Distinct subclassification of DRG neurons innervating the distal colon and glans penis/distal urethra based on the electrophysiological current signature

Kristofer K. Rau,1,4 Jeffrey C. Petruska,2 Brian Y. Cooper,3 and Richard D. Johnson4
1Department of Anesthesiology, Department of Anatomical Sciences and Neurobiology, and Kentucky Spinal Cord Injury Research Center, University of Louisville College of Medicine, Louisville, Kentucky; 2Department of Anatomical Sciences and Neurobiology, Department of Neurological Surgery, Kentucky Spinal Cord Injury Research Center, University of Louisville College of Medicine, Louisville, Kentucky; 3Department of Oral and Maxillofacial Surgery, Division of Neuroscience, J. Hillis Miller Health Center, University of Florida College of Dentistry and McKnight Brain Institute, Gainesville, Florida; and 4Department of Physiological Sciences, University of Florida College of Veterinary Medicine and McKnight Brain Institute, Gainesville, Florida

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Rau KK, Petruska JC, Cooper BY, Johnson RD. Distinct subclassification of DRG neurons innervating the distal colon and glans penis/distal urethra based on the electrophysiological current signature. J Neurophysiol 112: 1392–1408, 2014. First published May 28, 2014; doi:10.1152/jn.00560.2013.—Spinal sensory neurons innervating visceral and mucocutaneous tissues have unique micrometrical distribution, peripheral modality, and physiological, pharmacological, and biophysical characteristics compared with those neurons that innervate muscle and cutaneous tissues. In previous patch-clamp electrophysiological studies, we have demonstrated that small- and medium-diameter dorsal root ganglion (DRG) neurons can be subclassified on the basis of their patterns of voltage-activated currents (VAC). These VAC-based subclasses were highly consistent in their action potential characteristics, responses to algesic compounds, immunocytochemical expression patterns, and responses to thermal stimuli. For this study, we examined the VAC of neurons retrogradely traced from the distal colon and the glans penis/distal urethra in the adult male rat. The afferent population from the distal colon contained at least two previously characterized cell types observed in somatic tissues (types 5 and 8), as well as four novel cell types (types 15, 16, 17, and 18). In the glans penis/distal urethra, two previously described cell types (types 6 and 8) and three novel cell types (types 7, 14, and 15) were identified. Other characteristics, including action potential profiles, responses to algesic compounds (acetylcholine, capsaicin, ATP, and pH 5.0 solution), and neurochemistry (expression of substance P, CGRP, neurofilament, TRPV1, TRPV2, and isolectin B4 binding) were consistent for each VAC-defined subgroup. With identification of distinct DRG cell types that innervate the distal colon and glans penis/distal urethra, future in vitro studies related to the gastrointestinal and urogenital sensory function in normal as well as abnormal/pathological conditions may be benefitted.

viscera; sensory neuron; nociception; dorsal root ganglion; classification

THE DISTAL COLON AND GLANS PENIS/DISTAL URETHRA are innervated by a complex network of sensory neurons that encode and transmit information to the central nervous system regarding the state of the gastrointestinal and urogenital tracts, respectively. Although they share many aspects of cellular anatomy, physiology, and function similar to those of neurons found in the somatic regions of skin and muscle, the ultrastructural and environment of visceral (colon) and mucocutaneous (glans penis/distal urethra) tissues present unique stimuli and conditions to their embedded sensory terminals.

Sensory neurons innervating these tissues conduct nociceptive and nonnociceptive information to the spinal cord, including responses to chemical and mechanical stimuli in both tissues and thermal stimuli in the glans penis. In both the colon and penis, sensory neurons integrate with various interneurons, pseudomotor neurons, sympathetic and parasympathetic neurons, and somatic motoneurons to coordinate regulation, reflexes, and general neuroendocrine functions (for review see Brookes et al. 2009; Furness 2006a, 2006b; Janig 1996; Janig and Morrison 1986; Johnson 2006; Robinson and Gebhart 2008). Although many of these activities are subconscious as part of the autonomic nervous system, conscious sensations from these regions do arise via spinal sensory neurons, with cell bodies in the segmental dorsal root ganglia (DRG).

In the colon, stretch and distention may result in nonpainful sensations (e.g., fullness, pressure, and urge to defecate) and acutely painful or uncomfortable sensations (e.g., bloating). Chronic pain states and altered peristalsis may also occur due to hypersensitivity of visceral sensory neurons in neurogenic and nonneurogenic pathologies, such as inflammatory bowel syndrome (IBS; Kanazawa et al. 2011; Zhou et al. 2010), inflammatory bowel disease (IBD; e.g., Crohn’s disease and ulcerative colitis; Baumgart and Carding 2007; Xavier and Podolsky 2007), substance abuse (Dimitrijevic et al. 2008), surgical intervention, and cancer (including tumor, chemotheraphy, and radiation therapy related; see Slavik et al. 2004 for review). Viscerosensory processes of the DRG that have nociceptive functions are of great importance from the perspective that disorders such as IBS, previously thought to be the result of motor dysfunction (McMahon 1997), may be partially explained by altered sensory function (Cervero 1994; Holzer 1991; McMahon 1994, 1997; Tougas 1999).

In the distal urethra and glans penis, the rich innervation (Halata and Munger 1986; Johnson and Halata 1991) relays a variety of stimuli that give rise to sensations similar to those which arise by stimulation of visceral tissues in the urethra (i.e., nonpainful and painful distention, chemoreceptor-based burning sensations, etc.) and mucocutaneous tissue of the glans surface epithelium (i.e., touch, pressure, movement, vibration, and pain). Pressure associated with passage of urine and semen
typically results in nonpainful sensations, unless neurons innervating the urethral lining are sensitized. In addition, these mucocutaneous afferents have unusual properties. For example, the process of erection causes single afferent fibers 1) to fire to the cavernous space engorgement in the absence of external stimuli while also sensitizing the response to tactile stimulation (Johnson 1988) and 2) to change the physical structure of the intraepithelial ending by uncoiling as the epithelium is stretched (Johnson and Halata 1991). As with the colon, various conditions may induce hypersensitivity and pain, such as cancer (Rigor 2000; Russo 2000), external trauma of the groin, passage of kidney stones (Wasserstein 1998), and catheterization (Wilson 2008). Urethral stricture, in which the urethral lumen narrows due to congenital defect or buildup of scar tissue after disease or injury, can result in hypersensitivity and feelings of pressure (Lumen et al. 2009; Vorvick 2012). Irritation of the glans penis (balanitis) can occur due to afflictions that commonly affect cutaneous tissues, including psoriasis and contact dermatitis (Buechner 2002; Das and Tunuguntla 2000). Also, infections due to various viral, bacterial, fungal, and parasitic sources of sexually and non-sexually transmitted diseases can affect sensitivity in both the distal urethra and glans penis.

Whereas many somatic sensory neurons within the skin and muscle do have intricate receptive structures (e.g., Pacinian corpuscles, Ruffini corpuscles, Merkel disks, muscle spindles, Golgi tendon organs, etc.), very few specialized receptive endings have been identified in visceral tissue, including intra-muscular arrays (Wang and Powley 2000), intraganglionic laminar endings (Lynn et al. 2003), and structures resembling Pacinian bodies (Janiq and McLachlan 1986; Janig and Morris 1986; Sheehan 1933; Willis and Coggshall 1991). These endings are less intricate than those found in somatic tissues and appear to be involved with nonnociceptive low-threshold mechanoreception. Most stimuli in the colon, however, are detected through unencapsulated free nerve endings (Blackshaw et al. 2007; Grundy 2006). Mucocutaneous tissue is located in the transition areas between somatic and visceral tissue and has a unique morphology and embryology (Halata and Munger 1986). The axons of the pudendal nerve and dorsal nerve of the penis (DNP) terminate in the mucocutaneous tissue of the glans penis and distal urethra in a network of free nerve endings and, to a lesser extent, genital end bulb-type receptors having lamellated corpuscles (Halata and Munger 1986; Johnson and Halata 1991). Compared with the somatic skin areas, however, very few Paciniform or Ruffini corpuscles are observed, and no Merkel disks or Meissner’s corpuscles are present (Halata and Spaehe 1997; Johnson and Halata 1991).

Previously, we have characterized small- and medium-diameter sensory afferents that innervate glabrous skin, hairy skin, and gastrocnemius muscle, using a technique that combines retrograde tracing from the peripheral target with DRG whole cell patch-clamp recordings of voltage-activated current (VAC) patterns (Jiang et al. 2006; Rau et al. 2005b, 2007). With an extension of procedures introduced by Scroggs (Cardenas et al. 1995), DRG cells have been subtyped by three classification protocols that use hyperpolarization and depolarization step commands. These step commands activate a variety of VACs that can then be recognized by their characteristic voltage dependence and kinetics (Petruska et al. 2000b, 2002). This method of classification appears to be a reliable system for identifying distinct DRG cell groups in vitro, because these subtypes have uniform properties with regard to action potential shape and afterhyperpolarization, sensitivity to laser-induced heat stimuli and heated solutions (Jiang et al. 2007; Rau et al. 2007), and responses to algesic compounds (i.e., capsaicin, ATP, acetylcholine, and acidic solutions; Petruska et al. 2000b, 2002). Furthermore, immunocytochemical phenotype is also remarkably consistent, including neurofilament, calcitonin gene-related peptide (CGRP), substance P, and binding of isoleucin B4 from Griffonia simplicifolia (IB4; Petruska et al. 2000b, 2002), P2X purinergic receptors (Petruska et al. 2000a), nicotinic acetylcholine receptors (Rau et al. 2005b), opioid receptors (Rau et al. 2005a), TWIK-related acid-sensitive K+ channels (TASK; TWIK: two-pore-domain weak inwardly rectifying K+ channel; Cooper et al. 2004; Rau et al. 2006), acid-sensing ion channels (ASIC; Jiang et al. 2006), and the transient receptor potential cation channel vanilloid subfamily members 1 and 2 (TRPV1 and TRPV2; Rau et al. 2007).

To determine some of the characteristics of spinal sensory afferents innervating the distal colon and glans penis/distal urethra of the adult male rat, we examined the patterns of VAC expressed by DRG neurons labeled by the retrograde tracer DiI, which was either injected into the distal colonic wall or applied to the glans penis/distal urethra. Based on observed VAC patterns, we have grouped cells into individual cell types. This multitechnique approach enables the documentation of a recorded neuron’s peripheral target, membrane cell properties and size, response to algesic chemicals, and immunocytochemical phenotype. Identification and characterization of the specific cell types that participate in normal functions will serve to further our understanding of normal physiology and also provide a vital baseline from which to identify changes involved in pathological conditions such as visceral diseases (Crohn’s, IBS, etc.) and spinal cord injury (Johnson 2006; Johnson et al. 2011).

**MATERIALS AND METHODS**

Animals were housed in quarters that conform to ALAAC standards. All procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Florida and the University of Louisville, and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

**Surgical procedures.** This study used 4- to 6-wk-old male Sprague-Dawley rats, including 8 animals for control studies and 31 animals for DRG recording studies. The rats weighed 60–70 g at the time of tracer injection and 100–150 g when euthanized for in vitro cell typing. For tracer injections, animals were anesthetized with a mixture of ketamine and xylazine (intraperitoneal injection, 80 mg/kg ketamine, 10 mg/kg xylazine). The following vital signs were monitored: heart rate, respiratory rate and ventilatory status (end-expired PCO2), and body temperature. Animals were placed on a heating pad to maintain body temperature (36–37°C).

DiI injections into the distal colon (n = 15; Fig. 1, A and B) were similar to those detailed in other studies (Gold et al. 2002; Ohtori et al. 2001; Su and Gebhart 1998; Su et al. 1999; Ueno et al. 2001; Wang and Scott 1999; Wang et al. 1998). A small longitudinal incision was made through the ventral midline hairy skin and linea alba into the peritoneal cavity. The bladder was aseptically evacuated using autoclaved sterile cotton swabs to gently press on the lateral surfaces of the bladder, thereby expelling urine onto autoclaved sterile cotton gauze held at the external urethral orifice without allowing the urine...
to enter the open abdominal region. The intestine and mesentery were then gently pushed aside to expose the most distal portion of the colon rostral to the rectum. Injections of the fluorescent retrograde tracer FastDiI oil (DiI, 1,1’-dilinoleyl-3,3’,3’-tetramethylindocarbocyanine perchlorate, 4% in methanol; Invitrogen) were then made into the lining of the distal colon, ~2 cm rostral to the anus, with a beveled 33-gauge needle coupled to a Hamilton microsyringe (10-μl volume per animal divided into 10 injections of 1 μl each). After each injection, the needle was slowly removed, and any leakage was removed by cotton-tipped applicators and the injection site surface sealed with a microdrop of cyanoacrylate and surgical staples.

DiI application was also made onto the mucocutaneous epithelium of the glans penis in a separate group of animals (n = 16; Fig. 1, C and D). By manual manipulation, the prepuce was retracted, and the glans penis/distal urethra was delicately exposed. Since the mucocutaneous epithelium in this region is very thin and underlying tissue is highly vascularized, DiI was not injected directly into this tissue. Instead, the lipophilic dye was absorbed through the epithelium and spread into the distal urethral tissue through the external urethral orifice. All rats were allowed to recover for 7–10 days and then euthanized to retrieve tissue for in vitro electrophysiological studies, target tissue histology, and immunochemical studies, as described below.

Preparation of cells for patch-clamp electrophysiology. DRG extraction and dissociation methods have been previously described in detail (Petruska et al. 2000b, 2002). Briefly, rats were decapitated following isoflurane anesthesia, and the spinal column was removed and bisected in the midsagittal plane. DRG (right and left L4–S2) were then dissected free, with the use of fine forceps and microdissecting scissors, and digested in a tube containing dispase (neutral protease, 5 mg/ml; Boehringer Mannheim) and collagenase (type I, 2 mg/ml; Sigma). The tube was rocked for 70 min in a heated (35°C) bath, and the cells were then gently triturated to dissociate the cells. The tube was returned to the bath for an additional 15 min. The cells were then gently triturated to dissociate the cells. The tube was returned to the bath for an additional 15 min. The cells were then gently triturated to dissociate the cells.

Whole cell patch recording. The electrophysiological techniques used in this study have been previously described in detail (Petruska et al. 2000b, 2002). Electrodes were prepared (1.8–4.2 MΩ) from glass pipettes using a Brown and Flaming-type horizontal puller (Sutter model P87). Each dish was briefly illuminated using epifluo-

Fig. 1. DiI labeling in the distal colon and glans penis/distal urethra. Bright field (A and C) and fluorescent images (B and D) are shown 7 days after DiI was injected into the distal colon (A and B) or painted onto the external surface of the glans penis and internal surface of the distal urethra (C and D). An asterisk indicates the injection site in the distal colon. DiI labeling (E and G) and activating transcription factor 3 (ATF3) expression (F and H) are also shown in the dorsal root ganglion (DRG) 7 days after DiI was either injected into the distal colon (E and F) or painted onto the external surface of the glans penis and internal surface of the distal urethra (G and H). NeuN labeling (I and K) and ATF3 expression (J and L) are shown at 10 h (I and J) and 24 h (K and L) post-DRG retrieval. ATF3-expressing neurons are indicated with arrows, and DiI-labeled cells are indicated with arrowheads. Scale bars are shown in B (for A and B), D (for C and D), F (for E and F), H (for G and H), J (for I and J), and L (for K and L).

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rescence microscopy to reveal tracer-labeled cells (total exposure of field <1 min). Whole cell recordings were made with an Axopatch 200B (Molecular Devices). Stimuli were controlled and digital records captured with pClamp8.1 software and a Digipack 1322A converter (Molecular Devices). Series resistance (R_s) was compensated 30–60%. Whole cell resistance was assessed by pClamp software from voltage transients associated with small step commands (10 mV). All experiments were conducted at room temperature. Only cells having a resting membrane potential (RMP) of −45 to −70 mV were included in this study.

**Cell classification protocols and action potential generation.** Cell diameter was estimated with an eyepiece micrometer scale by averaging the cell's longest and shortest axes. Cells were designated as small (<30 μm), medium (30–45 μm), or large (>45 μm). Cells were then classified according to their patterns of VACs recorded from three classification protocols (Cardenas et al. 1995; Petruska et al. 2000b). Classification protocol 1 (CP1) was used to examine the pattern of hyperpolarization-activated currents within a cell. From a holding potential (V_H) of −60 mV, the cell membrane was hyperpolarized to a final potential of −110 mV in 10-mV steps (500-ms duration, 4-s interstimulus interval). Classification protocol 2 (CP2) was used to produce inward current patterns. From a V_H of −60 mV, the cell was initially hyperpolarized to −100 mV (500-ms duration), followed by a depolarization to −40 mV in 20-mV steps (200-ms duration). Classification protocol 3 (CP3) was used to produce inward current patterns. From a V_H of −60 mV, a 500-ms conditioning pulse to −80 mV was followed by a series of depolarizing command steps (10-mV steps, 2.0-ms duration) to a final potential of +10 mV.

After classification using CP1–CP3, cells were brought into current-clamp mode. Action potentials were evoked at threshold through a 1-ms, 1,500- to 5,000-pA current step. The average of three action potentials was used to determine afterhyperpolarization (AHP) and action potential duration at the base (APDb). To quantify AHP, a criterion of 80% recovery to baseline was used (AHP80; Djouhri et al. 1998). APDb was measured as the time from the first upward deflection of the AP waveform to its return to baseline (−60 mV).

**Algesic responses of cells innervating the colon and penis.** After action potential generation, the cell was brought back into voltage-clamp mode. Solutions of algesic compounds were sequentially applied to evoke action potentials (2-N-morpholino)ethanesulfonic acid (MES), ACh solutions were prepared to a final concentration of 640 μM with Tyrode’s solution.

**Measurements.** All electrophysiological measurements were performed using Clampfit analysis software. APDb and 80% recovery afterhyperpolarization duration (AHP80) were obtained for each cell. The amplitudes of peak currents evoked by capsaicin (5 μM), ATP (50 μM), ACh (640 μM), and acidic solution (pH 5.0) were obtained for each cell. Currents were normalized by dividing by whole cell capacitance, an indicator of cell size (pF). Rates of decay (τ) were determined for peak currents evoked by ATP (50 μM), ACh (640 μM), and acidic solution (pH 5.0) using the equation $f(\tau) = A \cdot \exp(-e^{-t/\tau})$, where $A$ is amplitude and $C$ is capacitance. Single and multiple exponential fits were examined. Results are reported as means ± SE.

**Immunocytochemistry of cells innervating the colon and penis.** Immunocytochemistry was performed on dishes containing recorded cells within 2 h of recording. The cells were incubated at room temperature for 1 h in a solution of 1:30 normal goat serum in PBS with 0.4% Triton X-100 (GS-PBS-T) to block nonspecific antibody binding. Cells were then incubated over sequential evenings in solutions of primary antisera [guinea pig anti-substance P (diluted 1:3,000 in PBS; Peninsula Labs); rabbit anti-CGRP (1:2,000 in PBS; RBI); mouse anti-neurofilament M (NF; 1:500 in PBS; Dianova); rabbit anti-TRPV1 (1:1,000 in PBS; Chemicon); and rabbit anti-TRPV2 (1:1,000 in PBS; Chemicon) or in IB4 isolecitin (1:100 in PBS; conjugated to Alexa 594; Sigma). Species-specific goat-conjugated secondary antibodies (1:100 dilution in PBS) to Pacific blue (Invitrogen), AlexaFluor 488 (green; Invitrogen), and Cascade yellow (Invitrogen) allowed for multilabel fluorescent histochemical phenotyping for each cell examined. All steps were followed by multiple rinses with 1% GS-PBS-T. Incubations in primary antisera or IB4 were overnight (14–18 h), whereas secondary antibody incubations were 3 h in length. Anti-mouse secondary antisera were preadsorbed before use for 1 h against serum prepared from normal Sprague-Dawley rats. If necessary, certain signals were amplified through incubation with the Vectastain Elite ABC reagent [avidin-biotin-horseradish peroxidase (HRP) complex; Vector Labs] and tyramide-signal amplification (TSA) kits (Invitrogen).

Before dishes that contained physiologically categorized cells were tested, a dilution series for each primary antibody was run in control dishes to determine optimum concentrations for identifying positive vs. negative cells. As a negative control, competitive inhibition of the primary antibodies was performed by preadsorption of the antibody with excess synthetic blocking peptides (1 μg of peptide per 1 μg of antibody), which were supplied by the vendor. This procedure completely prevented the immunofluorescence in all cases. Control dishes to ensure antibody specificity were tested in which the full protocols were run with omission of one of the steps (i.e., the primary antibody, secondary antibody, streptavidin-HRP, or TSA kit). When dishes that contained typed cells were tested, a control dish was also always run with omission of the primary antibody.

**Histology of colon, penis, and adjacent tissues.** The distal colon, glans penis/distal urethra, and adjacent tissues (i.e., mesocolon, sublumbar skeletal muscles, bladder, and prepuce) were harvested to examine injection sites and monitor any potential leakage. Tissues were incubated at 4°C overnight in 4% PFA in PBS and then transferred to a PBS solution containing 30% sucrose. After incubation in the sucrose solution overnight, the tissue was sectioned on a cryostat at 10 μm and adhered to sequential slides. Slides were viewed under a fluorescence microscope to assess the spread of DiI.

**Additional histological controls for DRG viability during recording.** As previously mentioned, recordings were made 7–10 days after DiI injection, and within 10 h following DRG retrieval from an animal. To address the possibility that recorded cells may be injured due to 1) the initial injection of retrograde tracer or 2) DRG retrieval/dissociation on the day of recording, we assessed DRG neurons for expression of activating transcription factor 3 (ATF3). ATF3 is
Electrophysiological subtyping of colon and urogenital neurons

essentially absent from adult neurons but is expressed if they are stressed or injured, making it a reliable and robust indicator of neuronal stress/injury (Averill et al. 2004; Braz and Basbaum 2010; Dahlin et al. 2008; Hill et al. 2010; Kataoka et al. 2007; MacGillavry et al. 2009; Seijffers et al. 2007; Tsujino et al. 2000).

The distal colon and glans penis/distal urethra are bilaterally innervated by DRG sensory neurons. Thus, in 8 control animals (4 per target tissue), 7–10 days after DiI application, the left-side L4–S2 DRGs were harvested for acute dissociation and the right-side L4–S2 DRGs were placed in PFA for cryosectioning. After the acutely dissociated cells were plated as described above, samples were removed at select time points (3, 4, 5, 6, 7, 8, 9, 10, and 24 h post-DRG retrieval) and the Tyrode’s solution was immediately replaced with 4% PFA for 10 min, followed by repeated rinsing with PBS. DRGs used for cryosectioning were incubated over sequential days at 4°C, first in 4% PFA in PBS, and were then transferred to a PBS solution containing 30% sucrose. The tissue was then sectioned on a cryostat at 14 μm and adhered to sequential slides. After the slides/dishes were briefly heated on a slide warmer (35°C, 10 min), the dissociated cells/tissue sections were tested for ATF3 expression by using rabbit anti-ATF3 primary antibody (1:1,000 in PBS; Santa Cruz) and goat anti-rabbit secondary antibody conjugated to Alexa 488 (1:100 in PBS; Jackson ImmunoResearch). Nuclear localization of ATF3 in neurons was verified by using an antibody for NeuN (mouse anti-NeuN; 1:1,000 in PBS; Millipore) and goat anti-mouse secondary antibody conjugated to Alexa 350 (preadsorbed in rat serum; 1:100 in PBS; Jackson ImmunoResearch). Blocking steps, wash steps, and controls were similar to those listed above. Cells were then examined for labeling of ATF3 and DiI (see Microscopy).

Microscopy. Sections of peripheral tissue and dishes were viewed with an epifluorescence Zeiss Axiohot microscope. Individual channels were viewed with Vivid filter sets (Omega Optical) appropriate for the spectra of the tracer and fluorophores. DiI was viewed with XF102, AlexaFluor 488 was viewed with XF100, and Cascade yellow was viewed with XF1041. Spectra from Pacific blue were viewed with XF119. Digital images were captured using a Dage 72S or Photometrics CoolSnap camera (Roper Scientific) coupled to a personal computer running the Analytical Imaging Station software package from MCID. The control studies that examined expression of ATF3/NeuN/ DiI were visualized using a Nikon TiE automated inverted microscope with a Photometrics CoolSnap camera (Roper Scientific) and Nikon Elements BR analysis software. ATF3-AlexaFluor 488, NeuN-AlexaFluor 350, and DiI were viewed with GFP, DAPI, and TRITC filter sets (Semrock), respectively.

Statistics. Student’s t-tests, analysis of variance (ANOVA), and post hoc tests (i.e., Mann-Whitney’s rank sum test and Tukey’s pairwise multiple comparisons test) were used to examine the possibility of significant differences between the electrophysiological characteristics of selected groups. For all tests, a difference was considered significant if $P < 0.05$.

RESULTS

DiI was injected into the mucosa of the distal colon (Fig. 1, A and B) or applied onto the surface of the glans penis/distal urethra (Fig. 1, C and D). Whole cell recordings were subsequently made from DiI-positive DRG neurons. Recordings were made from 190 cells (105 from the colon and 85 from the penis) harvested from 31 animals (15 from the colon and 16 from the penis). Based on the current patterns evoked by CP1–CP3, these cells were grouped into the previously characterized types 5, 6, 7, and 8 (Petruska et al. 2002; Rau et al. 2007, 2011) as well as novel types 14, 15, 16, 17, and 18 (see Figs. 2 and 3). Of the 90 recorded cells, 4 cells from the colon and 9 cells from the penis could not be suitably classified. For the sake of brevity, afferents originating from the distal descending colon and glans penis/distal urethra are hereafter often generalized as neurons of the colon and penis, respectively, although we acknowledge that this study does not take into account the possible diversity of neurons innervating other regions of these organs (e.g., ascending and transverse colon, penile body, prepuce, etc.).

ATF3 expression after injection/application of DiI in the colon and penis. To determine whether our procedures (injections, tracer incorporation, dissociation) induced an injury/stress response in the neurons, which could influence their electrophysiological and neurochemical properties (Djouhrri et al. 2012), we assessed the expression of ATF3 in neurons prepared according to the same protocols used for electrophysiological recordings. Following DiI injections into the colon, we found only the rare ATF3-expressing neuron (Fig. 1, E and F), whereas after DiI application to glans penis tissue, we found no upregulation of ATF3 (Fig. 1, G and H). Interestingly, the few ATF3-expressing neurons from the colon were never colabeled with DiI. ATF3 expression was also assessed in dissociated/plated neurons at the 3–10 and 24-h postretieval time points. Significant numbers of ATF3-expressing neurons were observed, but only at the 24-h time point (Fig. 1, I–L).

Although the recorded neurons were clearly injured by the dissociation process, considering all cell classification recordings were made within 10 h of DRG retrieval from an animal, it was unlikely that they were in the midst of executing the molecular/genetic injury/stress response defined by the expression of ATF3 (Seijffers et al. 2006). These assessments together suggest that the characteristics of the tissue-specific neurons described are not likely to be affected to any significant degree by the molecular/genetic changes associated with the cellular injury/stress response.

Electrophysiological characteristics of DRG cell types innervating the colon and penis. Cell types 5, 8, 15, 16, 17, and 18 were identified among the neurons traced from the colon (Fig. 2), and cell types 6, 7, 8, 14, and 15 were identified among the neurons traced from the penis (Fig. 3). Electrophysiological properties for all cell types, including cell diameter (μm), cell size (pF), RMP (mV), APD50 (ms), and AHP80 (ms), are listed in Table 1. A portion of the identified cells were similar to those recorded from other tissues (types 5, 6, and 8; Jiang et al. 2006; Rau et al. 2005b, 2007), but a number of novel cell classes were identified with either 1) distinct current signature patterns (types 14, 15, 16, 17, and 18) or 2) an identified target (type 7) that appeared to represent physiologically distinct nociceptive and nonnociceptive afferents with unique distribution into visceral and mucocutaneous tissues.

Type 5 cells, which are the most abundant cell type innervating muscle tissue, were also identified among the neurons traced from colon ($n = 7$; Fig. 2; Table 1; Rau et al. 2007). Similar to previously described type 5 cells (Petruska et al. 2002), these cells had medium-sized cell diameters of 35–45 μm and small nuclei. CP1 evoked hyperpolarization-activated inwardly rectifying current ($I_h$) that was considered "medium" in size (200–1,000 pA) and was similar in scale to type 6 and type 8 cells (see below; Figs. 2 and 3). CP2 produced three distinct A-type rapidly inactivating K+ current peaks ($I_A$; Fig. 2).

Type 6 cells identified from the penis ($n = 18$; Fig. 2; Table 1) had medium cell diameters of 40–45 μm. As previously identified from hairy skin (Jiang et al. 2006; Petruska et al. 2002; Rau et al. 2007), this cell type exhibited "medium" $I_h$...
(200–1,000 pA) and a lack of transient outward current from CP1 but could be distinguished from other subtypes by the appearance of five $I_A$ peaks in CP2.

Novel type 7 cells identified from the penis only ($n = 18$; Fig. 3; Table 1) had very small cell diameters (15–25 μm) and relatively large nuclei. The signatures evoked by CP1 and CP2 were virtually indistinguishable from those of type 3 cells (Petruska et al. 2000b, 2002), with small $I_H$ (<200 pA) and small rounded outward currents with no $I_A$ peaks. In this case, CP3 is used to identify this cell type, in which slowly inactivating inward Na currents are approximately twice the duration as those evoked in type 3 cells (Petruska et al. 2000b).

Type 8 cells were identified from both the colon (Rau et al. 2007) and penis. Interestingly, this cell type has also been traced from muscle, glabrous skin, and hairy skin (Jiang et al. 2006; Rau et al. 2007). Similar to the type 8 cells found in all of the other tissues previously examined, colonic type 8 neurons ($n = 21$; Fig. 2; Table 1) and penis type 8 neurons ($n = 6$; Fig. 3; Table 1) had medium cell diameters (35–45 μm) and expressed current signatures with “medium” $I_H$ (200–1,000 pA) in CP1 and four $I_A$ peaks in CP2 with sloping currents. As suggested by previous studies (Petruska et al. 2002), type 8 cells appear to include multiple subpopulations. Although currents evoked by CP1–CP3 remain consistent for all type 8 cells, certain algesic traits diverge into two subpopulations (see below). The AHP duration of colonic type 8 neurons was significantly shorter than that of type 8 cells from mucocutaneous penile tissue ($P < 0.01$).
Novel type 14 cells were among those identified by tracing from the penis only \((n = 12; \text{Fig. 3; Table 1})\). These neurons had cell diameters ranging from 40 to 50 \(\mu\text{m}\) and large nuclei. CP1 and CP3 current signatures were similar to those found in type 15 cells. CP2, however, generated current patterns that consisted of four \(I_A\) peaks, rather than the five peaks observed in type 15 cells.

Type 15 cells were found in both colon \((n = 39; \text{Fig. 2; Table 1})\) and penis \((n = 22; \text{Fig. 3; Table 1})\) and were the most numerous recorded cell type. These cells characteristically had the largest diameter in this study \((45–65 \mu\text{m})\) with large \(I_H\) \((>1,000 \text{pA})\), five \(I_A\) peaks with sustained current, and a CP3-evoked current that was similar to the rapid inward and outward currents from hairy skin-traced type 4 cells \(\text{(Jiang et al. 2006; Petruska et al. 2000b; Rau et al. 2005b)}\). In the penis-traced neurons, action potentials had an AHP80 that was significantly shorter than that of other neurons traced from mucocutaneous sites (types 6, 7, 8, and 14: \(P < 0.05\), \(P < 0.05\), \(P < 0.01\), and \(P < 0.05\), respectively). Of all of the cells types traced from the colon, type 15 cells had action potentials with the shortest APDb \((4.1 \pm 0.2 \text{ms}; P < 0.05)\) and a fairly short AHP80 \((67.1 \pm 3.8 \text{ms})\).

Colonic cells that fit the novel current signature pattern of type 16 \((n = 13; \text{Fig. 2; Table 1})\) had cell diameters of 30–40 \(\mu\text{m}\). In response to CP1 and CP2, cells evoked large \(I_H\) \((>1,000 \text{pA})\), a slowly desensitizing outward current, and four small \(I_A\) peaks. For CP3, the signature looks similar to type 3,
### Table 1. Properties of DiI-traced penis and distal colon cells

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<tr>
<th>Distal Colon Cell Type</th>
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</tr>
<tr>
<td>Diameter, μm</td>
<td>37.9 ± 2.1</td>
<td>40.7 ± 1.1</td>
<td>44.4 ± 0.6</td>
<td>33.5 ± 1.1</td>
<td>29.4 ± 1.5</td>
<td>44.6 ± 0.7</td>
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<td>Size, pF</td>
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<td>73.7 ± 3.4</td>
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<td>37.4 ± 2.4</td>
<td>86.6 ± 4.0</td>
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<tr>
<td>RMP, mV</td>
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<td>−64.1 ± 1.5</td>
<td>−57.2 ± 1.1</td>
<td>−48.3 ± 1.8</td>
<td>−50.7 ± 2.3</td>
<td>−621.2 ± 1.2</td>
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<tr>
<td>Duration, ms</td>
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<td>5.6 ± 0.4</td>
<td>6.7 ± 0.5</td>
<td>7.0 ± 0.5</td>
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<tr>
<td>AHP, ms</td>
<td>63.2 ± 11.1</td>
<td>106.5 ± 11.7</td>
<td>90.4 ± 7.5</td>
<td>63.9 ± 8.3</td>
<td>116.3 ± 16.0</td>
<td>115.8 ± 16.0</td>
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<td>13/13</td>
<td>7/8</td>
<td>12/12</td>
</tr>
<tr>
<td>Peak, pA/pF</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>10.6 ± 3.0</td>
<td>25.3 ± 9.7</td>
<td>1.8 ± 0.7</td>
<td>0.8 ± 0.2</td>
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<tr>
<td>τ, ms</td>
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<td>id</td>
<td>2395.8 ± 755.4</td>
<td>3293.3 ± 703.7</td>
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<tr>
<td>Responders</td>
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<td>37/39</td>
<td>12/13</td>
<td>9/9</td>
<td>12/12</td>
</tr>
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<td>11/11</td>
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<td>86.5 ± 28.0</td>
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<td>79.1 ± 16.1</td>
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<td>95.4 ± 13.0</td>
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<td>τ, ms</td>
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<td>12/13</td>
<td>9/9</td>
<td>12/12</td>
</tr>
<tr>
<td>Peak, pA/pF</td>
<td>130.1 ± 21.6</td>
<td>130.2 ± 17.3</td>
<td>107.0 ± 15.3</td>
<td>113.0 ± 22.9</td>
<td>107.0 ± 22.5</td>
<td>127.0 ± 14.8</td>
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</table>

### Table 2. Glass Penis/Distal Urethra Cell Type

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<tr>
<td>Diameter, μm</td>
<td>43.1 ± 0.7</td>
<td>27.5 ± 1.1</td>
<td>34.3 ± 1.5</td>
<td>45.0 ± 0.9</td>
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<td>Size, pF</td>
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<td>RMP, mV</td>
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<td>−596.1 ± 1.5</td>
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<td>Duration, ms</td>
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<td>AHP, ms</td>
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<td>id</td>
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<td>4/5</td>
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<td>6/6</td>
<td>11/11</td>
<td>21/21</td>
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<td>Peak&lt;sub&gt;prep&lt;/sub&gt; pA/pF</td>
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<td>153.9 ± 23.0</td>
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<td>5/6</td>
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<td>4/4</td>
<td>0/3</td>
<td>0/9</td>
<td>0/12</td>
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</tbody>
</table>

Physiological properties, including resting membrane potential (RMP), action potential characteristics, algesic response amplitude, and response kinetics, are presented with immunocytochemical profiles of the same recorded cells. Cell properties include cell diameter and capacitance (size). Action potential characteristics include the base duration of an action potential (duration) and afterhyperpolarization duration recovery to 80% (AHP). Values are means ± SE; id, insufficient data; τ, time constant of decay; Peak<sub>prep</sub> and Peak<sub>prep</sub>, peak of desensitizing and non-desensitizing component of current; NF-M, TRPV1 and TRPV2, transient receptor potential vanilloid type 1 and 2; neurofilament-M; IB4, isolecin B4.

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with rapid Na⁺ currents (Petruska et al. 2000b). Action potentials from these cells had an AHP80 that was significantly shorter than that of all other colon afferents (P < 0.05).

Novel type 17 cells (n = 9; Fig. 2; Table 1) were the smallest cells recorded among those traced from colon, with cell diameters of 25–35 μm (P < 0.01). Relatively small Iᵢᵥ (<200 pA) were expressed during CP1, whereas in CP2, there were three small Iᵢₐ peaks. In CP3, the signature is similar to that found in type 1 cells (Petruska et al. 2000b). The mean AHP80 for this cell type measured the longest of all of the cells recorded during this study (P < 0.05).

Finally, novel type 18 cells (n = 12; Fig. 2; Table 1) were traced from the colon. These cells had diameters of 40–50 μm and the largest cell sizes (86.6 ± 4.0 pF; P < 0.05). These cells had current signatures with large Iᵢᵥ (>1,000 pA), four Iᵢₐ peaks with sustained current, and a CP3-evoked current that was similar to, but larger than, the inward currents in type 8 cells (Fig. 2; Petruska et al. 2002). Action potentials from this cell type had the largest average APDb and relatively long AHP80 (P < 0.05).

Algesic responses of DRG cell types innervating the colon and penis. Responses to ATP (50 μM), ACh (640 μM), protons (pH 5.0 solution), and capsaicin (5 μM) were recorded for most cells innervating the distal colon (Fig. 4; Table 1) and penis (Fig. 5; Table 1). The normalized peak amplitudes (in pA/pF) and the numbers of insensitive and/or sensitive cells are indicated for each algesic compound. Time constants of current decay (τ) are also indicated for responses to ATP, ACh, and protons (Table 1). Novel cell types from penis and colon exhibited distinct patterns of algesic reactivity but were similar within tissue types. We found that all cell types innervating the colon and penis appear to respond to ATP to some degree (Figs. 4 and 5; Table 1). For colon-traced neurons, ATP evoked large-amplitude currents in type 16 cells and small-amplitude currents in type 5, 8, 15, 17, and 18 cells. For penis-traced neurons, all cell types with the exception of type 8 displayed relatively weak currents to ATP. All of the colon and penis cell types in this study also consistently responded to an acidic solution in which there was a strong rapidly desensitizing component along with a sustained, non-desensitizing component. All colon-traced cell types were robustly responsive to capsaicin. In contrast, with the exception of type 8 cells, all penis-traced cell types were either insensitive (types 6, 14, and 15) or weakly sensitive (type 7) to this compound. ACh elicited an inward current in all recorded cell types traced from the colon, with the strongest responses observed in types 8, 15, and 16. For neurons traced from the penis, only types 6 and 14 displayed large currents in response to ACh. In other cell types, currents were relatively small and transient (see Figs. 4 and 5).

Immunocytochemistry of cells innervating the colon and penis. After electrophysiological analysis, recorded cells were saved for subsequent immunocytochemistry. Examples of this are shown in Fig. 6, in which type 15 cells innervating the colon (A–L) or penis (M–X) were tested for NF, CGRP, substance P, TRPV1, and TRPV2 immunoreactivity and for IB4 binding. Although some cells were lost during the fixation and immunocytochemical processes, most surviving cells were located and their immunoreactivity assessed. A summary of expression patterns for all of the cell types is listed in Table 1. Immunocytochemistry suggested that traced neurons were derived from both

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**Fig. 4.** Algesic sensitivity of DRG cells innervating the distal colon. Evoked responses to the application of ATP, pH 5.0, capsaicin, and ACh are shown for each cell type. Duration of algesic application is indicated by the bar overlying each trace. Scale bars for the currents evoked by ATP, pH 5.0, capsaicin, and ACh for each cell type are shown at bottom.
Identification of cell types innervating the colon and penis.

In this study, DRG cells have been subclassified into cell types on the basis of their patterns of VACs. As with previous studies from our laboratory, each cell type has a consistent response to algesic compounds, has a characteristic cytochemical phenotype, and innervates tissues in specific combinations (Jiang et al. 2006; Petruska et al. 2000b, 2002; Rau et al. 2005a, 2005b, 2006, 2007, 2011). Novel cells described in this study can be easily distinguished from types published previously through the use of their characteristic current signature patterns and their cell size.

Following injection/application of the retrograde tracer DiI into the parenchyma of the distal colon or glans penis/distal urethra of the rat, we identified nine groups of small to medium-diameter DRG neurons. Cell types 5, 8, 15, 16, 17, and 18 were traced from the colon, and cell types 6, 7, 8, 14, and 15 were traced from the penis. We identified several cell types that appear to be unique to the colon (types 16, 17, and 18) or penis (types 7 and 14) because they were not found in skeletal muscle (Jiang et al. 2006), hairy skin (Rau et al. 2007), or glabrous skin (Rau et al. 2005b) in our previous studies using the same methodology. The rest of the cell types identified in the present study also innervate other types of tissue (types 5, 6, 8, and 15; Jiang et al. 2006; Rau et al. 2005b, 2007). Although these groups may eventually prove to include distinct populations that can be separated with the use of additional voltage protocols, they may be neuron types that serve physiological functions common to all of these different tissues. For instance, the type 5 cell innervates skeletal muscle (Rau et al. 2007) and may also innervate the smooth muscle associated with the colon. Both the colon and distal urethra share similar anatomic characteristics as visceral tubular structures, which have the capacity to detect similar stimuli (stretch/distention/chemical), a sensitivity that may be subserved by colonic and penile type 15 cells (Delcambre et al. 2011). The properties of the type 8 cells innervating the colon and penis were also highly similar to those traced from skeletal muscle (Rau et al. 2007), hairy skin (Jiang et al. 2006; Rau et al. 2005b, 2007), and glabrous skin (Jiang et al. 2006). Finally, the ubiquitous type 8 neurons may subserve sensory functions common to all tissue types injected and/or may represent innervation of tissue components common to all tissues injected, such as the vasculature.
Cell properties of sensory neurons innervating the colon and penis. Previous studies suggest that there is a difference in afferent fiber types between visceral, mucocutaneous, and somatic (cutaneous and muscle) tissues. Unlike typical cutaneous nerves, in which 20–25% of total fibers are the large myelinated Aβ-fibers, 10–15% are the small myelinated A-fibers, and 60–70% are unmyelinated C-fibers (Gasser and Grundfest 1939), the visceral afferent population is composed of very few large myelinated fibers (Janig and McLachlan 1986; Janig and Morrison 1986; Sheehan 1933; Willis and...
C-fibers (type 5), A-insensitive or “silent” nociceptors (see Meyer et al. 1991; Sato et al. 2002) to associate the presence of a long AHP with mechanically sensitive properties. For example, Lawson et al. (2002) were able to functionally classify DRG cells. For instance, certain action potential shapes (e.g., presence of a “hump” or “shoulder” on the action potential) were not able to characterize in this study. In the glans penis, large DiI-labeled cells in whole DRG sections (Ferrero et al. 2011) and Aβ-fiber low-threshold mechanoreceptors (Johnson and Murray 1992) are present.

Previous in vivo studies utilizing intracellular sharp electrode recording have also examined characteristic features of functionally classified DRG cells. For instance, certain action potential shapes (e.g., presence of a “hump” or “shoulder” on the repolarization phase; Harper and Lawson 1985; Koerber et al. 1988), long AHP durations (Djouhri et al. 1998; Villiere and McLachlan 1996), and large action potential overshoots (Djouhri and Lawson 2001) are associated with nociceptive cells. These attributes were also seen in the present study. There are, of course, notable differences between our in vitro study and the aforementioned in vivo studies that focused on somatic (skin, muscle, joint) sensory cells, which prevent conclusions as to whether the function of our visceral and mucocutaneous cell types are restricted to nociception. Furthermore, given that in vitro and in vivo studies indicate that many colon nociceptors respond to low-threshold mechanical stimuli but are polymodal and therefore may respond to algogenic substances (e.g., Lynn and Blackshaw 1999, Raybould et al. 1999, Sengupta and Gebhart 1994, and Su and Gebhart 1998), these cell types may not necessarily be classified as strictly “nociceptors” per se. Nonetheless, all of the cells recorded in this study do fit within the in vivo classification scheme of nociceptive-type cells, based on AP and AHP duration criteria. For example, Lawson et al. (2002) were able to associate the presence of a long AHP with mechanically sensitive or “silent” nociceptors (see Meyer et al. 1991; Sato et al. 1985). Colon-innervating cell types possibly included C-fibers (type 5), Aβ-fibers (types 15 and 16), and putative Aδ-silent fibers (types 8, 17, and 18). In the penis-innervating populations, type 7 cells are likely C-fibers and may fit into the C-silent nociceptive family, whereas types 6, 8, 14, and 15 shared properties with the Aδ nociceptive group by virtue of their long AHP characteristics (Djouhri et al. 1998; Lawson et al. 2002).

Algesic responses of sensory neurons innervating the colon and penis. Sensory neurons innervating the distal colon have previously been shown to respond to several of the algogenic compounds examined in this study, including ATP (Wynn et al. 2004), ACh (Darko et al. 1997), and capsaicin (Su et al. 1999). Although there have been numerous algesic response studies focused on other regions of the rat lower urinary tract (e.g., detrusor and urothelium of the bladder, proximal urethra) showing the effects of ATP (e.g., Brown et al. 1979; Dang et al. 2005; Pinna et al. 2006; Yu and de Groat 2008), ACh (e.g., Hashitani and Suzuki 1996; Kanai et al. 2007; Pinna et al. 2006), protons (Dang et al. 2005; Sadananda et al. 2009), and capsaicin (e.g., Dang et al. 2005; Masuda et al. 2006; Shea et al. 2000), few have examined algesic responses within the distal urethra and glans penis. However, in neurons innervating the colon and penis of the rat, some of the channels that mediate responses to algogenic compounds have been identified immunohistochemically, including those for ATP-responsive purinergic P2X receptors (colon: Van Crombruggen et al. 2007; Wynn et al. 2003; and penis: Lee et al. 2000), and proton- and capsaicin-sensitive TRPV1 (colon: Christianson et al. 2006; Rau et al. 2007; and penis: Stein et al. 2004). In agreement with other generalized studies on sensory neurons (Burnstock and Wood 1996; Doyle and Forrester 1985; Rang 1994; Rang et al. 1991), the majority of cells within each colon and penis subtype responded to ATP and ACh, although the frequency and intensity of the response was greater in colon-innervating cells. Responses to protons (pH 5.0 solution), however, were seen in all recorded cells.

Capsaicin responses, while seen in all colon cells, were present in penis cells types 7 and 8, but not in types 6, 14, and 15. Yet even these capsaicin-insensitive types had AHPs characteristic of somatic nociceptors (Lawson 2005). Capsaicin, a chemical component of “hot” chili peppers, elicits the perception of noxious heat by activating sensory neurons (Bevan and Szolcsanyi 1990; Holzer 1991) via TRPV1 channels (Caterina et al. 1997; Cesare et al. 1999; Tominaga et al. 1998). Capsaicin sensitivity is generally taken to indicate nociceptive cell types (Baumann et al. 1991; Belmonte et al. 1991; Chen et al. 1997; Szolcsanyi et al. 1988). However, capsaicin-insensitive nociceptive cell types (including heat-sensitive types) have also been shown to exist (Magerl et al. 2001; Ringkamp et al. 2001). Capsaicin sensitivity is found in 25% of cutaneous afferents versus 60% of visceral afferents (Hu-Tsai et al. 1992). Although the majority of colon cells in this study responded to capsaicin, there was a distinct population (12/39) of type 15 cells that were insensitive to this compound. A study by the Gebhart laboratory made a similar determination in that only a portion of DiI-labeled DRG cells innervating the colon responded to capsaicin (Su et al. 1999).

Nociceptive sensory neurons also respond to acidic solutions (Bevan and Geppetti 1994; Chesler 1990; Issberner et al. 1996; Jiang et al. 2006; Reeh and Steen 1996; Wood and Docherty 1997). Depending on their concentration and the types of proton-sensitive channels present within the cell membranes, protons evoke currents with diverse kinetics. The increase of protons after tissue injury results in the activation of some channels, including TRPV1 receptors (Caterina et al. 1997; Tominaga et al. 1998).
al. 1998), and the closure of others, such as TASK channels (Duprat et al. 1997; Kim et al. 1999; Leonoudakis et al. 1998; Reyes et al. 1998), both of which result in sustained, nondesensitizing currents. Alternatively, when ASIC channels are present, there is typically an inward current that rapidly desensitizes (Babinski et al. 1999; Chen et al. 1998; Garcia-Anoveros et al. 1997; Ishibashi and Marumo 1998; Jiang et al. 2006; Lingueglia et al. 1997; Price et al. 1996; Waldmann et al. 1997). Cells that express combinations of ASIC channels and TRPV1/TASK channels will have evoked responses that contain both desensitizing and nondesensitizing components. In previous studies, only type 1, 2 and 4 cells from the hairy skin expressed solely nondesensitized current patterns (Jiang et al. 2006; Petruska et al. 2000b; Rau et al. 2007). All other cells, many of which also express TRPV1, had distinct ASIC-like responses. Interestingly, all of the cell types in this particular study also had ASIC-like responses to a proton solution, along with a nondesensitizing component. It is likely that such cells express ASIC proteins. The positive proton responses were not surprising considering that all colon and penile afferents are normally exposed to mucosal fluids. Recordings from single Aδ-fibers innervating the distal urethra show responsiveness to protons in addition to mechanical stretch of the mucosa (Delcambre et al. 2011).

**Immunocytochemical characteristics of sensory neurons innervating the colon and penis.** Previous studies have identified the cytochemical markers we assessed here (substance P, CGRP, IB4, neurofilament, TRPV1, TRPV2) within neurons innervating the colon, penis, and other tissues, including skin and muscle. In previous reports by our laboratory, the patterns of these cytochemical markers were remarkably consistent for each of the cell types (Petruska et al. 2000b, 2002; Rau et al. 2007). This was generally true for the present study, as well (see below).

The peptides substance P and CGRP have been found in colon (Christianson et al. 2006; Dockray and Sharkey 1986; Keast and De Groat 1992; Mitsuji 2010; Sterrini et al. 1995; Su et al. 1987) and penis (Keast and De Groat 1992; McNeill et al. 1992; Properzi et al. 1992; Vanhatalo et al. 1996; Wimalawansa et al. 1987; Wu et al. 2011). Neuropeptides CGRP (Lawson 1996; McCarthy and Lawson 1997) and substance P (Lawson et al. 1997; McCarthy and Lawson 1997) are released in many nociceptive cells in response to noxious thermal stimuli and inflammation. CGRP may be present in nonnociceptive cells, as well (Lawson 1996; McCarthy and Lawson 1997). In this study, types 5, 7, 8, 14, and 18 expressed both substance P and CGRP, whereas types 15, 16, and 17 were nonpeptidergic. In penis type 6 cells, CGRP, but not substance P, was expressed. Functionally, substance P has been suggested as an important mediator of ejaculation, a process that requires intact innervation from the glans penis and distal urethra (Johnson 2006).

The markers NF and IB4 have been localized in the colon (Caldero et al. 1988; Chadi et al. 2004; Mestre et al. 1992; Mitsuji 2009; Qian et al. 2009) and penis (Mihaly et al. 1996; Petruska et al. 1997; Yoshimura et al. 2003). The binding of isolectin B4 has been used to identify a subgroup of neurons with unmymelinated axons, which include a subset of nociceptive cells (Silverman and Kruger 1988, 1990; Streit et al. 1985, 1986; Wang et al. 1994). Alternatively, expression of NF largely indicates neurons with myelinated axons, regardless of soma size (Harper and Lawson 1985; Lawson et al. 1984; Lawson and Waddell 1991; McCarthy and Lawson 1990; Perry et al. 1991). Regional differences in DRG cell labeling by IB4 (Petruska et al. 1997, 2000b, 2002) are reported in cutaneous and visceral tissue but not in gastrocnemius muscle (Wang et al. 1998). In this study only type 7 cells expressed IB4 binding. This population, representing 28.9% of the DRG cells labeled from the penis, may comprise many of the IB4-binding neurons found in other studies to innervate the distal urethral epithelium (Petruska et al. 1997; Yoshimura et al. 2003) and glans penile epithelium (Petruska et al. 1997). In the latter study, IB4+ profiles extend into the surface epithelium, but many glans unmyelinated intraepithelial endings have been shown to emanate from small myelinated axons (Johnson and Halata 1991), which may explain why some type 7 neurons were both IB4 and NF positive. Whereas some populations of unmyelinated afferents appear unresponsive to innocuous or noxious mechanical stimuli, it is speculated that these fibers may respond to pathophysiological states, such as inflammation (McMahon 1994), as has been demonstrated in urinary bladder (Habler et al. 1990) and colon (Janig and Koltzenburg 1990). Most cells in this study appear to be at least thinly myelinated. Among these populations, it is interesting to note that type 5 and type 8 cells in this study were IB4 negative, whereas they were IB4 positive in a prior study (Petruska et al. 2002). We have previously recognized the presence of subphenotypes of type 5, based on their algesic response profiles (Jiang et al. 2006; Petruska et al. 2002; Rau et al. 2005b). This may occur in their immunocytochemical content, as well, and reflect an association of sensory terminals with the peripheral cells that are unique to these particular tissues. Alternatively, the absence of positive staining may reflect the same cell type whose levels of marker expression are below the level of detection. Random failures of the staining method cannot be excluded but are not likely to be cell type specific; however, procedural difference could have contributed to the staining pattern we observed in these cell classes. One possible reason for this is the use of two different staining methods between these two studies. In the previous report, IB4 binding was amplified with the application of HRP-conjugated IB4, using a tyramide-based amplification technique for visualization. In this study, IB4 binding was directly tested with a less sensitive method, fluorophore-conjugated IB4. Therefore, the absence of IB4 binding may simply be an issue of not being able to detect the presence of such binding without signal amplification. Conversely, the previous study primarily examined cells located in thoracic and upper lumbar regions, so these type 5 and 8 cells may in fact be subtypes. Further analysis of this cell type is required to ascertain the correct histochemical pattern and family assignment. Regardless, it is possible that additional classification protocols will be necessary to sort out the wide variety of sensory afferents that innervate peripheral tissues.
identified the expression of TRPV1 in most type 5, 8, 16, 17, and 18 cells (which represent 61% and 8% of the cells that we recorded in the colon and penis, respectively) and TRPV2 in type 5, 6, and 8 cells (which represent 28% and 24% of the cells that we recorded traced from the colon and penis, respectively). The type 15 cell was generally capsaicin insensitive/TRPV1 negative in cells traced from the penis, but was a mix of capsaicin insensitive/TRPV1 negative and capsaicin sensitive/TRPV1 positive within the total group of cells traced from the colon, which was a notable exception to the consistency of the other cell types. This apparent lack of homogeneity likely requires development of additional VAC markers to fully separate the subpopulations.

**Conclusion.** The classification of cells by their patterns of current signatures has important advantages that have been discussed in previous reports (Petruska et al. 2000b, 2002). By identifying cells solely by their current signature patterns, the experimenter can then focus on the cell types that express certain markers or cells that are sensitive to certain neuroactive agents without having to test for all of these properties directly. Understandably, identifying the specific tissue to which these cell types innervate is also beneficial. Since nociceptive cells with distinct properties are shown to innervate particular tissue regions, researchers making recordings from randomly selected cells in the DRG should not assume that their results would be the same for all tissues. Also, the combined use of current signature cell typing and retrograde tracing methods is a powerful technique for characterizing cells involved in nociception in peripheral tissues such as the colon and penis.

In this study, we have demonstrated how unique DRG cell types innervating the visceral tissue of the distal colon and the mucocutaneous tissue of the glans penis and distal urethra may be identified and sorted on the basis of their VAC patterns. These cell types have action potential features (e.g., long AHP80), algesic responsiveness, and histochemical phenotypes that suggest they may function as thermoreceptors, multimodal mechanonociceptors, or “silent” nociceptors that have Aδ- and C-fibers (see Lawson 2005). By identifying distinct DRG cell types that innervate the distal colon and glans penis/distal urethra, we facilitate future in vitro studies related to the gastrointestinal and urogenital sensory function in normal as well as abnormal conditions (e.g., inflammation, disease, spinal cord injury, etc.). Future studies linking cell types identified by in vitro classification of VAC with functional characterization identified through in vivo and ex vivo preparations may be possible with the use of immunocytochemical reactivity patterns or gene expression analysis of single-cell PCR.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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