somatostatin (18 ± 3.7 μm) (P < 0.001; fig. 3).

**TRPV1**

Cells staining positively for TRPV1 were small- to medium-diameter neurons (figs. 1 and 3). TRPV1 staining was numerically more common in fluorogold-labeled neurons than unlabeled neurons in all groups, although the difference was only significant in estrogen-treated animals (P < 0.001; fig. 4).

**P2X3**

Cells staining positively for P2X3 receptors were also small to medium size (figs. 1 and 3). Interestingly, not a single fluorogold-labeled uterine cervical afferent neuron colabeled with the P2X3 receptor in any group. In contrast, DRG neurons that were not traced from the uterine cervix expressed the P2X3 receptor with no differences among treatment groups (19 ± 5.4% in ovariectomy, 21 ± 3.7% in estrogen replacement, and 27 ± 3.6% in control animals; P > 0.05).

**CGRP**

Calcitonin gene–related peptide-immunostained neurons were also of small to medium size (figs. 1 and 3). Within each group, the proportion of neurons with CGRP staining was significantly greater in fluorogold-labeled neurons (P < 0.001; fig. 5). There was no effect of ovariectomy or estrogen replacement on the proportion of uterine cervical afferent or control neurons expressing CGRP (fig. 5).

**Somatostatin**

Very few DRG neurons expressed somatostatin (fig. 1), with no differences among groups (4.2 ± 1.0% in ovariectomy, 3.9 ± 1.6% in estrogen replacement, and 6.5 ± 0.7% in control animals; P > 0.05). As with the P2X3 receptor, there were no fluorogold-labeled neurons expressing somatostatin.

**Discussion**

Stimulation of the uterine cervix, whether from chronic inflammation, cancer, or acute dilatation, is a common cause of pelvic pain in women, yet its anatomic and neurophysiologic bases have received little previous...
attention. Berkley et al.\(^2\) characterized hypogastric afferents that innervate the uterine body of the rat and their responses to distension, heat, and bradykinin, but the relevance of these afferents to acute nociception is unclear, because conscious animals display remarkably little response to uterine body distension in the absence of inflammation.\(^28\) Papka et al.\(^36–39\) described the presence of some neurotransmitters and enzymes in nerve fibers in the rat cervix as well as in L6–S1 DRGs and the influence of pregnancy and estrogen treatment on their expression. These studies, however, focused primarily on pelvic afferents which terminate in low lumbar and upper sacral cord and did not trace uterine cervical afferents in the DRG, so the studies could not determine whether estrogen- or pregnancy-related changes occurred specifically in these afferents. Therefore, the current study fills an important gap in our knowledge regarding the sensory phenotype of hypogastric nerve afferents innervating the uterine cervix and lower uterine segment. Because all identified uterine and uterine cervical afferents in electrophysiologic studies conduct at C-fiber velocity,\(^2,6,8\) we focused our initial characterization on neurotransmitters and ion channels that are commonly expressed in C fibers and considered important in nociception. The results indicate a remarkably different phenotypic profile of uterine cervical hypogastric nerve afferents compared with other visceral or somatic C fibers.

Transient receptor potential vanilloid type 1 is a non-selective ion channel expressed in normal conditions by C fibers that responds to noxious heat, low pH, and vanilloid compounds, including capsaicin.\(^10\) Its expression is regulated by nerve growth factor\(^13\) and increases in uninjured neurons in models of neuropathic pain.\(^40\) Expression of TRPV1 in bladder urothelium in animals\(^11\) and pain and hyperalgesia from topical application of capsaicin to gastrointestinal endothelium in humans\(^41\) suggests that TRPV1 signaling is important to pain from

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**Fig. 1.** Photomicrographs of typical fluorogold labeling and immunostaining. Images in the left column show fluorogold labeling, those in the middle column show immunostaining, and those in the right column show combinations of the two. Arrows indicate cells with fluorogold labeling. A, B, and C depict staining of transient receptor potential vanilloid type 1 (TRPV1); note that the fluorogold-labeled cell colocalizes with TRPV1. D, E, and F depict staining of P2X3 receptor; note lack of colocalization between P2X3 and fluorogold labeling. G, H, and I depict staining for calcitonin gene–related peptide (CGRP); note colocalization of CGRP with fluorogold labeling. J, K, and L depict staining for somatostatin; note lack of colocalization of somatostatin with fluorogold labeling.

**Fig. 2.** Distribution of fluorogold-labeled neurons (n = 727 cells) at dermatomal level of T12–L2.
these viscera. Importantly, TRPV1 channel activation can, including colonic visceral afferents, transduce responses to mechanical stimuli and sensitization to distension after visceral inflammation.9,16 We previously demonstrated that some uterine cervical afferents respond to heat,8 consistent with expression of this channel. The current study suggests that approximately one third of uterine cervical hypogastric nerve afferents express TRPV1. Female sex hormones, at least at levels seen in the nonpregnant state, do not seem to regulate expression of this channel, as evidenced by no effect of ovariectomy or estrogen treatment. However, there was a difference in proportion of uterine cervical afferent neurons expressing TRPV1 compared with untraced neurons in the presence of estrogen. Therefore, these results indicate it is possible that increased TRPV1 expression could explain estrogen-mediated sensitization of uterine cervical afferents.8

Of course, immunocytochemistry studies only indicate expression of the antigen to which the antibody was raised at a level great enough to provide a signal above background and do not necessarily indicate the presence of functional protein or its quantitative level of expression. Other techniques, including single-cell proteomics or neurophysiologic recording, could examine more subtle changes in level of expression or in function. We used a stringent, population-based approach to determine whether a cell would be considered immunostained, and the proportion of cells meeting this criterion was somewhat less for TRPV1 and the other markers in the current study than in previous publications.42–44

P2X3 is one of a family of adenosine triphosphate–gated receptors on C fibers that is regulated by glial-derived nerve growth factor14 and is important in sensitization of somatic responses after inflammation and nerve injury.15 Unlike TRPV1, P2X3 receptor expression is unchanged in undamaged afferents after peripheral nerve injury.17 P2X3 receptors are present and functional in urinary bladder12,18 and gastrointestinal afferents19 and are believed to play a key role in visceral nociception. Thus, it is surprising that we observed a total lack of P2X3 receptor immunostaining in uterine cervical afferents in the current study. As noted above, we used a stringent criterion for indicating positive immunoreactivity and could have missed weakly stained cells. We consider this unlikely, because the proportion of control DRG neurons meeting the criterion for P2X3 receptor staining, 20–25%, is similar to that previously observed.45 Lack of P2X3 receptors on uterine cervical

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afferents of hypogastric nerve in the nonpregnant state represents an important distinction between this and other viscera and suggests that targeting this receptor would be unlikely to yield analgesia from acute uterine cervical nociception.

Calcitonin gene-related peptide is an important pain neurotransmitter expressed by a subset of C fibers and that increases in expression in states of sensitization, including inflammation, nerve injury, or chronic opioid exposure. CGRP also plays an important effenter function and is released in areas of inflammation, including the gastrointestinal mucosa, to result in vasodilatation and recruitment of leukocytes. The uterus and uterine cervix contain neuronal fibers that express CGRP, and some have speculated that CGRP plays an important effenter role in the cervical ripening process preceding labor. As previously reported, uterine cervical afferent neurons were more likely than untraced DRG neurons to express CGRP in the current study, consistent with an important role of this neurotransmitter in sensation and possibly peripheral signaling in the uterine cervix. Lack of effect of ovariectomy or estrogen suggests that changes in expression of this neurotransmitter are unlikely to explain estrogen-mediated sensitization of uterine cervical afferents, although the effects of pregnancy are worthy of exploration.

Somatostatin is present in a small subset of C fibers that express c- and, paradoxically, inhibit dorsal horn and peripheral responses of somatic nociceptors, in part by modulating TRPV1 channel activity. Inflammation of the gastrointestinal mucosa leads to a reduction in somatostatin content in colonic afferents, consistent with its presence and regulation in afferents to this visceral structure. The current study did not identify any afferents that innervate the uterine cervix and express somatostatin, suggesting targeting somatostatin receptors might not reduce responses to nociception from this organ.

In summary, despite the importance of the uterine cervix in acute and chronic pain states, little is known regarding the hypogastric afferents that innervate this structure. The current study demonstrates a unique phenotype of these afferents. The common expression of CGRP likely participates in both sensory and effenter functions in this tissue, and expression of TRPV1 may play a role in mechanosensitivity and, particularly, sensitization from inflammation or estrogen exposure. Interestingly, uterine cervical afferents in the hypogastric nerve do not express P2X3 receptor, which is thought to play a key signaling role in pain from the urinary bladder, nor do they express the inhibitory neuropeptide, somatostatin. Female sex hormones, at levels present in the nonpregnant state, do not seem to regulate expression levels of these neurotransmitters and channels, with the possible exception of estrogen dependence of TRPV1. These results provide testable hypotheses regarding novel strategies to treat pain from the uterine cervix and lay the groundwork for future studies examining the effect of pregnancy and labor on the neurophysiology of these afferents.

References

PHENOTYPE OF UTERINE CERVICAL AFFERENTS


