The effect of spinal cord injury on the neurochemical properties of vagal sensory neurons

April N. Herritya,b, Jeffrey C. Petruskaa,b,c, David P. Stirlingb,c,d, Kristofer K. Raua,b,e and Charles H. Hubscherab.

aDepartment of Anatomical Sciences & Neurobiology, University of Louisville School of Medicine, Louisville, KY 40202
bKentucky Spinal Cord Injury Research Center, University of Louisville, Louisville, KY 40202
cDepartment of Neurological Surgery, University of Louisville, Louisville, KY 40202
dDepartment of Microbiology & Immunology, University of Louisville School of Medicine, Louisville, KY 40202
eDepartment of Anesthesiology, University of Louisville, Louisville, KY 40202

Running head: SCI-induced vagal sensory plasticity

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PROOFS AND CORRESPONDENCE TO:
Dr. Charles H. Hubscher
Dept. of Anatomical Sciences & Neurobiology
University of Louisville
511 S. Floyd Street
Louisville, KY 40202
Telephone: (502)852-3058
FAX: (502)852-6228
E-Mail: chhubs01@louisville.edu

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Abstract

The vagus nerve is comprised primarily of non-myelinated sensory neurons whose cell bodies are located in the nodose ganglion (NG). The vagus has widespread projections that supply most visceral organs, including the bladder. Due to its non-spinal route, the vagus nerve itself is not directly damaged from spinal cord injury (SCI). Because most viscera, including bladder, are dually innervated by spinal and vagal sensory neurons, an impact of SCI on the sensory component of vagal circuitry may contribute to post-SCI visceral pathologies. To determine if SCI, in male Wistar rats, might impact neurochemical characteristics of NG neurons, immunohistochemical assessments were performed for P2X3 receptor expression, IB4 binding, and Substance-P expression, three known injury-responsive markers in sensory neuronal subpopulations. In addition to examining the overall population of NG neurons, those innervating the urinary bladder also were assessed separately. All three of the molecular markers were represented in the NG from non-injured animals, with the majority of the neurons binding IB4. In the chronically injured rats, there was a significant increase in the number of NG neurons expressing P2X3 and a significant decrease in the number binding IB4 compared to non-injured animals, a finding that held true also for the bladder-innervating population. Overall, these results indicate that vagal afferents, including those innervating the bladder, display neurochemical plasticity post-SCI that may have implications for visceral homeostatic mechanisms and nociceptive signaling.

Keywords: Vagus nerve; Nodose ganglion; Spinal Cord Injury; Bladder; Immunohistochemical phenotype
Introduction

Spinal cord injury (SCI) results in deficits to sensorimotor systems and profoundly affects the functionality of the autonomic nervous system. Basic research focusing on improving pelvic-visceral outcomes following SCI is of great clinical importance, since complications such as bladder, bowel and sexual dysfunction affect health and quality of life for this population (4, 47, 67). Despite the direct immediate impact of injury to the spinal-derived autonomic supply of the pelvic viscera, most of the body’s visceral organs also are supplied by a non-spinal source through the vagus nerve. Since the vagus nerve does not travel directly through the spinal cord, its neurocircuitry is often considered intact following SCI. Nevertheless, there is some degree of indirect involvement of both vagal afferents and efferents. For example, following SCI, subsequent neuroplastic-responsive changes have been extensively described within the dorsal vagal complex controlling gastric function (79). Gastrointestinal (GI) alterations after human upper-thoracic SCI include conditions such as dysphagia (160), esophagitis (133), peptic ulcerations (62, 138), gastroparesis and overall dysmotility (87, 123, 125, 159). Although the mechanisms of GI dysfunction in humans after SCI are not thoroughly understood, experimental studies in rats suggest that many of the delays in gastric emptying and transit may in part be attributed to vagally-mediated pathways (60, 61, 79, 140). In fact, subdiaphragmatic vagotomy has been shown to prevent much of the SCI-induced GI sequelae (61).

The vagus nerve, with sensory cell bodies primarily located in the nodose ganglia (NG), provides innervation to the thoraco-abdominal structures. Despite the view that the vagus nerve does not innervate viscera caudal to the transverse colon, numerous
experimental studies have demonstrated that it also provides sensory innervation to the majority of the pelvic viscera (2, 28, 38, 57, 77, 81, 84, 111, 149). Even though the vagus nerve has such widespread projections, SCI does not disconnect the anatomical relationship the nerve has with the tissue it innervates. However, SCI does lead to pathological changes and dysfunction of below-level target organs, such as the bladder (45, 46, 94), and can thereby influence neuronal phenotype (122, 145, 158, 167, 176). Furthermore, various classes of primary sensory neurons, including vagal afferents, have been shown to alter their phenotype and the expression of different receptors in response to nerve injury and tissue inflammation (11, 78, 103, 107, 155, 173).

In this experiment, P2X3 receptor and SP immunoreactivity (ir) as well as IB4 binding was examined in NG neurons. These particular markers were selected based on their presence in the NG, involvement in the spinal and vagal circuitry, responsiveness in other sensory neurons to injury and/or inflammation, and the potential physiological role these markers may play in nociceptive signaling (11, 25, 30, 40, 42, 43, 83, 97, 103, 109, 146, 154, 163, 169, 172). In addition, anatomical evidence that the vagus nerve provides sensory innervation to the bladder in male rats (77) and the presence of these cellular markers in bladder tissue (6, 19, 100) adds to the importance of understanding the relationship between target-organ tissue and its innervating neurons. It is therefore hypothesized that following a spinal transection injury which removes any potential sources of spinal input rostrally and isolates vagal afferent fibers, the expression profile of known injury-responsive factors P2X3, IB4 and SP would be altered in the NG in general and also for the subset of NG neurons innervating the bladder.
Materials and Methods

Animals

All experimental procedures were carried out according to NIH guidelines and protocols reviewed and approved by the Institutional Animal Care and Use Committee at the University Of Louisville School Of Medicine. All adult male Wistar rats (n=16, Harlan Sprague Dawley, Inc, Indianapolis, IN), approximately 250 grams in weight, were individually housed in an animal room with a 12-hour light and dark cycle. They had ad libitum access to water and food (Laboratory Rodent Diet). Groups were either naïve (n=8) or spinal cord injured (n=8). Each group had a subset (n=4 each) which received retrograde neural tracer injected into the bladder to enable identification of single NG neurons which innervated the bladder.

Spinal cord injury

Half of the animals (n=8) were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (10mg/kg), injected intraperitoneally, for spinal transection. All surgeries were performed under aseptic conditions and the body temperature was maintained within the range of 36-37°C via a warm water recirculator (Gaymar T/Pump, Gaymar Industries Inc, Orchard Park, NY) throughout the surgery and recovery period. Following our previously published protocol (86), a dorsal longitudinal incision was made to expose the T7 vertebra and a laminectomy was performed in order to expose the underlying T8 spinal cord. The overlying dura was reflected laterally and the spinal cord cut using a pair of surgical microdissecting scissors. Gentle suction with an air vacuum was used to carefully elevate the cut stump in order to verify the completion of the
lesion. Gelfoam (Pharmacia & Upjohn Company, Kalamazoo, MI) soaked in topical hemostat solution (Henry Schein Inc., Melville, NY) was placed in the lesion cavity. The incision was closed using 4-0 nylon suture for the muscle layers and fascia and surgical clips for the skin. Animals were given subcutaneous injections of ketoprofen (Ketofen, 2.5mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) for analgesia twice a day for 2 days, 0.5ml of dual penicillin (Penicillin G coupled with Procaine, PenJect®, The Butler Company, Columbus, OH) in a single dose peri-operatively as a general prophylactic and 5mg/kg gentamicin (GentaFuse®, Butler Schein, Dublin, OH) once per day for 5 days to prevent bladder infections. After surgery each animal was housed individually. The urinary bladder was emptied by manual crede every 8 hours until the micturition reflex occurred automatically, typically 6–12 days after surgery (82). Animals survived for six weeks followed by euthanasia and tissue removal.

Retrograde tracer injection

At five weeks post injury, four spinally-transected rats and four age-matched naïve control rats were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (10mg/kg). They received a ventral/caudal midline peritoneal incision to expose the urinary bladder which was subsequently manually voided by pressure. Using an established protocol (77, 124), the fluorescent tracer FAST Dil™ oil (1,1’-dilinoleyl-3,3,3’,3’-tetramethylindo-carbocyanine perchlorate, 5mg dye dissolved in 0.1ml methanol, Molecular Probes, Inc., Eugene, OR) was injected into the bladder wall with a dye-dedicated 33-gauge needle coupled to a Hamilton microsyringe (Fisher Scientific, Pittsburgh, PA). Note that the abbreviation Dil is used throughout the
Dye injections were made to the circumference of the trigone, body and dome areas (10μl volume per animal divided into 10 injections of 1μl each; (77)).

Animal body temperature was maintained within the range of 36-37°C during surgery via a warm water recirculator (Gaymar T/Pump, Gaymar Industries Inc, Orchard Park, NY). After each injection, the needle was removed slowly and any dye-leakage was removed by cotton-tipped applicators. After injections were completed, the exposed viscera were hydrated as necessary with 5% Dextrose Lactated Ringers, the abdominal musculature was sutured closed (Ethicon 4-0 non-absorbable surgical suture), the skin closed with Michel clips (Fine Science Tools, Foster City, CA), and a topical antibiotic (Bacitracin, Actavis Mid Atlantic LLC, Lincolnton, NC) applied. Following surgery, animals were placed on a heating pad and core temperature monitored. Post-operative medication was provided as per spinal transection surgery. All animals were monitored daily to inspect the surgical incision and identify any changes in an animal’s general condition.

**Perfusion and Tissue Collection**

All 16 animals were deeply anesthetized with a ketamine (80mg/kg body weight)/xylazine (10mg/kg) mixture and transcardially exsanguinated with heparinized saline, followed by 4% paraformaldehyde perfusion. Each vagus nerve was identified adjacent to the carotid artery and gently separated from surrounding tissues and traced rostrally to the NG, which was excised. The bladder was dissected free from the prostate and extracted at the most distal aspect of the neck. Before weighing the
bladder, any residual intravesicular urine was allowed to flow out. Superior cervical ganglia were identified on both sides at the bifurcation of the common carotid artery and removed to be used as control tissue. For the transected group of rats, following a dorsal spinal incision, removal of the spinal cord tissue 1 cm above and below the transection site was performed. All tissues were placed in individually-labeled tubes of 4% paraformaldehyde for at least 48 h, followed by immersion in a cryoprotectant solution of 30% sucrose/phosphate buffer solution with 1% sodium azide for at least 24 h. Following removal from the cryoprotectant solution, NG’s were embedded in OCT® compound (Baxter Scientific) and 12-14 μm sections were cut on a cryostat (Leica CM 1850). During retrieval of the bladder, the abdominal cavity and surrounding viscera were inspected for tracer leakage.

**Histology of the lesion epicenter**

The lesion cavity, coated in embedding media, was cut into 18 μm sagittal sections using a Leica CM 1850 cryostat and mounted onto gelatin-coated histological slides (Azer Scientific, Morgantown, PA). The slides were then stained with both Luxol fast blue and cresyl violet (Kluver-Barrera method) to observe myelin and Nissl substance, respectively. Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) and the Nikon E400 microscope were used to image the lesion cavity and verify the completeness of the spinal transection (86). The percentage of spared white matter from the transection lesion was calculated using Nikon Elements software. The boundary between spared tissue at the ventral portion of the cord and the lesion cavity was identified. The first anatomical region of interest (ROI) outlined was the portion of
spared tissue at the ventral aspect of the spinal cord and the second ROI outlined was the entire lesion cavity, encompassing both spared and non-spared tissue and extending from both the rostral and caudal cord stumps. Area was determined for each ROI and the percentage of spared tissue was calculated by dividing these areas (95).

Hindlimb assessment for lesion completeness

The Basso-Beattie-Bresnahan (BBB) scale (14), an open-field locomotor assessment, was used to evaluate hindlimb function as an assessment of post-injury spinal cord function. Each animal was placed in an open-field and tested for 4 minutes by the same two scorers, who were presented with injured and non-injured animals in random order. The 21-point BBB scale was used to assess hindlimb coordination and rated parameters such as individual joint movements (0-7), weight support (8-13) and paw placement (14-21). Intact animals should demonstrate a locomotor score of 21. Animals that receive a complete T8 transection have been shown to exhibit BBB scores of 3 (extensive movement of 2 joints) on average (13, 130). To prevent any functional connections across the lesion site from potential spared tissue, gelfoam was placed between the two cut stumps. It has been demonstrated that as little as 4-5% sparing (primarily in the ventrolateral funiculi) was sufficient for attaining a BBB score of 7 following “complete” spinal transection (no gelfoam used across lesion) (53).

Immunoﬂuorescence histochemistry

Sections were thaw-mounted onto slides and allowed to air-dry. They were then encircled with hydrophobic resin (PAP Pen, Research Products International Corp).
Slide-mounted sections were incubated at room temperature for 2h in a solution of 2% Triton X-100® in phosphate buffered saline (PBS). This pre-treatment step improves the quality of P2X3-ir (116). The slides were rinsed in distilled water and then incubated for 30min in a solution of 10% normal donkey serum (Jackson Immuno Research, West Grove, PA) in PBS with 0.3% Triton X-100 (MP Biomedicals, LLC, Solon, OH) to block non-specific antibody binding. The immunohistochemical reagents and the labeling procedures are summarized in Table 1. Incubations in primary antisera were performed overnight (14–18 hours) at 4ºC. All steps were followed by multiple rinses with PBS. All fluorescent secondary antisera were diluted 1:100 and incubations were 2 hours at room temperature. The tyramide signal-amplification (TSA™) reagent kit (Sigma-Aldrich, St. Louis, MO) (20-22) was used at 1:100 and incubations were for 4-5min (124). Once all steps were completed, the slides were coverslipped with a glycerol-based photobleach-protective medium (Fluoromount-G, Southern Biotech).

**Cell Quantification**

To view labeled sections, imaging was performed using the Nikon Eclipse TiE inverted microscope with NIS Elements software. Initially, images were captured using a 10x lens (APo DIC N1 10x/0.45 NA, Nikon) with consistent exposure times and computationally stitched together to visualize whole ganglion sections. Imaging of individual fluorophores was achieved with a mercury-arc lightsource and the following filter sets: for Cy3 [543/22nm excitation, 593/40nm emission, 562nm dichroic, Semrock]; for Alexa Fluor 488 [470/40nm excitation, 525/50nm emission, 495nm dichroic, Nikon]; for Alexa Fluor 350 [350/50nm excitation, 460/50nm emission, 400nm dichroic, Nikon]; for Cy5 [615/70nm excitation, 700/74nm emission, 660nm dichroic, Nikon].
In order to obtain unbiased percentages of P2X3, SP and IB4 positive neurons in the NG, the physical dissector method was applied (37, 113). Across the entire ganglion, assembled by automated stitching, counts of all singly-, multi- as well as non-labeled/other (collectively comprising total neuronal counts) NG neurons were made by a scorer blinded to treatment groups. Starting with a random section, neurons with a clearly visible nucleus and definable soma were counted only if they were not present in an adjacent “look up” serial section. As an added measure to avoid double counting single neurons, the counting-sections were at least 60 microns apart (every 5th section). To differentiate background from foreground pixels, threshold values also were obtained based off the image histogram for each marker and held constant for each image quantified. Note that non-labeled/other cells (NeuN+ only) were quantified and represented the total neuron population (59, 75, 105). Images of tissue without application of the primary antibody were taken and utilized as baseline controls (images not shown). Using Nikon Elements software, cell diameter between groups also was determined by estimating the average of two cross-sectional diameters (longest and shortest axis).

Positive neuron counts were expressed as a percentage of the total number of neurons (NeuN+ only) from within the entire stitched ganglion as well as a percentage of all labeled neurons. An Olympus 3 Laser scanning confocal microscope with Fluoview 500 software (Mellville, NY) and a Nikon A1R MP+ confocal microscope with Elements software were used to collect high resolution, serial optical sections of NG neurons.
Statistics

Analyses were performed using SPSS v19-20 (IBM, North Castle, NY). Levene’s statistic was applied for homogeneity of variances and data are expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) with Tukey HSD post hoc t-tests was performed for the assessment of all histochemical markers and NG bladder labeling within groups. For the analysis of the histochemical markers between groups, data were normalized as percentages of total NG cells and analyzed via a two-way ANOVA with Tukey HSD post hoc t-tests. For all other analyses, two-tailed Student’s t-tests were performed assuming equal variance. Statistical significance was defined as p≤.05.

Results

Immunohistochemical signature of NG neurons

IB4 binding in NG neurons was localized to the plasma and axonal membranes as well as the Golgi complex. Immunoreactivity (-ir) for P2X3 and SP was present in the cytoplasm and P2X3-ir also could be found in the plasma and axonal membranes. All of the patterns of staining for these markers were consistent with previous studies in both NG and DRG (8, 10, 83, 116, 151). When examining the total percentage of labeled NG neurons, all of the markers were well represented (Figure 1A), with the majority of NG neurons binding IB4 (IB4, 65.5 ± 6.2% versus SP, 31.1 ± 10.3%; IB4 versus P2X3, 51.2 ± 7.9%). Overall, there were 8 different histochemical signatures represented in the NG (Figure 1B). The most prevalent combinations were neurons that were IB4+ only followed by the P2X3+ only and IB4+/P2X3+/SP- combinations (IB4+ only versus P2X3+ only, 33.3 ± 7.1% versus 15.0 ± 3.3%, p<.001; IB4+ only versus IB4+/P2X3+...
only, $33.3 \pm 7.1\%$ versus $15.0 \pm 5.0\%, p<.001$). When considering specific co-localization patterns in the NG, about a third of all IB4 neurons contain P2X3 ($30.9 \pm 9.2\%$) and about half of all P2X3 neurons contain IB4 ($49.1 \pm 8.6\%$). A typical example of the quadruple immunohistochemical staining in the NG is provided in Figure 2.

Effect of SCI on NG neurons expressing P2X3, SP and binding IB4

Following a chronic transection injury at T8, BBB locomotor assessments at the 6 week time point for all transected animals revealed an average score of $0.9 (\pm 0.9)$. Only one animal had a BBB score greater than zero (score of $4.5$: 6 on the left hindlimb and 3 on the right hindlimb). Post hoc histological assessments of the lesion site revealed that this same animal in the spinal cord-transected group had a small percentage of area spared within the lesion site ($16.1\%$ - located at the most ventral extent of the epicenter). The data from this incomplete transection rat is provided, but as a separate group with an n of 1. Although statistical analyses could not be performed with n=1, the immunohistochemical expression pattern in this SCI rat appeared to more closely resemble that of the naïve group (P2X3+ only, $11.5 \pm 5.2\%$; IB4+ only, $44.0 \pm 2.2\%$). An example indicating a typical complete lesion is provided in Figure 3. Note that it was also found that the average cell diameter for NG neurons was $29.6 \pm 5.7 \mu m$. This morphological feature was not affected by spinal transection injury ($28.5 \pm 6.04 \mu m$).

In rats with a complete transection (n=3; 6 ganglia), there was a significant increase in the percentage of total NG neurons expressing P2X3 ($p<.001$) as well as a significant decrease in the percentage of total NG neurons binding IB4 ($p<.05$) relative to non-injured controls (Figure 4). There were no significant differences in SP or NeuN
expression between groups. P2X3 expression and IB4 binding in the NG are
demonstrated in Figure 5. Within the spinal cord-transected group, the percentage of
neurons expressing P2X3 and the percentage of neurons binding IB4 were each
significantly greater than the percentage of neurons expressing SP, which was
unchanged from the non-injured group of animals (P2X3, 27.3 ± 4.8% versus SP, 6.5 ±
2.8%, p<.01; IB4, 23.7 ± 6.5% versus SP, 6.5 ± 2.8%, p<.01; SP, 6.5 ± 2.8% versus SP-
non-injured, 5.8 ± 3.8%). From the total population of NG neurons, SCI did not
significantly impact the subset of NG neurons that co-expressed IB4 and P2X3 (Naïve,
15.0 ± 5.0 versus TX, 13.5 ± 5.7, p>.05), nor did it impact the subset of all IB4 neurons
that contained P2X3 (Naïve, 30.9 ± 9.2% versus TX, 35.9 ± 11.2%, p>.05). However,
following SCI, the subset of all P2X3 neurons that contained IB4 was significantly lower
compared to non-injured animals (Naïve, 49.1 ± 8.6% versus TX, 32.2 ± 10.5%, p<.01).

With respect to the total number of labeled neurons within their individual
populations (i.e. all P2X3+ neurons and all IB4+ neurons), the distribution of the
P2X3+/IB4-/SP-only subset represents 50.7% of all P2X3+ neurons, while the
IB4+/P2X3-/SP-only subset represents 42.6% of all IB4+ neurons. Overall, these
populations of neurons comprised about half of the total population of NG neurons
examined.

Immunohistochemical profile of bladder non-injured and injured NG neurons

The retrograde tracer Dil, was injected into the bladder wall in order to determine
if the subsets of NG neurons affected by spinal cord transection include those that
supply the bladder. Initially, we found that the percentage of NG neurons traced from
the bladder in both groups in this study is similar to our previous study of spinally intact animals (22.2 ± 3.6% versus 21.4 ± 4.0%, (77)). Assessment of the superior cervical ganglion, which is located adjacent to the NG, did not reveal evidence of Dil tracer, indicating the tracer did not spread non-specifically (Figure 6).

With respect to the total population of P2X3+ NG neurons after chronic SCI (50.7 ± 8.2%), bladder-innervating neurons (Dil+/P2X3+/IB4-) represented 32.8 ± 1.1%, while with respect to the total population of neurons that were IB4+ after injury (42.6 ± 5.1%), bladder-innervating neurons (Dil+/P2X3-/IB4+) represented 21.5 ± 7.4%. Overall, in these two distinct subsets of NG neurons, more than half of the neurons are traced from the bladder (Figure 7). Images of the Dil+/P2X3+ and Dil+/IB4+ subsets following transection are demonstrated in Figure 8. Note that in this study, the proportion of NG neurons traced from the bladder in spinally-intact rats (23.7 ± 3.6%) did not differ significantly from the proportion traced from the bladder after chronic spinal transection injury (20.2 ± 3.0%). Transection injury did however, result in a significant increase in bladder size (wet weight) compared to non-injured controls (.267 ± .118g, SCI vs .149 ± .35g, Naïve; p<.05), which can impact overall total bladder capacity and voiding efficiency, potentially leading to an alteration in bladder function.

Discussion

Visceral organs including those in the pelvic region have a dual sensory innervation from spinal and non-spinal (i.e., the vagus nerve) sources (28, 38, 57, 77, 81, 84, 111). This study, using immunohistochemical techniques, examined the impact of SCI on the vagal component of that sensory innervation by assessing changes in the
presence of P2X3, IB4 and SP in NG neurons. Following spinal transection at T8, potential plasticity was evaluated in subsets of NG neurons which contain projections that bypass the spinal cord from visceral organs, including those projections that specifically supply the bladder. A major traumatic event to the nervous system, such as SCI, leads to dysfunction in multiple organ-systems, and ultimately influences the neurons that innervate these tissues. The findings of the current study indicate that vagal sensory cell bodies displayed an increase in P2X3 expression and a decrease in IB4 binding, which also held true for many neurons innervating the bladder. These results suggest that NG neurons, including the bladder subset, are sensitive to a spinal injury and are capable of responding by modifying their phenotype.

**Immunohistochemical phenotype of NG neurons**

Overall, from the cellular markers examined in this study, the majority of NG neurons were IB4+. Isolectin B4 has been shown to label primarily a subpopulation of non-peptidergic spinal sensory afferents that are thought to be functionally distinct from peptidergic neurons or neurons that are IB4 negative (135). Even though IB4+ neurons, in general, are widely expressed within the NG (83, 98, 129, 169, 172), the meaning of IB4 binding in vagal afferents and whether or not these neurons share common characteristics based on their IB4 binding is still unclear. Vagal afferents are largely known for their involvement in conveying information about the physiologic state of the viscera to the brain as part of homeostatic regulation (32, 66, 85). In the gastrointestinal tract, vagal afferent fibers are responsive to stretch and tension as well as to locally released hormones following the ingestion of food (16, 18, 119, 156). Although they are typically not responders to visceral stimuli within the noxious range (112), previous data
from our lab and that of others suggest otherwise. For example, while spinal afferents may be responsible for relaying mechanical nociceptive information, vagal afferents may play more of a role in conveying chemical nociceptive stimuli, thus contributing to disease-related conditions stemming from visceral hyperalgesia (44, 80, 86, 136). Therefore, besides the known role of vagal afferents in relaying homeostatic information to the brain, the population of vagal afferents that also are IB4+ may be involved in visceral nociceptive processing (based on the putative role of the majority of DRG IB4 binding neurons (117, 151)).

In the current study, it was shown that many vagal visceral afferents innervating the bladder also were IB4+. In other NG visceral afferents and in line with the results here, labeled vagal afferents from the stomach and duodenum demonstrated a substantial percentage of IB4 binding (172). Furthermore, numerous studies identify a low percentage of calcitonin gene related peptide (CGRP)-ir or peptide-containing NG neurons projecting to thoraco-abdominal viscera (63, 175). Despite the fact that a large proportion of visceral NG neurons appear to be IB4+, the functional significance of these specific subsets also has yet to be determined, as evidence of particular markers for the coding of vagal afferent subtypes are limited (17). One exception may be calretinin (calcium binding protein), which appears to be expressed specifically by cervical esophageal vagal afferents (49).

Similar to other studies reporting P2X3 receptor expression in vagal sensory cell bodies using immunohistochemical techniques, P2X3 receptors were highly prevalent and distributed throughout the NG in the current study (11, 154). These findings suggest that large populations of vagal afferents are sensitive to ATP and thus, vagal pathways
may be activated through purinergic signaling mechanisms. When considering individual subsets of neurons based on the cellular markers examined in this study, P2X3 receptor expression was present in about half of the IB4+ NG neurons, a co-expression subset previously reported by our group in the NG (83) and others in the DRG (25, 150, 152, 153, 164) and trigeminal ganglion (TG) (131). However, while the percentage of overlap of IB4+ neurons expressing P2X3 is relatively higher in the DRG (67.5%, (25)), this co-expression pattern was similar in the TG (32%, (131)) to our findings in the NG (31%). Furthermore, the percentages for all P2X3+ neurons containing IB4 are both greater in the DRG (98%) and TG (64%) compared to our findings in the NG (49%). The lower percentage of overlap between IB4 and P2X3 overall in the NG compared to other sensory ganglia may be attributed to differences in embryological origin (NG, epibranchial placode derived vs. DRG, neural crest derived vs. TG, both placode and neural crest), which appears to influence the neurochemical phenotype of visceral afferents. For instance, DRG afferents innervating different viscera such as the stomach (63, 64, 128, 172), duodenum (172) and pancreas (54, 128) are primarily peptidergic, expressing TRPV1, CGRP or SP, whereas NG afferents innervating those same tissues display limited expression of these peptidergic markers. In addition, DRG afferents innervating bladder (168), colon (35) and gastroduodenal (172) tissue exhibit low IB4 binding, which is in contrast to the substantial degree of bladder- innervating IB4+ neurons reported here in the NG and the significantly greater percentage of IB4+ NG afferents innervating the stomach and duodenum reported previously (172).
In regards to the overall population of NG neurons, the percentage of SP+ only neurons we found was similar to an earlier report, around 30% (161). While it is noted that SP+ neurons are abundant in the NG (175), their distribution has been reported to be located near the rostral pole of the ganglion (72, 161, 175). We and others have previously found a homogenous distribution of visceral labeling throughout the NG (1, 77, 127, 172). Although we did not assess the existence of an organotypic distribution of labeling for the histological markers of interest within the NG in this study, the presence of many SP-ir neurons in the rostral region may be anticipated due to the fact that it is anatomically close to the jugular ganglion, containing neuropeptide-rich neurons that primarily project to rostrally located viscera such as the esophagus and lungs (120, 143, 157) and is embryologically distinct (neural crest-derived) from the NG (placode-derived) (9). The proximity of neurons with similar neurochemical phenotypes may be important for performing like functions and even for sensory afferent integration (26). Furthermore, given the embryological distinction between the vagal ganglia, the phenotype of vagal afferents also may be influenced by whether or not the cell body is located in either the nodose or jugular ganglion (170).

Effect of SCI on P2X3-ir in NG neurons

Following chronic SCI, two significant changes were observed in subsets of NG neurons. The first finding was a significant increase in the number of neurons expressing P2X3-ir in the spinal-transected group relative to non-injured controls. In the somatosensory system, alterations in P2X3 expression following nerve injury have been mixed. Both down-regulation (25) and up-regulation (50, 109) of the receptor have been
documented in various peripheral nerve injury models such as axotomy, ligation and chronic constriction. In both studies where there were increases in P2X3 expression, the injury model used resulted in some neurons that would be potentially “uninjured”. To assess these differences, activating transcription factor 3 (ATF3), a marker of peripheral nerve injury and absent from intact neurons (141), identified decreased P2X3 (mRNA) in ATF3-ir neurons, while the increased expression was evident in the intact subset of neurons (142). The significant increase in NG P2X3-ir found in the present study was consistent with the ATF3-ir findings (142), since the vagal afferents are likely not directly injured given they by-pass the SCI, though this has not yet been directly tested.

Even though contact between the vagus nerve and its peripheral targets has not been severed, there is an overall effect of injury on the vagal afferent neurocircuitry (81, 86) and our discovery of the increased P2X3 expression seen in the NG here may help to improve our understanding of the indirect effect on the vagal system after injury. For instance, the increased P2X3-ir following SCI may be attributed to an inflammatory reaction of the system due to the nature of the injury itself. Acutely, SCI triggers an inflammatory response characterized by various resident (i.e. CNS origin) (55, 90, 121) and blood-derived (134) cellular events such as the synthesis of cytokines, chemokines and the infiltration of leukocytes, neutrophils and monocytes, which, over time, systemically may affect tissues outside the central nervous system leading to organ dysfunction. Released inflammatory cells from the blood stream can impact the functionality of different viscera due to the intimate relationship these organs have with the vascular system (12, 31, 65). In addition, both acute and chronic SCI induce significant changes in organs with spinal innervation from segments below the lesion-
level. Organs such as the bladder experience substantial stress and histopathology, which can lead to alterations in the integrity of the lining of the bladder (5) making the bladder more susceptible to chronic inflammation (76).

Structural changes after SCI also include bladder (detrusor muscle) hypertrophy, which triggers a release of neurotrophic factors such as NGF from the urothelial lining (56, 126, 147, 148). Increases in NGF following SCI (147, 158, 165) or inflammation (110, 132) as well as other excitatory neurotransmitters such as ATP (137), play a major role in neuro-epithelial interactions. For example, in a migraine headache model, retrograde transport of NGF from the periphery to the trigeminal ganglion or exposure of trigeminal afferents to NGF led to an upregulation of P2X3 receptor protein in the cell bodies (41, 58). Given that the vagus nerve provides a substantial degree of innervation to the bladder (77), the fact that we found many co-labeled Dil+/P2X3+ NG neurons after injury in this study, the presence of the high affinity receptor for NGF (TrkA) (70, 96, 174) and low affinity (p75) receptor (144, 175) and that vagal afferents have the capability to transport NGF (70), the phenotypic changes with respect to P2X3 -ir in the bladder-innervating NG neurons have the potential to be mediated through the actions of NGF. Importantly, in a manner distinct from the actions of NGF (41), CGRP-mediated insertion of P2X3 into the cell-surface membrane is an alternative mechanism, which has been demonstrated in sensitized trigeminal ganglion neurons (52). However, since the majority of CGRP expressing neurons appear to reside in other cranial ganglia (petrosal, trigeminal, glossopharyngeal and jugular) compared to the NG (68, 69, 71, 72) this molecular mechanism may indirectly affect NG neurons, perhaps acting at a distance through en passant synaptic contact (91).
The P2X3 receptor, predominantly expressed on sensory afferents (33, 97), including vagal fibers (88), also can be separately retrogradely transported from the periphery to the cell body via endosomes (34). This retrograde transport is thought be important for maintaining neuronal activity and cell excitability through activation of transcription factors (34). In disease states, such as SCI, the extracellular milieu of ATP may be relatively high compared to healthy states, where excesses are rapidly hydrolyzed (29, 89, 108). Large amounts of ATP (likely released from damaged tissue (39)), can signal through P2X3 receptors and may show that P2X3 has a more extensive role in the NG besides normal visceral afferent transduction, perhaps contributing to nociceptive signaling following injury or tissue inflammation.

Effect of SCI on IB4 binding in NG neurons

The second change following chronic transection injury was a decrease in NG IB4 binding relative to controls. This finding is similar to that of others in cases where decreases in the total number of IB4 binding DRG neurons on the contralateral side (uninjured side) also have been demonstrated following L5 spinal nerve transection (99). Importantly, the numbers remain reduced at the chronic time point (5 weeks post-injury), suggesting the effect was not transient. Since many neurons in general in the NG were found to bind IB4, the significant post-SCI decrease in the co-expression subset of P2X3 neurons containing IB4 may be attributed to the overall decrease in this relatively large population of IB4 neurons.

An explanation for the decrease in IB4 binding may be attributed to a stress response to the system following transection. Since glial cell-line derived neurotrophic
factor (GDNF) supports and aids in the regulation of IB4 neurons post-natally (104), perhaps some disruption to its availability or receptor complex as well as alteration to the IB4 binding glycoconjugate could explain the observed decrease (15, 118). However, in response to peripheral nerve injury, spared IB4 neurons also demonstrate the capability to sprout, forming perineuronal nets with both satellite and adjacent cells within the ganglion (99). It has been suggested that a mechanism behind this sprouting in response to nerve injury may involve inflammatory environmental changes that create a chemotactic gradient, attracting various chemokines (23). This communication between injured and non-injured “neighbors” within the ganglion may serve as a basis for cross-excitation and could eventually induce hyperalgesia or allodynia (3, 24). Even though the injury model used in this study does not directly injure vagal neurons, they could be considered “spared” neurons that also demonstrate a phenotypic switch in response to CNS damage and have the potential to drive visceral nociceptive signaling.

**Effect of SCI on SP expression in NG neurons**

SP is one of the main neuropeptides released from a proportion of primary afferent terminal endings that express SP, in response to irritation or inflammation (7, 27, 48) and is present in NG neurons (175). No significant differences in SP-ir were present in this study between transected and non-injured groups. A previous report assessing changes in NG neurotransmitters found that SP-ir was unaffected by vagal axotomy (74). The lack of changes in the NG with respect to SP-ir following injury does not preclude any particular alterations at terminal endings, either peripherally in target organs or centrally (solitary nucleus). For instance, there is a high concentration of SP
afferent terminals, primarily of vagal origin, present in solitary nucleus (73, 102, 175).

Alternatively, there may be molecular pathway alterations involved in the release of SP and translation at the cell body (114, 139). An acute SCI or direct tissue inflammation model (such as acetic acid instillation into the bladder) may provide more insight to vagal SP expression in the rat (10, 114).

Alterations to bladder NG neurons following SCI

SCI did not result in differences compared to non-injured controls in the number of NG neurons labeled from bladder, confirming these vagal afferents remain intact after cord transection. Although vagal afferents exhibit a high degree of neurochemical and electrophysiological plasticity in response to trauma and inflammation (26, 106, 115, 171), it is likely that the observed neurochemical changes in this study are a result of interactions with the target organs these vagal fibers innervate rather than direct neural damage. It should be noted, however, that other, extrinsic sources of ATP can reach P2X3 receptors through release from sympathetic neurons, tumor cells or from vascular endothelial cells associated with ischemia (30).

Plasticity related changes in bladder vagal afferents falls in line with evidence from the spinal system after SCI. Spinal sensory neurons innervating the bladder exhibit both morphological and physiological changes after SCI (93, 166). Given the important transduction role of P2X3 receptors in spinal bladder afferents (36) and the fact that many vagal neurons traced from the bladder expressed P2X3 suggests that the vagus nerve may participate in the sensory portion of micturition function. Our collective recent data indicating extensive vagal afferent innervation of mammalian urinary bladder (77)
and SCI-induced changes in a transduction channel like P2X3, may have important clinical applications. Such changes could contribute to whatever plasticity underlying reports of altered sensations stemming from the below level viscera, such as sensations of bladder filling or fullness in clinically complete SCI patients above T10 (51, 92, 162).

A large proportion of the IB4 neurons were traced from the bladder which is complementary to an earlier study showing that IB4 binds different types of visceral afferents in the NG (172). Although the distal urethra was not examined in the current study, a large proportion of spinal neurons which innervate this region of the lower urinary tract include IB4+ afferents (168).

**Perspectives and Significance**

Due to the chronic extent of multi-system functional impairment and disability, SCI presents a significant economic burden for the patient, family unit and society overall with high direct and indirect costs estimated in the billions (101). Apart from paralysis, some of the major complications of SCI affecting quality of life, include deficits to urological function (4, 47, 67). The vagus nerve, with the majority of its cell bodies located in the NG, is an extraspinal pathway through which information from regions below the level of a spinal lesion can directly travel to the brainstem, bypassing the spinal cord entirely. Work from our lab previously identified an anatomical connection to the male rat urinary bladder through the vagus nerve (77). The current study examined the immunohistochemical phenotype of vagal sensory neurons overall as well as in those that innervate the bladder. The results from this study demonstrate that vagal afferents are responsive to spinal injury and further assessments of their functional
nature may provide insight on how to take advantage of this route that by-passes the spinal cord in order to improve therapeutic interventions for SCI patients.

Conclusion

The present study demonstrated neurochemical changes in the NG, a site remote from the injured spinal cord. Through target-organ neural interactions, vagal afferent fibers are influenced by their connections to the viscera. Therefore, overall changes in these organs following SCI could impact the neurochemical properties of vagal afferents innervating them. The increased expression of P2X3 and the decreased binding of IB4 in the sensory cell bodies of vagal afferents post-SCI indicates an indirect effect of injury on the vagal neurocircuitry. A majority of neurons in the P2X3 subset after spinal transection were Dil+ indicating many NG bladder afferents have the potential to respond to alterations in ATP, perhaps even playing a role in generating specific sensations associated with the bladder such as fullness. In addition, the considerable proportion of bladder IB4+ NG neurons demonstrates that vagal afferents may participate in visceral nociceptive processing. Whether or not SCI induces changes in NG neuron function will require an examination of their electrophysiological properties in bladder populations.
Role of the Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: A.H., J.P., K.R., C.H. Acquisition of data: A.H., C.H. Analysis and interpretation of data: A.H., J.P., D.S., K.R., C.H. Manuscript editing and approval: A.H., J.P., D.S., K.R., C.H. The authors report no conflict of interest with this project.

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Abbreviations

1 SCI, Spinal cord injury; NG, Nodose ganglion; DRG, Dorsal root ganglion; IB4, Isolectin B4; SP, Substance P; -IR, Immunoreactivity; GI, Gastrointestinal; BBB, Basso Beattie Bresnahan; FAST Dil oil/Dil, 1,1'-dilinoleyl-3,3',3'-tetramethylindo-carbocyanine perchlorate; ATP, Adenosine tri-phosphate; GDNF, Glial cell-line derived neurotrophic factor; NGF, Nerve growth factor; CGRP, Calcitonin gene-related peptide;
References


101. Ma VY, Chan L, and Carruthers KJ. Incidence, prevalence, costs, and impact on disability of common conditions requiring rehabilitation in the United States: stroke, spinal cord injury, traumatic


### Table 1. Immunohistochemical Reagents

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary Detection</th>
<th>Dilution/Vendor/Catalog #</th>
<th>Secondary Detection</th>
<th>Dilution/Vendor/Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X3</td>
<td>GP-anti P2X3 1:1000/Neuromics/GP10108</td>
<td>Dky anti-GP-AF®488 1:100/Jackson/706-545-148</td>
<td></td>
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<tr>
<td>Biotinylated Lectin from Bandeiraea Simplicifolia Isolectin B4 (IB4)</td>
<td>HRP-SA 1:500/Sigma-Aldrich/L2140</td>
<td>Tyramide-AF®350 1:100/Molecular Probes/T20937 TSA™ Kit#27</td>
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<td>SP</td>
<td>RBT-anti SP 1:1000/Abcam/ab67006</td>
<td>Dky anti-RBT-Cy™3 1:100/Jackson/711-165-152</td>
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<td></td>
</tr>
<tr>
<td>NeuN</td>
<td>MS-anti NeuN 1:1000/Chemicon/MAB377</td>
<td>Dky anti-MS-Cy™5 1:100/Jackson/715-175-151</td>
<td></td>
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AF, Alexa Fluor; Dky, Donkey; GP, Guinea Pig; HRP, Horseradish peroxidase; MS, Mouse; RBT, rabbit; SA, Strepavidin; SP, Substance P; TSA, Tyramide Signal Amplification
**Figure 1. Immunohistochemical representation of P2X3, IB4 and SP in NG neurons.**

**Figure 1. A.** Following staining of the immunohistochemical markers P2X3, SP and IB4, the bar graph demonstrates that all three were well represented in the NG, with the majority being IB4+ (IB4 vs P2X3, #p<.05; IB4 vs SP, **p<.001; P2X3 vs SP, *p<.01). B. A pie graph depicting all possible combinations of the molecular targets in the NG. Note that neurons that were IB4+ only were the most prevalent and most NG neurons were labeled with at least one of the three cellular targets examined. (One-way ANOVA with Tukey post hoc t-tests, n=3, 6 ganglia, values are expressed as mean ± S.D.)

**Figure 2. Quadruple immunohistochemical staining in the NG**

**Figure 2.** A confocal image displays the typical staining within the NG. NeuN was used to label all neurons. Different histochemical combinations include neurons that were IB4+, P2X3+, but SP- (white arrows) and neurons that were IB4-, P2X3+ and SP- (yellow arrows). Scale bar indicates 25µm (20X objective).

**Figure 3. Spinal transection injury at T8**

**Figure 3.** An 18µm thick sagittal section stained with the Kluver-Barrera method illustrates a complete spinal transection at T8. Gelfoam is placed in the lesion cavity to prevent contact from between rostral and caudal spinal cord tissue. The scale bar indicates 500 µm (4X objective).

**Figure 4. The effect of SCI on number of NG neurons expressing the individual markers**

**Figure 4.** Following SCI, there was an increase in P2X3-ir in the transected group relative to intact/normal (SCI, 27.3 ± 4.8% versus non-injured, 15.0 ± 3.3%, *p<.001) and a decrease in IB4 binding in the transected group relative to intact/normal (SCI, 23.7 ± 6.5% versus non-injured, 33.3 ± 7.1%, #p<.05). No changes were apparent for SP. The “Other” category represents neurons that were NeuN+, but did not express or bind any of the markers. (N=6, 12 ganglia)
Figure 5. The effect of SCI on P2X3 and IB4 in the NG

Figure 5. An example displaying P2X3-ir (A) and IB4 binding (C) in the NG and following chronic spinal cord transection injury at T8 (B and D, respectively). Note the presence of increased P2X3-ir and decreased IB4 binding post-SCI. Images of sections from both SCI and non-injured animals were stained and captured with the same protocols and at the same time (10X objective).

Figure 6. Superior Cervical Ganglion

Figure 6. There was no evidence of Dil punctate labeling present in the SCG. The scale bar represents 20µm (20X objective).

Figure 7. Effect of SCI on bladder-traced NG neurons

Figure 7. Demonstrating that out of the total percentage of either P2X3 or IB4 subsets after injury, more than half of the neurons were traced from bladder. Bladder innervating neurons in the P2X3+ subset represent (32.8 ± 1.1%) while in the IB4+ subset, they represent (42.6 ± 5.1%). (N=3, 6 ganglia)

Figure 8. P2X3-ir and IB4 binding in bladder-traced NG neurons after transection

Figure 8. A confocal image illustrating a Dil+ neuron in panel A that is also IR for P2X3 in panel B (white arrows). Panel C demonstrates the overlay. An image from the inverted Nikon microscope illustrating a Dil+ neuron in panel D that also binds IB4 in panel E (white arrowhead). Panel F demonstrates the overlay. One example of each is displayed. In both images, the scale bar indicates 25 µm (20X objective).