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IB4 afferent sprouting contributes to bladder dysfunction in spinal rats

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ABSTRACT

We investigated the role that nonpeptidergic isolectin-B4 (IB4) positive, primary afferent sprouting plays in bladder dysfunction after spinal cord transection (SCT). Rats were implanted with an indwelling bladder cannula and subjected to a complete spinal cord transection at T9/T10. In one group of rats IB4-positive terminals increased below the level of the injury in L6 cord in laminae I and III–VI as early as 3 days after transection, and remained increased 8 and 21 days after transection. Growth associated protein 43 (Gap-43) was expressed on IB4-positive neurons 3 days post-transection and the number of L6 dorsal root ganglia (DRG) neurons expressing IB4 did not change after injury. In another set of experiments IB4-saporin or saporin alone was administered intrathecally to L6/S1 cord. IB4-positive afferents sprouted in L6 cord of saporin only treated rats but IB4 afferent labeling was decreased by 42 and 33% in L6 cord and DRG 21 days after IB4-saporin treatment. IB4-saporin treated rats voided with an efficiency of 28.3% 10–14 days after transection whereas one week later voiding efficiency increased to 86.1%. Inefficient voiding by saporin and 10–14 day IB4-saporin treated rats was linked to voiding that occurred after the peak in micturition pressure. On the other hand, increased voiding efficiency in 20–30 day IB4-saporin treated rats was associated with voiding occurring before the peak of the micturition pressure. These results suggest that IB4-positive afferent sprouting plays a role in the generation of bladder dysfunction following SCT.

Introduction

The central pathway controlling micturition requires a neural pathway that includes caudal midbrain, pons, and lumbosacral spinal cord. Complete suprasacral spinal cord injury interrupts this circuit and initially results in bladder areflexia. However, bladder function does emerge after injury. The emergent bladder function is inefficient due to bladder sphincter dyssynergia. This results in high bladder pressure that can induce autonomic dysreflexia and cause kidney failure. What accounts for bladder activity after spinal cord injury is not fully understood. Previous studies suggest that the creation of a spinal circuit, by way of primary afferent sprouting, may play a role in the bladder dysfunction that is seen after spinal cord injury (Mitsui et al., 2005; Zinck and Downie, 2005). L6/S1 primary afferents sprout to deeper regions of the dorsal horn after complete thoracic SCT. The timing of this sprouting response correlates well with emergence of bladder dysfunction after spinal cord injury (Zinck et al., 2007). However, this study examined those afferent neurons that respond only to nerve growth factor and contain the neuropeptide calcitonin gene-related peptide (CGRP) (Molliver et al., 1997). Glial derived neurotrophic factor (GDNF) supports a separate group of primary afferent neurons referred to as nonpeptidergic afferents. These neurons do not contain CGRP but are distinguished by their ability to bind the plant isolection B4 (IB4) (Stucky and Lewin, 1999). The bladder body and urethra are innervated by IB4-positive neurons and these bladder afferents have been shown to display different functional properties when compared to CGRP containing afferents (Yoshiyama et al., 2003). In addition, GDNF null mice have significantly smaller bladders and lack kidneys and an enteric nervous system, indicating the importance of this growth factor in the development of the lower urinary tract (Moore et al., 1996). IB4-positive afferents sprout in response to peripheral nerve injury and this has been implicated in the development of neuropathic pain, a common consequence of spinal cord injury (Li and Zhou, 2001). Saporin, when complexed to IB4, is neurotoxic and therefore can be used to selectively eliminate IB4-positive neurons. Saporin tagged antigens have been used in a number of studies to inhibit the contribution of specific neuronal populations to various pathologies, including bladder dysfunction (Nishiguchi et al., 2004; Tarpley et al., 2004). Saporin enters neurons through receptor-mediated endocytosis and inhibits ribosomal function ultimately resulting in cell death (Wiley and Lappi, 2003). Selective targeting of IB4-positive afferents through the use of IB4 conjugated saporin revealed that IB4 afferents contribute to overactive bladder function induced by bladder irritation (Nishiguchi et al., 2004). This study aims to determine if IB4-positive neurons sprout after complete spinal cord transection (SCT) and, if they do sprout, at what timepoint relative to the emergence of

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bladder activity. Further, we will determine, by selective removal of IB4 afferents, if this population of afferents plays a role in bladder activity seen after spinal cord injury.

Methods

The experimental protocols for these experiments met the guidelines of the Canadian Council on Animal Care and were approved by the University Committee on Laboratory Animals, Dalhousie University. Throughout the study all attempts were made to reduce animal suffering.

Surgical procedures

All 39 female Wistar rats (200–300 g body weight) used in this study underwent surgery to implant an indwelling bladder cannula. Under isoflurane (2–3% in oxygen) anesthesia, a ventral midline abdominal incision was made to expose the urinary bladder. A cannula fashioned from silastic tubing was inserted and secured to the bladder dome with a purse string suture. The distal end of the tubing was then tunneled under the skin and exited from the back of the skull where it was connected to a stainless steel tube in a plastic assembly secured to the skull with dental acrylic and screws. Rats were given subcutaneous (s.c.) intraoperative injections of saline (5 ml), enrofloxacin (Baytril) (2.5 mg/kg) and bupenorphrine (0.03 mg/kg) and received enrofloxacin in their drinking water (0.02 mg/ml) throughout the course of the study.

At least one week after bladder cannulation, and after (see below) confirming normal bladder function, 30 of the 39 rats were again anesthetized with isoflurane and a dorsal midline incision was made to expose T8–T13 vertebral processes. Fascia and muscle were removed from T9 and T10 vertebrae. A laminectomy was then performed to visualize the whole width of the dural sac and a complete surgical SCT was performed at T10 using a scalpel. Completeness of transection was verified by observing retraction of the cord at the time of cutting and subsequent placement of gelatin sponge (Gelfoam) between the cut ends of the spinal cord. Rats were given s.c. injections of 0.9% saline (5 ml), Baytril (2.5 mg/kg) and bupenorphrine (0.03 g/kg) intraoperatively and s.c. injections of saline (2.5 ml) and bupenorphrine (0.03 mg/kg) every 8 to 12 h for 24–48 h after SCT.

In 16 rats undergoing SCT, an intrathecal injection step preceded transection. This injection was also done in 5 rats that were not subsequently subjected to SCT. Under the 25× objective of a Leica WILD M3C microscope, a puncture was made in the dura using a 26-gauge needle. An intrathecal (i.t.) cannula constructed of Tygon tubing (Cole Parmer) with a micro-renathane attachment (Braintree Scientific) was inserted through the hole and fed caudally approximately 1.8 cm to reach the L6/S1 cord. 2 μl of IB4-SAP (1.2 mg/ml, 53% saporin/mole IB4) (Advanced Targeting Systems) (n=9 SCT, 4 control rats), or 3 μl of SAP (1 mg/ml) (Advanced Targeting Systems) (n=3 SCT rats) was diluted in phosphate buffered saline (pH=7.4) to make total i.t. injectant volumes of 8 μl. Saline only injections were given (8 μl saline) in 2 SCT rats. After injection the cannula was left in place for approximately 10 min in an attempt to prevent leakage upon removal.

Post-surgical care

Rats were housed individually after SCT and were monitored daily for any signs of distress, autotomy, and failure to thrive. Injured animals were given a supplementary diet and both food and water intakes were monitored throughout the course of the study. Animals that were suffering from dehydration or were not eating were given subcutaneous saline and put on a high calorie diet. Animals were sacrificed if they lost more than 20% of their original body. Rats were also immediately sacrificed if autotomy resulted in injury to two digits or if one full digit was lost. During the course of this study 5 IB4-SAP treated rats (1 untransected, 4 with SCT) were euthanized due to autonomy. Three of these rats (1 untransected, 2 with SCT) did not contribute data to this study. Two SCT rats however, were sacrificed 3 days post-transection and were therefore included in the study at that timepoint. Bladders were drained by withdrawing urine from a tube connected to the implanted cannula through the assembly mounted on the skull. Draining was performed 3 times daily at approximately 8 h intervals. Bladders were not manually expressed unless cannula lines were blocked. 7 rats required manual expression. Of these, 2 were expressed once, 1 required expression 3 times, 3 required expression 4 times and one required expression 5 times.

Perfusions and tissue processing

Uninjured IB4-SAP treated rats (n=4), injured saline injected (n=2) and injured SAP injected (n=3) rats were perfused at 20–30 days post-injection. Rats were deeply anesthetized with sodium pentobarbital (Euthanyl) and perfused through the left ventricle with 0.9% saline followed by phosphate buffered 4% paraformaldehyde. Complete transection of T10 cord was again verified by inspection after perfusion and before removal of L6/S1 spinal cord. L6/S1 spinal cord and DRG were post-fixed overnight in 4% paraformaldehyde and then cryoprotected in ascending concentrations to 30% sucrose. Tissues were embedded in O.C.T. Compound (Tissue-Tek) and cut on a cryostat (spinal cord, 20 μm in three sequential series; DRG 40 μm in one series). For visualization of IB4 alone in spinal cord, coronal sections were blocked with 10% normal donkey serum in 0.3% Triton-X for 1 h at room temperature. Sections were then rinsed with 0.05 M PBS (pH= 7.4) and IB4-FITC (1:200, 5.5 mol FITC/mol lectin) (Sigma) in PBS and Triton-X was applied to the sections on slide for 3 h at room temperature. For dual CGRP/IB4 visualization in spinal cord or DRG, primary monoclonal antibody rabbit anti-CGRP (Peninsula Labs, 1:1000) was applied on slide at room temperature for 3 days. 10% normal goat serum and 0.3% Triton-X were added to the primary antibody solution. Sections were rinsed three times with 0.05 M phosphate buffered saline pH 7.4 and placed in biotinylated goat anti-rabbit (Jacksons Immuno Research Laboratories Inc., 1:500) secondary antibody overnight at room temperature. After rinsing sections again in PBS sections were incubated in streptavidin Alexa 568 conjugate (1:300) (Molecular Probes) for 3 h at room temperature. At the same time sections were also incubated with IB4-FITC (1:200) (Sigma). Sections were then rinsed with PBS and coverslipped using a solution containing 0.3% phenylenediamine in 50% PBS and glycerol.

Processing for IB4 and Gap-43 was conducted on longitudinally cut lumbosacral spinal cords at 8 days post-transection. Sections were rinsed three times with 0.05 M phosphate buffered saline pH 7.4 and placed in a solution containing primary monoclonal anti-Gap-43, (Chemicon, 1:1000), 10% normal goat serum and 0.3% Triton-X. After rinsing sections again in PBS sections were incubated in rhodamine goat anti-mouse IgG (1:200) (Chemicon) and IB4-FITC (1:200) (Sigma) for 3 h at room temperature. Sections were rinsed and coverslipped using the same procedure described above.

Analysis of histochemistry

For DRGs, double counting was avoided by counting only stained neuron profiles containing nuclei. For the initial study of IB4 distribution after SCT total cell counts of IB4 positive, CGRP positive and double labeled cells were conducted on the whole L6 ganglia. Analysis of the subsequent group receiving intrathecal injections was carried out from 5 randomly selected sections for each animal. To gauge the risk of counting error due to change in nuclear dimensions