

VISCERAL SENSORY NEURONS THAT INNERVATE BOTH UTERUS AND COLON EXPRESS NOCICEPTIVE TRPV1 AND P2X3 RECEPTORS IN RATS

Victor V. Chaban, PhD

In women, clinical studies suggest that functional pain syndromes such as irritable bowel syndrome, interstitial cystitis, and fibromyalgia, are co-morbid with endometriosis, chronic pelvic pain, and others diseases. One of the possible explanations for this phenomenon is visceral cross-sensitization in which increased nociceptive input from inflamed reproductive system organs sensitize neurons that receive convergent input from an unaffected visceral organ to the same dorsal root ganglion (DRG). The purpose of this study was to determine whether primary sensory neurons that innervate both visceral organs—the uterus and the colon—express nociceptive ATP-sensitive purinergic (P2X3) and capsaicin-sensitive vanilloid (TRPV1) receptors. To test this hypothesis, cell bodies of colonic and uterine DRG were retrogradely labeled with fluorescent tracer dyes micro-injected into the colon/rectum and uterus of rats. Ganglia were harvested, cryo-protected, and cut in 20- μ m slices for fluorescent microscopy to identify positively stained cells. Up to 5% neurons were colon-specific or uterus-specific, and 10%–15% of labeled DRG neurons innervate both viscera in the lumbosacral neurons (L1–S3 levels). We found that viscerally labeled DRGs express nociceptive P2X3 and TRPV1 receptors. Our results suggest a novel form of visceral sensory integration in the DRG that may underlie co-morbidity of many functional pain syndromes. (*Ethn Dis*. 2008;18[Suppl 2]:S2-20–S2-24)

Key Words: DRG, Colon, Uterus, P2X3, TRPV1

From the Department of Biomedical Sciences, Charles R. Drew University of Medicine and Science; Department of Neurobiology, David Geffen School of Medicine, University of California, Los Angeles, California.

Address correspondence and reprint requests to: Victor V. Chaban; Department of Biomedical Sciences; Charles R. Drew University of Medicine and Science; 1731 E 120th St; Los Angeles, CA 90095; 323-357-3672; 323-563-9363; victorchaban@cdrewu.edu

INTRODUCTION

The incidence of persistent, episodic, or chronic visceral pain disorders such as irritable bowel syndrome and other “functional” syndromes (fibromyalgia, interstitial cystitis, chronic pelvic pain, and others) are more prevalent in women.^{1,2} Indeed in most clinical studies, women report more severe pain levels, more frequent pain, and longer duration of pain than do men. Nociception is a balance of pro- and anti-nociceptive inputs that is subject to regulation depending on the normal state of the organism. The cell bodies of primary visceral spinal afferent neurons are located in dorsal root ganglia (DRG). Direct activation of chemosensitive receptors and ion channels on their peripheral terminals and modulation of neuronal excitability activates extrinsic primary afferent nerves. Nociceptors belong predominantly to small- and medium-size DRG neurons whose peripheral processes detect potentially damaging physical and chemical stimuli.

Defining the sites and mechanisms of pain transmission in visceral nociception is an important step in understanding the pain perception and in designing appropriate therapies. One such mechanism may be the convergence of nociceptive stimuli in the primary afferent neurons that innervate different viscera. Visceral nociceptive C-fibers are activated by ATP released by noxious stimuli from cells in target organs and have been implicated as mediators of noxious stimulus intensities.³ Alteration in signal transduction of primary afferent neurons can result in enhanced perception of the visceral sensation, which is common in patients with different disorders, resulting in elevated pain perception. Irritable bowel syndrome is currently defined as a chronic

functional syndrome characterized by recurring symptoms of abdominal discomfort or pain and alterations in bowel habits in the absence of detectable organic disease. In the context of visceral pain, the capsaicin-sensitive vanilloid (TRPV1) receptor is a sensory neuron-specific cation channel that belongs to the transient receptor potential subfamily 1 and plays an important role in transporting thermal and inflammatory pain signals. Evidence for TRPV1's role in the pathogenesis of many diseases come from studies showing that mice lacking the TRPV1 receptor gene have deficits in thermal- or inflammatory-induced hyperalgesia.⁴ Activation of both TRPV1 and ATP-sensitive purinergic (P2X3) receptors induce mobilization of $[Ca^{2+}]_i$ in cultured DRG neurons.⁵ Capsaicin-induced TRPV1 receptor-mediated changes in $[Ca^{2+}]_i$ may represent a level of DRG activation to noxious cutaneous stimulation, while ATP-induced changes in $[Ca^{2+}]_i$ may reflect the level of DRG neuron sensitization to noxious visceral stimuli, since ATP is released by noxious stimuli and tissue damage near the primary afferent nerve terminals.⁶ In this report, we investigated whether subsets of visceral DRG neurons that innervate both uterus and colon express nociceptive P2X3 and TRPV1 receptors in rats.

METHODS

Retrograde Labeling

Retrograde labeling was used to identify uterus-specific and colon-specific DRGs and sensory neurons innervating both visceral organs: uterus and colon. For colonic afferents, the descending colons of adult female ovariectomized Long-Evans rats (weighing

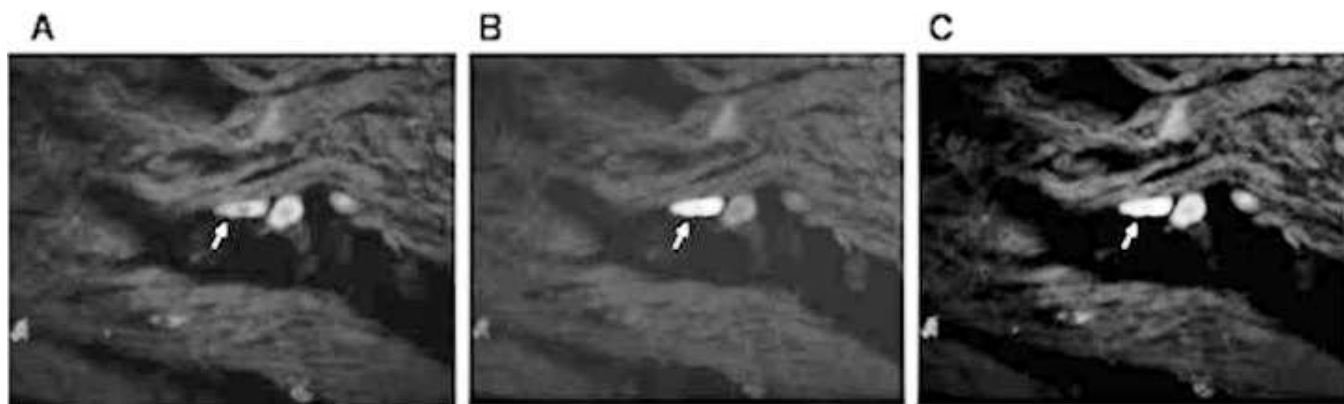


Fig 1. Visceral retrogradely-labeled dorsal root ganglion (DRG) neurons. A) Uterus-specific DRGs were injected with tetramethylrhodamine (red) into the uterus. B) Colonic DRGs were injected with Fluorogold (gold) into the distal colon. C) DRGs labeled with both dyes colocalized (orange). Sensory neurons innervating both uterus and colon indicated by arrows.

200-250g) were exposed under deep anesthesia with isoflurane. Fluorogold (FG) (12 μ L, 5% in .9% saline) was injected into the intestinal wall into four to eight different sites by using a Hamilton syringe (Hamilton Co., Reno, Nev) with a 26-gauge needle. The syringe was retracted .1 mm and held in this position for two minutes and then fully retracted during a two-minute period. Injection sites were carefully swabbed, and the colon was extensively rinsed with .9% sodium chloride solution and sealed with New Skin to prevent dye leakage. For uterine afferents, a laparotomy was performed to visualize the uterine horns, which were injected with tetramethylrhodamine (TMR, 6 μ L, 1.5% in .9% saline) into each lumen. Both dyes were injected into respective locations into the colon and uterine horns of the same animals. Immediately after surgery, rats were injected with sterile saline to prevent dehydration and were given analgesics for pain relief and antibiotics. All rats were allowed to survive for 10–14 days to allow for maximal transport of retrograde tracer. Rats were perfused with fixative, and the bilateral L₁-S₃ DRGs were postfixed overnight in 4% paraformaldehyde in .1 M phosphate buffer, pH 7.4, and cryoprotected in 30% sucrose for two days and sectioned (20 μ m thick) with cryostat. Sections

were mounted on glass slides and coverslipped with Aqua PolyMount (Polysciences, Inc, Warrington, Pa). All procedures were done in accordance with the National Institutes of Health and Charles R. Drew University of Medicine and Science Policy on Humane Care and Use of Laboratory Animals. The number and percentage of projection neurons to the DRG in each slice were analyzed by Olympus IX 51 microscope equipped for epifluorescence.

Immunocytochemistry

DRG sections were immunostained with rabbit-generated primary antibodies against P2X₃ and TRPV1 (NeuroMics, Northfield, Minn) for 48 hours, washed in .01 M phosphate-buffered saline (PBS) with .1% Triton X, and then incubated for 80 minutes in donkey anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (FITC, Molecular Probes, Eugene, Ore) followed by washes in PBS. Sections were mounted on slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, Calif). Immunostaining for FG and TMR was not performed since it is autofluorescent.

Statistics

Neuronal profiles from each rat were quantified for each tracer. Fluorescent

profiles of labeled neurons were discriminated from the background in the outlined cells. Retrogradely labeled and unlabeled cells were compared by two-way analysis of variance and Student *t* test by using Prism 3.0 software (GraphPrizm, San Diego, Calif).

RESULTS

Retrograde Labeling of Visceral DRG Neurons

Retrograde labeling was used to identify cultured DRG neurons innervating the colon by abdominal incision and Fluorogold (Molecular Probes) injection into the muscle wall. Animals were allowed to survive up to 10 days, and DRGs (L₁-S₃) were harvested. Cross-sections of the DRGs innervating distal colon and uterus were obtained from control and retrogradely labeled rats. Neuronal profiles from seven rats were quantified for each retrograde probe. Both colonic (labeled with FG, Figure 1A) and uterine (labeled with TMR, Figure 1B) afferents were indicated in L₁-S₃ DRGs. Cells that project to the uterus or colon were colocalized in the same DRG, indicating a subpopulation of DRG neurons that innervate both uterus and colon (Figure 1C). In control experiments, we sprayed TMR on uterine horns, FG on colon, and FG

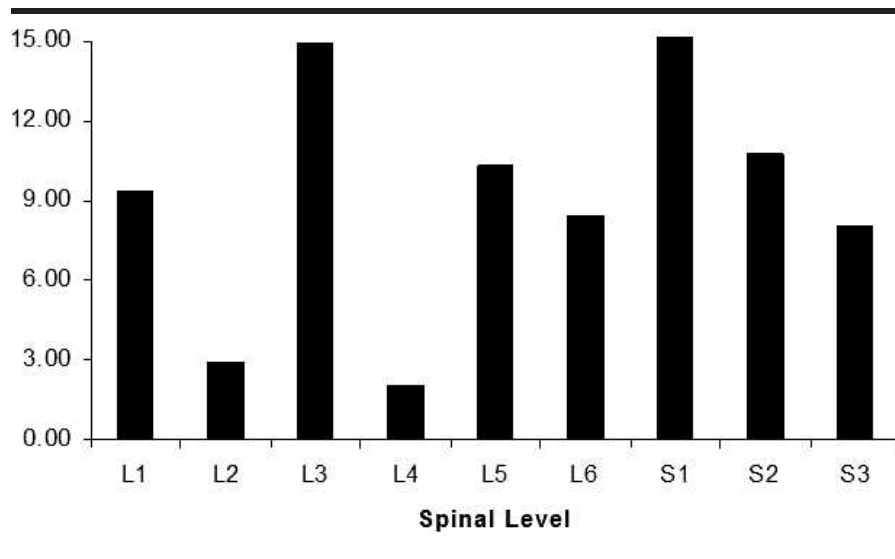


Fig 2. Percentage distribution of dorsal root ganglion neurons dually labeled for two visceral organs: the uterus and colon through the L₁, L₂, and L₆ and S₁, S₂, and S₃ levels.

and TMR dyes on both organs in the same animal (each procedure was performed on two different rats). Less than .5% cells were labeled with either FG and TMR or both tracers.

Our experiments revealed that the colon- and uterus-specific DRGs were located in all L₁-S₃ levels (Figure 2). These DRGs innervating both uterus and colon (labeled with both dyes) represent a new subset of dichotomizing afferent fibers (5%–15%) innervating both uterus and colon.

Expression of P2X₃ and TRPV1 Receptors in DRG Neurons

In another set of experiments, we studied whether visceral DRG neurons express nociceptive TRPV1 and P2X₃ receptors. DRGs sections were immunostained with primary antibodies against P2X₃ and TRPV1 (Neuromics, Northfield, Minn) for 48 hours and then incubated for 80 minutes in secondary antibodies conjugated to FITC. Neuronal profiles from five rats were quantified for each fluorescent probe. Both nociceptive-mediating P2X₃ and TRPV1 receptors were present in visceral DRGs (Figure 3).

DISCUSSION

The major finding of this study is that DRG neurons that innervate both uterus and colon express nociceptive capsaicin-sensitive TRPV1 and ATP-sensitive P2X₃ receptors. Our results indicate that sensory information to the DRG neurons may originate in different viscera. Although it is generally accepted that each primary afferent neuron is a single sensory channel, several studies have challenged that view and demonstrate that a population of DRG neurons can innervate both the visceral and somatic tissues.⁷ The design of these studies, using retrograde labeling from the uterus and colon, addressed the possibility that the same primary afferent can innervate both reproductive and gastrointestinal organs (DRG neurons had both retrograde tract tracing dyes). This new subset of dichotomizing fibers provides a novel pathway for sensitization of one viscus by another. The localization of P2X₃ and TRPV1 receptors in visceral DRG neurons in this report and the attenuation of ATP-induced [Ca²⁺]_i as we previously shown in DRG neurons *in*

*vitro*⁸ strongly suggest that nociceptive signals modulate visceral pain processing peripherally. Lumbosacral DRG neurons *in vitro* retain the expression of P2X₃ and TRPV1 receptors,⁹ which mediate the response to putative nociceptive signals. They continue to respond to P2X₃ and TRPV1 agonists, mimicking *in vivo* activation. ATP release from tissues during pathological conditions that cause tissue damage or inflammation activates P2X₃ receptors on primary afferent fibers innervating the afferent organs.¹⁰ Moreover, the predominant ATP receptor in small-diameter (nociceptive) DRG neurons that are capsaicin-sensitive is P2X₃.^{11,12} Opening P2X₃ channels results in membrane depolarization, sufficient to trigger action potential and Ca²⁺ influx through voltage-gated calcium channels.¹³ P2X₃-null mice lose ATP-induced currents in DRG neurons and have reduced pain-related behavior in response to noxious stimuli.^{14,15} The cloned TRPV1 receptor is a nonselective cation channel with a high permeability for Ca²⁺.¹⁶ TRPV1 receptors are distributed in peripheral sensory nerve endings and are involved in the transduction of different stimuli in sensory neurons. TRPV1 functions as molecular integrator of painful chemical and physical stimuli (noxious heat [$>43^{\circ}\text{C}$] and low pH). Various inflammatory mediators such as prostaglandin E₂ and bradykinin potentiate the TRPV1 channel. Significantly, a subset of DRG neurons responds to both capsaicin and ATP,¹⁷ indicating a potential cross-activation of these receptors that may underlie the sensitization of visceral nociceptors. In this report, we show that viscerally labeled DRG neurons that innervate both uterus and colon express P2X₃ and TRPV1 receptors.

Visceral pain is different from cutaneous pain based on clinical, neurophysiologic, and pharmacologic characteristics.¹⁸ The pathophysiology of visceral hyperalgesia is less well-known

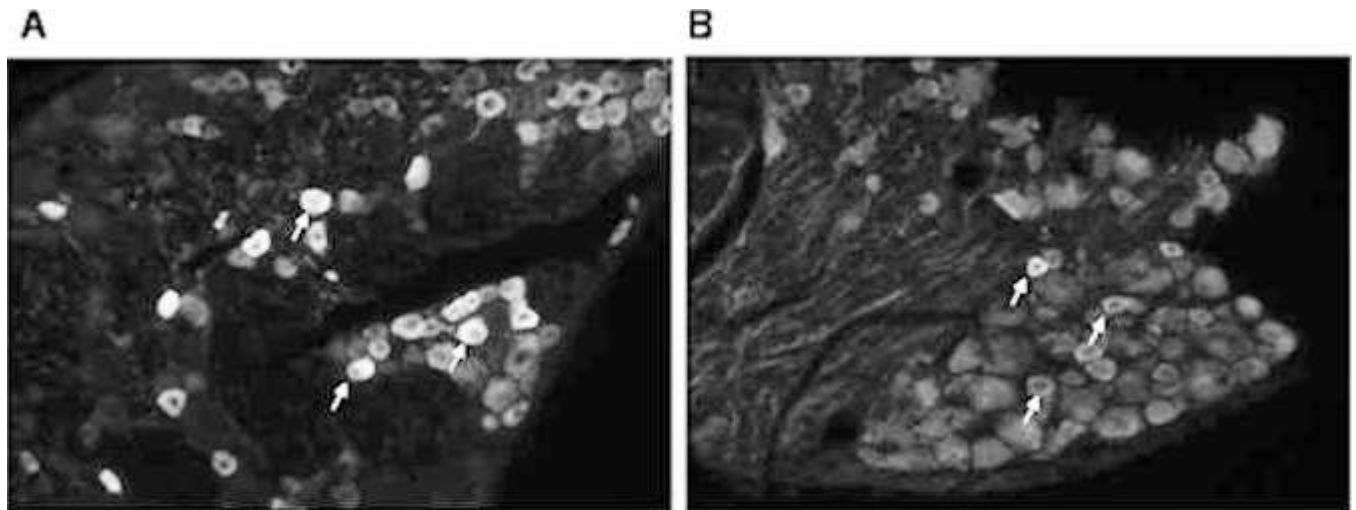


Fig 3. Visceral dorsal root ganglia (DRG) (L₆) express both nociceptive-mediating P2X₃ receptors (A) and TRPV1 receptors (B). DRG neurons innervating both uterus and colon indicated by arrows.

than its cutaneous counterpart, and our understanding of visceral hyperalgesia is colored by comparison to cutaneous hyperalgesia, which is believed to arise as a consequence of the sensitization of peripheral nociceptors from long-lasting changes in the excitability of spinal neurons.¹⁹ Peripheral sensitization can develop in response to sustained stimulation, inflammation, and nerve injury.

Nociceptive systems implicated in the etiology of functional disorders may be complicated by co-morbid disorders, all of which may pose health risks. The published literature suggests that >50% of individuals with irritable bowel syndrome have another pain-associated disorder. The fact that these changes are similar in all “functional” disorders suggests a model in which alterations in the central stress circuits in predisposed individuals are triggered by the similar pathophysiology. Acute and recurrent/chronic pelvic pain in women or abdominal pain from irritable bowel syndrome are illustrative examples of visceral pain that undergo sensitization.²⁰ Pain is the symptom that patients with irritable bowel syndrome list as the most depressing and is a major factor for consulting a physician. Thus, from a public health perspective, the

outcome of this study will have a substantial impact because it will increase our knowledge of nociceptive functional diseases such as irritable bowel syndrome, interstitial cystitis, and chronic pelvic pain and will help achieve a deeper understanding of sex differences in clinical aspects of these symptoms in various pain-associated disorders.

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VISCERAL DRGs EXPRESS NOCICEPTIVE RECEPTORS - Chaban

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