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Roles of isolectin B4-binding afferents in colorectal mechanical nociception

Jun-Ho La, Bin Feng*, Kaori Kaji, Erica S. Schwartz, and G. F. Gebhart

Center for Pain Research, Department of Anesthesiology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213

Abstract

Isolectin B4-binding (IB4+) dorsal root ganglion (DRG) neurons are distinct from peptidergic DRG neurons in their terminal location in the spinal cord and respective contributions to various classes and modalities of nociception. In DRG neurons innervating the mouse colon (c-DRG neurons), the reported proportion of IB4+ population is inconsistent across studies, and little is known regarding their role in colorectal mechano-nociception. To address these issues, in C57BL/6J mice, we quantified IB4-binding (IB4+) after labeling c-DRG neurons with Fast Blue (FB) and examined functional consequences of ablating these neurons by IB4-conjugated saporin (IB4-sap). Sixty one percent of FB-labeled neurons in the L6 DRG were IB4+, and 95% of these IB4+ c-DRG neurons were peptidergic. Intrathecal administration of IB4-sap reduced the proportion of IB4+ c-DRG neurons to 37%, which was due to the loss of c-DRG neurons showing strong to medium IB4+ intensity; c-DRG neurons with weak IB4+ intensity were spared. However, this loss altered neither nociceptive behaviors to colorectal distension nor the relative proportions of stretch-sensitive colorectal afferent classes characterized by single-fiber recordings. These findings demonstrate that more than one half of viscerosensory L6 c-DRG neurons in C57BL/6J mouse are IB4+ and suggest, in contrast to the reported roles of IB4+/non-peptidergic neurons in cutaneous mechano-nociception, c-DRG neurons with strong to medium IB4+ intensity do not play a significant role in colorectal mechano-nociception.

Introduction

Primary afferent neurons of small to medium size are heterogeneous in their morphological, anatomical, and stimulus-response functional features. One widely used classification is to subgroup them into two populations based on their neurochemical properties. One group is dependent on nerve growth factor (NGF), expresses tyrosine kinase receptor A (trkA), and contains neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) and thus designated as peptidergic [3]. The other group is dependent on growth factors belonging to the glial cell-derived neurotrophic factor (GDNF) family, is devoid of neuropeptides, and expresses binding sites for the plant lectin, isolectin B4 (IB4) [4; 17].

Correspondence to: Jun-Ho La, DVM, PhD, laj@upmc.edu, Address: 200 Lothrop Street, W1402 BSTWR, Pittsburgh, PA 15213, USA, Tel: 412-648-8123, Fax: 412-383-5466.

*Current address: Department of Biomedical Engineering, University of Connecticut, Storrs, CT 06269

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These two diverse afferent populations differentially project into the spinal cord dorsal horn; central terminals of peptidergic afferents terminate mainly in lamina I and outer lamina II, while those of IB4-binding (IB4+) afferents terminate in inner lamina II [26].

Adding to our knowledge of the contribution of peptidergic afferents to neurogenic inflammation [21] and central sensitization [16], roles of non-peptidergic IB4+ afferents in nociception have been investigated using animals whose IB4+ afferents were ablated by the IB4-conjugated neurotoxin saporin (IB4-sap). IB4+ afferents were found to contribute not only to baseline nociceptive responses to noxious cutaneous thermal and mechanical stimuli [29], but also to inflammatory, neuropathic, and cancer-induced hyperalgesia [12; 13; 27; 31].

In the viscerosensory system, comparatively little is known about roles of IB4+ afferents in responses to noxious stimulation, such as hollow organ distension. In fact, the majority of visceral afferents (70–90%) are peptidergic, and only a few are IB4+ (for review, [22]), leading to the presumption that the contribution of IB4+ afferents to visceral nociception is relatively insignificant. However, it should be noted that the reported proportion of IB4+ afferents in viscerosensory pathways significantly varies (6–49%) across studies. For instance, in dorsal root ganglia (DRG) neurons innervating the distal colon (c-DRG neurons) *via* pelvic nerves in C57BL/6 mice, Christianson et al [6] reported that only 10% bound IB4 in sections of whole DRG, whereas Robinson et al [23] observed that 49% were IB4+ in dissociated c-DRG neurons. In addition, virtually all IB4+ c-DRG neurons were also positive for CGRP-immunoreactivity, making the simple peptidergic *vs.* IB4+ afferent classification inapplicable in this viscerosensory pathway.

The present study was performed to clarify the role of IB4+ afferents in visceral nociception, specifically colorectal mechanical nociception, in C57BL/6 mice. We first quantified IB4-binding in L6 DRG sections (pelvic nerve afferent pathway) using Fast Blue (FB) as a tracer for estimation of their relative proportion in the c-DRG neuronal population, then examined the functional consequences of ablating IB4+ c-DRG neurons with IB4-sap.

Materials and Methods

Animal

Adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME; 25–30 g), housed under a 12/12-hr light/dark cycle in an AAALAC accredited facility were used throughout. Water and food were provided *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee, University of Pittsburgh.

c-DRG neuron labeling

The distal colon was surgically exposed while mice were under 2% isoflurane anesthesia (Hospira Inc., Lake Forest, IL). The retrograde tracer FB (1% in sterile saline, EMS-Chemie GmbH, Groß-Umstadt, Germany) was injected into the colon wall using a microsyringe (3–4 sites, each in a volume of ~3 μ L). Mice were used for experiments 2–3 weeks after FB injections.

Ablation of IB4+ afferents

IB4-sap (1.5 µg in 5 µl) or unconjugated saporin (0.65 µg in 5 µl, amount equivalent to that of saporin in 1.5 µg IB4-sap) was delivered intrathecally by lumbar puncture (between the L5 and L6 vertebrae) with a 30G needle-attached microsyringe under 1.5–2% isoflurane anesthesia. Insertion of the needle to the intrathecal space was confirmed by a tail flick upon penetration [25].

Immunohistochemistry

One day after final behavioral experiments, mice were euthanized by CO₂ inhalation, transcardially perfused with ice cold Lana's fixative (4% paraformaldehyde, 14% picric acid in 0.4 M phosphate buffer) and the spinal cord and the L6 DRG (bilaterally) were harvested. After cryoprotection in 20% sucrose, fixed tissue was embedded in OCT compound (Sakura Finetek, Japan), frozen, and sectioned at 20 µm (spinal cord) and 14 µm (DRG) thickness. Tissue sections from all experimental groups were processed simultaneously; incubated with rabbit anti-CGRP (1:3000, Sigma-Aldrich, St. Louis, MO), rabbit anti-TRPV1 (1:3000, Alomone, Israel) or chicken anti-NeuN (1:1000, Millipore, Billerica, MA) overnight at 4°C and then with Cy3-conjugated anti-rabbit or chicken IgG (1:200, Jackson Immunoresearch, West Grove, PA) for 1 hr at room temperature. IB4+ neurons were detected by incubating the immunostained sections with AlexaFluor® 488 or 647-conjugated IB4 (0.5 µg/ml in phosphate-buffered saline containing 1 mM Ca²⁺, Invitrogen, Grand Island, NY) overnight at 4°C. Immunostained tissue sections, separated by 42 µm (DRG) and 60 µm (spinal cord) between sections, were photographed using a microscope-mounted digital camera (DFC340FX, Leica Microsystems, Bannockburn, IL) or a FluoView™ FV1000 confocal microscope (Olympus, Japan). FB-containing, immunostained, and IB4+ neuronal structures were identified in DRG or spinal cords, the outlines of neurons (in DRG, based on FB-labeling) or dorsal horn laminae I to III (in spinal cords, based on NeuN staining) traced, counted (the number of DRG neurons), and quantified (the intensity and area of staining) after setting the threshold of 8-bit grayscale images to exclude at least 99.99% of background intensity based on its Gaussian distribution using ImageJ (v 1.48v, NIH) [10]. Specifically, the threshold value was calculated from the standard normal distribution (Z) equation: $\text{threshold} = 3.72SD + \mu$, where 3.72 is the Z-score at $p = 0.9999$, SD and μ are the standard deviation and mean of background intensities in 8-bit grayscale (0 to 255), respectively.

Colorectal distension

A pair of sterile wire electrodes was surgically implanted under aseptic conditions into the external oblique abdominal musculature with the tips of other ends exposed at the back of the neck for electromyographic recordings of the visceromotor response (VMR) to colorectal distension (CRD) as a measure of colorectal nociception. At least 7 days after electrode implantation, mice were briefly sedated (3% isoflurane) and a 2 cm-long balloon connected to a distension device was inserted trans-anally into the colorectum 0.5 cm proximal from the anus. The mouse was placed in a plastic cylinder to restrict movement and allowed 30 min to recover from the sedation. Baseline VMRs to an ascending series of graded, constant pressure CRD (30, 45 and 60 mmHg for 10 sec every 4 min, 3 repetitions at

each intensity) were measured one day before the intrathecal injection of IB4-sap or unconjugated saporin. VMRs were measured again 7 and 14 days after the injections and normalized to the maximum baseline response in each mouse.

Intracolonic application of inflammatory soup

Acidic (pH 6.0) inflammatory soup, containing bradykinin, serotonin, histamine, and prostaglandin E2 (all 10 μ M in 0.2 ml, Sigma-Aldrich) was instilled intracolonic to sensitize colorectal afferents [8; 11] after the final CRD session on day 14. Five min later, another series of CRDs (60 mmHg only, 3 repetitions) was performed to evoke VMRs.

Restraint stress-induced defecation

Seven and 14 days after the intrathecal injection of IB4-sap or unconjugated saporin, mice were restrained in a plastic cylinder for 30 min. The number of fecal pellets defecated during the 30 min was counted.

In vitro colorectal afferent recording

Mice were euthanized by CO₂ inhalation and subsequently exsanguinated by cardiac perforation. The distal colorectum with attached pelvic nerves was opened longitudinally along the mesenteric border, pinned flat mucosal side up in a tissue chamber filled with Krebs solution (in mM: 117.9 NaCl, 4.7 KCl, 25 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄(H₂O)₇, 2.5 CaCl₂, 11.1 D-glucose, 2 butyrate, and 20 acetate, 0.004 nifedipine and 0.003 indomethacin) and the pelvic nerve teased into fine bundles for single-fiber electrophysiological recordings. Colorectal afferent endings were located by systematic electrical stimulation of the colorectal surface and subsequently tested for mechanosensitivity to further class them as serosal, muscular, mucosal, muscular-mucosal or mechanically insensitive (MIAs) [9]. The proportions of the five afferent classes were calculated in mice treated with IB4-sap or unconjugated saporin (days 14–27).

Data Analyses

Data are expressed as mean $\pm$ S.E. with n, the number of samples and N, the number of mice. Fisher's exact test (FET) was used for analysis of 2X2 contingency tables. Student's t-test or Mann-Whitney U-test (MWU) was used to compare means of two groups. Two-way ANOVA with multiple comparison tests (Bonferroni's) was used to analyze data with two variables. Results were considered statistically significant when $p < 0.05$.

Results

In control mice injected with unconjugated saporin, 61% (n=563 of 929 cells, N=6) of FB+ c-DRG neurons in the L6 DRG bound IB4. IB4-binding was detected in c-DRG neurons with various somata sizes (Fig 1A). In naïve mice, a similar proportion (60%, 351 of 588 cells, N=3, $p=0.75$ vs. control) of c-DRG neurons were IB4+, suggesting that the intrathecal injection of unconjugated saporin had no significant neurotoxic effect.

The IB4+ intensity could be classed into weak, medium, and strong IB4+ intensity based on the 8-bit grayscale histogram (Fig 1B and C): 42% of IB4+ c-DRG neurons showed weak

binding (grayscale intensity <75), 47% medium (75 and <145), and 10% strong (>145). Further characterization of these IB4+ c-DRG neurons revealed that the majority (95%) of them were peptidergic (CGRP-immunoreactive, Fig 2A), confirming the previous finding [6; 23] that mouse IB4+ c-DRG neurons are also peptidergic. In a separate set of L6 DRG sections, 88% (n=173 of 197 cells, N=4) of IB4+ c-DRG neurons were TRPV1-immunoreactive (Fig 2B), different from their counterparts in non-visceral (primarily cutaneous) somatosensory pathways [5; 19; 24].

Following intrathecal injection of IB4-sap, the proportion of L6 IB4+ c-DRG neurons was significantly decreased to 37% (n=290 of 794, N=4), as compared to 61% in control group ($p<0.01$, Fig 3A), indicating an overall 40% loss in the IB4+ c-DRG neuronal population. In the mice treated with IB4-sap, IB4+ c-DRG neurons with strong IB4-binding were rare; only 3 of 290 neurons exhibited an 8-bit grayscale intensity greater than 145 ($p<0.01$ vs. 58 of 563 neurons in control, Fig 3B). In these mice, the relative proportions of c-DRG neurons with weak and medium IB4+ intensities were changed to 68% ($p<0.01$ vs. 42% in control) and 31% ($p<0.01$ vs. 47% in control), respectively. For an intuitive comparison, we set the total numbers of IB4+ c-DRG neurons in control and IB4-sap-treated mice at 100 and 60 (reflecting the 40% loss), respectively, and reconstructed the histograms by normalizing the neuronal numbers in each bin accordingly (Fig 3C). This normalization demonstrated that after IB4-sap treatment, c-DRG neurons with weak IB4+ intensity were essentially spared (n=42.3 in control vs. 40.6 in IB4-sap), but neurons with medium (n=47.4 in control vs. 18.8 in IB4-sap) to strong IB4+ intensity (n=10.3 in control vs. 0.6 in IB4-sap) were effectively ablated. This loss of IB4+ afferents was also evident in the spinal cord dorsal horn. When IB4+ terminals were quantified as a relative area in laminae I-III, terminal density was decreased to $43\pm4\%$ (N=11, $p<0.01$) of control ($100\pm4\%$, N=11) in the lumbosacral spinal cord after IB4-sap treatment (Fig 3D & E). The IB4-sap injected intrathecally by lumbar puncture between the L5 and L6 vertebrae effectively spread up to thoracolumbar regions, decreasing IB4+ areas in laminae I-III to $54\pm7\%$ ($p<0.01$) of control ($100\pm5\%$) (Fig 3E). We attempted to ablate more IB4+ afferents by injecting greater doses of IB4-sap. However, IB4-sap greater than 3 μg (in 5 μl) resulted in significant mortality during two weeks without greater reduction in IB4-binding. Given the ethical considerations surrounding the toxicity of the compound, we did not pursue additional greater doses.

Despite the loss of IB4+ afferents, however, mice treated with IB4-sap showed no defect in CRD-induced nociceptive behaviors on day 7 or 14 (Fig 4A & B). We considered it possible that the role of IB4+ colorectal afferents only becomes apparent in pathological states, as reported in rat muscle afferents where IB4-sap did not alter baseline mechanosensitivity but inhibited the development of acute mechanical hyperalgesia by intramuscular carrageenan or eccentric exercise [2]. We tested this possibility by instilling an inflammatory soup into the colorectal lumen, which is known to sensitize colorectal afferents [8; 11]. The IB4-sap-treated mice retained the ability to become hypersensitive to CRD after an acidic inflammatory soup enema (Fig 4C). In addition, IB4-sap-treated mice showed no significant impairment in defecation when peristaltic movement of the colorectum was stimulated by restraint stress (Fig 4D).

To further assess the unaltered CRD-induced nociception despite the significant loss of IB4+ c-DRG neurons, we hypothesized that the ablated IB4+ c-DRG neurons might be afferents that are insensitive to distension (i.e., mechanically-insensitive, serosal, or mucosal class) [9]. We tested this hypothesis by examining the proportions of stretch-sensitive (i.e., muscular and muscular-mucosal classes) and -insensitive colorectal afferent classes in single-fiber recordings *in vitro*. As shown in Fig 5B, however, the relative proportion of each colorectal afferent class in pelvic nerves was comparable between control and IB4-sap-treated mice, and thus the probability of detecting stretch-sensitive afferents in IB4-sap-treated mice was not different from that in control mice (0.27 vs. 0.36, $p=0.3$). Not only were their relative proportions the same, but their mean response thresholds (36.9 ± 4.4 mN, $n=31$, $N=7$ in control vs. 50.5 ± 9.2 mN, $n=19$, $N=6$ in IB4-sap, $p=0.14$) and magnitudes (123.2 ± 30.9 action potentials in control vs. 85.5 ± 35.7 in IB4-sap, $p=0.44$) of response to a ramp stretch (0 to 170 mN in 34 sec) also did not differ between control and IB4-sap-treated mice. Interestingly, in histograms, we noticed a trend in IB4-sap-treated mice toward a decrease in the sampling frequency of stretch-sensitive afferents that have relatively low-thresholds for response (<40 mN, 24 of 31 in control vs. 10 of 19 in IB4-sap, $p<0.12$, Fig 5C) and robust discharges (>50 action potentials, 18 of 31 in control vs. 6 of 19 in IB4-sap, $p<0.09$, Fig 5D).

Discussion

This study demonstrates that more than one half of c-DRG neurons in the pelvic nerve pathway (in C57BL/6J mice) are IB4+, and the partial, but significant loss (up to 40%) of these IB4+ c-DRG neurons does not affect colorectal mechanical nociception. The high proportion (61%) of IB4+ neurons in the L6 c-DRG neuronal population in this study is likely due to the high detection sensitivity (overnight IB4 incubation in 1mM Ca^{2+} -containing buffer) that enabled us to identify neurons with weak and medium IB4-binding; counting only the neurons with strong IB4+ staining reduces the proportion to 6% of c-DRG neurons, and excluding the weak IB4+ neurons reduces it to 35%, suggesting that the high variance of reported proportions of IB4+ visceral afferents in the literature could be attributed to the different staining/detection methods utilized. Two weeks after IB4-sap treatment, the normalized histograms, reflecting the 40% loss of IB4+ c-DRG neurons, revealed that c-DRG neurons with strong IB4+ intensity were virtually absent and those with medium IB4+ intensity were decreased in number, while the number of c-DRG neurons with weak IB4+ intensity remained unaffected, which is not surprising given that the degree of neurotoxic effect of IB4-sap would be positively correlated with the intensity of its binding to DRG neurons. In rat L6 DRG, similar to our observations, IB4+ neurons could be divided into weak and strong intensity IB4+ groups, and IB4-sap more effectively decreased the number of strong intensity IB4+ neurons [18].

In rat C-nociceptor type DRG neurons innervating the skin, IB4+ intensity was found to be positively correlated with the expression of Nav1.9 and TREK-2 channels, and action potential duration, rise time, and height but negatively correlated with the expression of trkA and resting membrane potentials [1; 7], suggesting that sensory neurons with different IB4+ intensities could function differentially, and thus, functional consequences of selective

ablation of ‘weak’ IB4+ c-DRG neurons could be different from the results of this study in which ‘medium to strong’ IB4+ c-DRG neurons were found to be ablated.

Almost all mouse IB4+ c-DRG neurons were CGRP-immunoreactive in this study, confirming that IB4-binding cannot be used as a classification criterion to identify a non-peptidergic afferent population in viscerosensory pathways [6; 23]. It should be noted that in *rat* C-nociceptor type DRG neurons mentioned above, a significant proportion (57%) of IB4+ neurons were immunoreactive to trkA (mostly those with weak IB4+ intensity), a classical marker for peptidergic afferent neurons. Likewise, ~30% of *rat* IB4+ trigeminal ganglia (TG) neurons were immunoreactive to CGRP, compared with only ~10% of their *mouse* counterpart [20]. These findings collectively suggest that differences in species and sensory pathways must be considered when non-peptidergic afferent neurons are identified based on IB4-binding.

In the mouse, the expression of TRPV1 (Fig 2B and C) makes IB4+ c-DRG neurons further divergent from non-peptidergic IB4+ afferents in cutaneous sensory pathways where IB4+ afferents and TRPV1-expressing afferents represent parallel sensory pathways that selectively mediate mechanical and thermal nociception, respectively [5; 24]. In visceral *mechanosensation* in the mouse, in contrast, TRPV1 plays a key role; CRD-induced nociceptive behaviors are attenuated in TRPV1 gene knock-out mice [11], and a selective antagonist for TRPV1 inhibits ~65% of stretch-sensitive mouse colorectal afferents [14]. Extrapolating the critical function of IB4+ afferents in cutaneous mechano-nociception to viscerosensation, and considering the involvement of TRPV1 in visceral mechanosensation in mice, one would expect that ablation of IB4+ c-DRG neurons would reduce colorectal mechano-nociception evoked by CRD in mice. However, the present findings clearly contradict this expectation; ablation of ~40% of IB4+ c-DRG neurons did not affect CRD-induced nociceptive behaviors. Further evidence from the *in vitro* single-fiber recordings suggests that it is not because IB4-sap preferentially ablated distension-irresponsive (i.e., stretch-insensitive) colorectal afferents that CRD-induced nociceptive behaviors were unchanged after IB4-sap treatment. Rather, it appears that the ablation of IB4+ colorectal afferents by IB4-sap occurred across all afferent classes to a similar extent because the relative proportions of each afferent class remained unchanged, suggesting that IB4+ c-DRG neurons do not represent a specific colorectal afferent class characterized in single-fiber recordings *in vitro* (likewise, a TRPV1-agonist capsaicin-sensitive afferents were found across all PN colorectal afferent classes [9; 11]). It is important to appreciate that the unbiased, electrical stimulus search strategy employed here would not favor locating or studying one class of colorectal afferent over any other. Thus, it may be noteworthy that there was a trend toward a reduction in the proportion of low-threshold, robust-firing stretch-sensitive afferents in IB4-sap-treated mice, suggesting that IB4-sap might have ablated such afferents more than high-threshold/low frequency-firing afferents within stretch-sensitive colorectal afferent classes. If high-threshold/low frequency-firing afferents were more important for colorectal mechano-nociception than low-threshold/robust-firing afferents, the preferential ablation of the latter by IB4-sap could account for the unaltered CRD-induced nociceptive behaviors in IB4-sap-treated mice.

As an alternative explanation for the failure of IB4-sap to alter colorectal nociceptive behaviors to CRD, a compensatory adaptation in the viscerosensory pathway could be supposed. In rats treated with IB4-sap, their baseline mechanical sensitivity in the hind paw was reduced two weeks after treatment, returning to normal at week three despite sustained loss of IB4+ afferents at that time [27], suggesting compensatory changes in that sensory pathway to overcome the loss of IB4+ afferent inputs. Although we also conducted our behavioral experiments focusing on day 14, when the inhibitory effects of IB4-sap on nociceptive behaviors are reportedly apparent both in rats and mice [27; 31], we cannot exclude the possibility that a compensatory adaptation to the loss of IB4+ afferent inputs could occur faster in viscerosensory pathways. The colorectum is innervated by pelvic (PN) and lumbar splanchnic nerves (LSN); the PN sensory pathway mediates baseline colorectal nociceptive VMRs to CRD [15] and simultaneously inhibits the spinal processing of sensory inputs transmitted through the LSN pathway [30]. The LSN pathway is able to mediate VMRs to CRD in the absence of the PN pathway when sensitizing stimulation is given into the colorectum [28]. Therefore, it may be that the loss of IB4+ afferent inputs in the PN pathway is compensated by engagement of the LSN pathway in mediating colorectal mechano-nociception. Because IB4+ afferents were also significantly reduced in the thoracolumbar spinal cord (where the LSN afferents project) after intrathecal injection of IB4-sap (Fig 3E), if there was any compensation by the LSN pathway, IB4+ LSN colorectal afferents must not be pivotal for the process.

Collectively, these results point to the absence of a contribution of IB4+ colorectal afferents (with strong to medium IB4+ intensity) to colorectal *mechanical* nociception. These afferents could respond to modalities of visceral stimulation other than mechanical, mediate non-nociceptive sensations, and/or be involved in other physiological responses. Addressing these possibilities in detail is well beyond the scope of this study; we did find, however, that a partial loss of IB4+ PN colorectal afferents does not impair the function of neural circuits controlling peristaltic colorectal movements for stress-induced defecation.

In summary, more than one half of colorectal afferents in the PN pathway are IB4+ in C57BL/6J mice, most, if not all, of which are also peptidergic, in contrast to the widely held distinction between peptidergic and IB4+/non-peptidergic DRG neurons and their respective roles in cutaneous nociceptive processing in mice. These IB4+ colorectal afferents do not belong to any one of the five different functional afferent classes that have been characterized in single-fiber recordings of the mouse PN. Within the two stretch-sensitive PN colorectal afferent classes (i.e., muscular and muscular-mucosal), IB4+ afferents with low-threshold/robust-firing responses appear to be more sensitive to IB4-sap-induced ablation, which might account for the unaltered nociceptive behaviors to CRD after ablation of IB4+ afferents. The possibility of adaptive changes compensating for the loss of IB4+ afferent inputs and their roles in (patho)physiology other than colorectal mechanical nociception deserves future investigation.

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Fig 1. Isolectin B4-binding (IB4+) in L6 dorsal root ganglia neurons innervating the mouse distal colon (c-DRG neurons)

(A) Sixty one percent of L6 c-DRG neurons (labeled by retrograde tracer Fast Blue, FB) were IB4+ in control mice injected with unconjugated saporin. These IB4+ c-DRG neurons were of various (A) somata sizes and (B & C) IB4+ intensities classed into weak (W), medium (M), or strong (S) intensity. FB-containing neurons that did not bind IB4 (-) are outlined with a broken line in panel B (fluorescence images were inverted for clear representation of staining intensity; calibration bar=25 μm). Arrowheads in the lower panel indicate corresponding neurons containing FB. (C) The 8-bit grayscale histogram indicates that most L6 IB4+ c-DRG neurons bound IB4 with weak (grayscale <75) and medium (grayscale <145) intensity.

Fig 2. Neurochemical characterization of L6 IB4+ c-DRG neurons

L6 IB4+ c-DRG neurons were frequently (A) CGRP- or (B) TRPV1-immunoreactive, resulting in the conclusion that (C) more than one half of c-DRG neurons were both IB4+ and CGRP-immunoreactive (55%) or IB4+ and TRPV-immunoreactive (57%). This leads to the estimation that ~83% (54/65) of IB4+ c-DRG neurons would express both CGRP and TRPV1. Arrowheads indicate IB4+ c-DRG neurons. Double arrowheads indicate c-DRG neurons that are both target-positive and IB4+. Calibration bar=50 μ m.

Fig 3. Ablation of IB4+ c-DRG neurons two weeks after intrathecal injection of IB4-conjugated saporin (IB4-sap)

Fourteen days after IB4-sap treatment, (A) the proportion of IB4+ c-DRG neurons was significantly reduced from 61% (N=6) to 37% (N=4, 40% reduction, ** $p < 0.01$ by MWU test), and (B) the mean IB4+ intensity of c-DRG neurons was significantly decreased (** $p < 0.01$ by t-test). (C) The normalized histograms, reflecting 40% loss of IB4+ c-DRG neurons after intrathecal IB4-sap treatment, indicate that the treatment preferentially ablated c-DRG neurons with medium to strong IB4+ intensity, leaving neurons with weak IB4+ intensity relatively unaffected. (D & E) This partial but significant loss of IB4+ afferents was evident in both thoracolumbar (TL) and lumbosacral (LS) spinal cords (** $p < 0.01$ by 2-way ANOVA). The LS spinal cord dorsal horn (laminae I-III) with IB4+ afferent terminals are indicated by broken lines (traced based on NeuN staining) in D.

Fig 4. Behavioral consequences of the ablation of IB4+ afferents

(A & B) Colorectal mechanical nociception (i.e., visceromotor response, VMR) to colorectal distension (CRD) was not altered 7 or 14 days after IB4-sap treatment. The mice showed no impairment in (C) inflammatory soup-induced sensitization or (D) restraint stress-induced defecation.

Fig 5. Properties of pelvic nerve colorectal afferents in mice treated with IB4-sap
 (A) A representative trace of stretch-sensitive afferent (muscular-mucosal, Mus-Muc) discharging action potentials (AP) in response to a ramped circumferential stretch of the colorectal wall (0–170 mN, 5 mN/sec). (B) The relative proportion of each colorectal afferent class (four mechanically sensitive colorectal afferent classes and a mechanically insensitive (MIA) class) did not differ between control and IB4-sap-treated mice. There was a trend in stretch-sensitive afferent classes from IB4-sap-treated mice toward a decrease in (C) the proportion of afferents with low response thresholds (<40 mN, $p < 0.12$) and (D) the proportion of afferents discharging AP at high frequency (>50 action potentials for 34 sec, $p < 0.09$).