Spinal cord injury leads to atrophy in pelvic ganglia neurons

Arshdeep Marwaha\textsuperscript{a,1}, Rahul Sachdeva\textsuperscript{a,b,1}, Diana Hunter\textsuperscript{a}, Matt Ramer\textsuperscript{a,b,c,*}, Andrei V. Krassioukov\textsuperscript{a,b,*}

\textsuperscript{a} International Collaboration on Repair Discoveries (ICORD), University of British Columbia (UBC), Vancouver, British Columbia, Canada
\textsuperscript{b} Department of Medicine, Division of Physical Medicine and Rehabilitation, UBC, Vancouver, British Columbia, Canada
\textsuperscript{c} Department of Zoology, UBC, Vancouver, Canada

1 Introduction

Spinal cord injury (SCI) leads to the loss of communication between supraspinal centers and the spinal cord, and loss of coordination of motor, sensory and autonomic functions. Of the numerous debilitating consequences of SCI, amelioration of genitourinary and gastrointestinal dysfunctions are among the highest priorities for recovery (Anderson, 2004; Hammell, 2010). These pelvic organ dysfunctions are commonly manifested as lower urinary tract (LUT) complications such as failure in urine storage/ bladder emptying, detrusor sphincter dysynergia (Hamid et al., 2018), neurogenic bowel dysfunctions such as decrease in colonic motility, altered tone leading to constipation, fecal incontinence, and bowel impaction (Lynch et al., 2001; Lynch et al., 2000; Tate et al., 2016), and sexual dysfunction such as impaired arousal, erection/ejaculation failure (Benevento and Sipski, 2002; Elliott, 2006). Recent recommendations from an expert panel comprising of key international SCI clinicians, researchers and funding organizations also encourage more mechanistic understanding of these dysfunctions after SCI (Wheeler et al., 2018).

While considerable strides have been made in the management of symptoms, targeting recovery of reproductive functions in males as well as voiding functions in both sexes has remained a challenge- largely due to the physiological and anatomical complexity of these systems. Of significant interest among these is the pelvic ganglion (PG), which supplies innervation and integrates neural control of nearly all pelvic viscera (Keast, 1999). The target organs include the penis and internal reproductive tissues in males, the uterus and vagina in females, as well as the bladder, colon and anal accessory muscles in both sexes. Compared to other autonomic ganglia, PG are relatively less well-investigated, owing to the sexual dimorphism and dramatic inter-species variability in their location and anatomy (Keast, 2006). To further add to this complexity, the PG is the only site in the autonomic nervous system where sympathetic and parasympathetic neurons are located in the same ganglion (Keast, 1999).

A previous report from our group suggests that high thoracic spinal cord injury results in significant central and peripheral plastic changes in neurons associated with the development of autonomic and sensory dysfunctions (Inskip et al., 2009). In the present study, we took a straightforward approach to evaluate the time course of changes in size distribution of PG neurons, a defining phenotypic feature, following an...
experimental high thoracic SCI. We hypothesize that SCI leads to maladaptive plasticity in the PG neurons and that these effects will be more pronounced in the chronic stage.

2. Methods

2.1. Ethical approval

Animal surgery and post-surgical care were conducted according to guidelines established by the Canadian Council for Animal Care. Institutional ethics approval was obtained from the University of British Columbia (approval certificate: A18-0183).

2.2. Surgical procedures

Twenty, adult male Wistar rats (300-325 g, from Harlan Laboratories, Indianapolis, IN) were divided into four groups (n = 5 each). Three groups underwent SCI and were euthanized at one-, four-, and eight-weeks post-SCI, while one group served as the uninjured control. Surgical procedures and post-surgical care were performed as previously described (Phillips et al., 2018). Briefly, rats were administered a prophylactic dose of enrofloxacin (Associated Veterinary Purchasing, Langley, Canada, 10 mg/kg) via a subcutaneous (s.c.) injection for 3 days prior to surgery. On the day of surgery, animals were anesthetized in an induction chamber by 5% isoflurane in 2 L min⁻¹ oxygen and maintained at surgical plane with 2% isoflurane. Subsequently, animals were administered enrofloxacin (10 mg/kg, s.c.), buprenorphine (Temgesic; 0.02 mg/kg, McGill University s.c.), and warm lactated Ringers solution (10 mL, s.c.). Following a dorsal midline skin incision and blunt dissection of superficial muscle overlying the C8-T5 vertebrae, a T2 laminectomy was performed to expose the T3 spinal cord. The dura mater was incised using a 30G needle and the spinal cord was transected using micro-scissors and gentle vacuum aspiration to clean excess blood and cerebrospinal fluid (Sachdeva et al., 2020; Sachdeva et al., 2016). Lesion completeness was confirmed by visualization of a clear separation between the lesioned ends. A pledget of Gelfoam (Pharmacia & Upjohn Company, Pfizer, New York, USA) was transiently placed between the lesioned ends to achieve hemostasis. The dura mater was closed using 9–0 sutures. Muscle and skin were closed with continuous, 4–0 Vicryl sutures, and interrupted, 4–0 Prolene sutures, respectively.

Following surgical procedures, animals were injected with warmed Lactated Ringer’s solution (5 mL, s.c.) and allowed to regain consciousness in a warm, temperature-controlled environment (Animal Intensive Care Unit, Lyon Technologies, Los Angeles, CA). Enrofloxacin (10 mg/kg, s.c.) and Buprenorphine (0.02 mg/kg, s.c.) were given once and twice a day, respectively, for 3 days and after spinal cord injury. Warmed lactated ringers (10 mL, s.c.) was administered twice per day for 7 days following surgery and 5 mL (s.c.) thereafter, only as needed. The bladder was manually expressed twice a day until spontaneous bladder voiding returned. Monitoring of body weight, activity level, social behaviour, healing at the surgery site and clinical signs of morbidity were completed daily for 14 days post-surgery and every two days thereafter.

2.3. Tissue processing

Rats were euthanized with chloral hydrate (1 g/kg, i.p.) and perfused transcardially with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. PG were dissected out bilaterally, post-fixed in 4% parafomaldehyde overnight and cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Tissue was immersed in cryomatrix (Thermo Scientific) and frozen using liquid nitrogen. PG were sectioned at 16 μm thickness using a cryostat at −23 °C and mounted on Superfrost Plus Slides (VWR Micro Slides). Slides were allowed to dry at room temperature (RT) overnight and stored at −20 °C.

2.4. Immunohistochemistry

PG tissue sections were circumscribed with a hydrophobic barrier and washed 3 times with 1× PBS for 15 min at RT. Subsequently, sections were blocked in 1% Bovine Serum Albumin (Sigma) and 10% Normal Goat Serum (Sigma) for 1 h at RT (solutions were diluted in 0.2% Triton in PBS, PBS-Tx). Sections were then incubated at RT overnight in primary antibodies diluted with 10% NGS in 0.2% PBS-Tx, followed by secondary antibodies for 2 h. Primary antibodies used were: rabbit-Neurofilament-medium (NFM; Millipore 1:500) and chicken- Microtubule associated protein 2 (MAP2; Millipore 1:4000). Secondary antibodies used were: goat anti-chicken (Invitrogen 1:500) and goat anti-rabbit (Invitrogen 1:500), both conjugated with Alexa Fluor® 488 (excitation peak at 493 nm and emission peak at 519 nm) in order to achieve complete filling of neuronal soma. Sections were washed 3 times with 1× PBS for 15 min at RT prior to being cover slipped with Cytoseal 60 (Thermo Scientific).

2.5. Imaging and analysis

Size-frequency analysis was performed as previously established protocol (Ramer et al., 2012). Briefly, five non-consecutive PG sections were randomly selected and immunostained per rat. Images were captured at 20× magnification (Zeiss LSM 800) with increased pinhole size to reduce z-resolution and were analyzed using the image processing package Fiji (https://fiji.sc/). Five sections were randomly selected from either one of the left or right ganglia The neuronal size-frequency distributions were determined using recursive translation, which uses the diameters from neuronal profiles in section to mathematically reconstruct the cell population from which the profiles were traced, as described previously (Ramer et al., 2001). The perimeters of the profiles were manually traced and the Feret diameters were subsequently calculated using the Fiji software. A histogram of the cell profiles was produced for each ganglion by separating them into 4 μm bins. Recursive translation was performed on these binned measurements using a Microsoft Excel program constructed from the corrected Rose and Rohrligh algorithm (Rose and Rohrlieh, 1988). All analyses were performed by experimenters blinded to the animal groups.

2.6. Statistical analysis

Kolmogorov-Smirnov (KS) goodness-of-fit tests were performed on size-frequency distribution data to determine the difference in the cumulative size-frequency distributions across the experimental groups. This test has the advantage of making no assumption about the distribution of data. Statistical significance was determined by comparing the maximum difference between cumulative size-frequency distributions (the D-statistic) to a critical value given by 1.36/√(N), where N is the combined number of cells for groups being compared. When D > the critical value, p < .05. The number of neurons analyzed per group were compared using one-way ANOVA.

3. Results

Representative images of neurons stained with neuronal markers in PG at one-, four-, and eight-weeks post-SCI are shown in (Fig. 1A–D). The dotted-white line represents an example of the perimeter of a neuron profile in the PG traced manually (Fig. 1A). Following quantification of profiles, recursive translation was performed to mathematically reconstruct the neuronal population. The total number of neurons/rat/group generated from profiles was not significantly different across groups (Uninjured 114.2 ± 32.59, 1-week 154.8 ± 12, 4 weeks 120.8 ± 49.31, 8 weeks 188.9 ± 90.67, p = .15; one-way ANOVA). Individual neurons were grouped into bins to generate histograms of proportional frequency (Fig. 1E–G). The KS goodness-of-fit test was subsequently used to assess the significance of the maximum
Fig. 1. Complete high-thoracic SCI results in atrophy of PG neurons. Representative images of neuronal cell bodies in the PG of (A) uninjured (B) one week (C) four weeks (D) and eight weeks post-SCI rats. Neurons were co-stained with neuronal markers, NFM and MAP2. Left-ward shift in proportional frequencies of neuronal size at (E) one week (F) four weeks and (G) eight weeks post-SCI, compared to uninjured control. (H) Leftward shift in cumulative sum of neuron-size proportions increases in severity over eight weeks. Error bars represent standard error. (n = 5 per group).
differences between the population distributions at distinct timepoints following SCI compared to the control group. At one- and four-weeks post-injury, the size-frequency distribution of neurons showed a significant but subtle leftward shift. However, at eight weeks post-injury, the left-ward shift was significant and much more pronounced than the earlier time-points (Fig. 1H). Of the neurons that demonstrated a significant size-frequency shift, the proportional frequency of neurons undergoing atrophy was 4-fold greater at eight weeks post-SCI, compared to one- and four-weeks post-SCI.

4. Discussion

We hypothesized that SCI is associated with maladaptive neuroplastic changes in key structures responsible for normal functioning of pelvic organs. The present study demonstrates an injury-induced atrophy of PG neurons. The SCI-dependent atrophy, although observable at as early as one-week post injury, does not become pronounced for at least four-weeks post injury. This delayed impact of SCI on neuronal morphology may indicate a possible therapeutic time window for interventions limiting or preventing the onset of these observed structural changes, potentially preventing or mitigating subsequent functional deficits.

Complete spinal transection at T3 was a model of choice as it represents the upper limit of severe SCI manifesting clinically relevant autonomic dysfunctions, while ensuring maximum survival rate and satisfactory recovery post-SCI in chronic experimental conditions. Of the two studies investigating SCI-dependent changes within PG (Krusz et al., 1995; Takahara et al., 2007), only one quantified the neuron size distribution, interestingly reporting no effect of SCI on PG neuron size (Krusze et al., 1995). This may be due to a much smaller sample size ($n = 2$). Furthermore, upper thoracic SCI offers relatively less ambiguity as it most certainly spares the PG circuitry that could be affected by a lower thoracic lesion (e.g. sympathetic innervation from T10-L2 spinal segments travelling via hypogastric nerve).

Although we observed a significant effect of SCI on PG neuronal size at 1 and 4 weeks post-SCI, the atrophic changes were more severe at 8-weeks timepoint. This may be attributed to the trophic support (e.g. nerve growth factor, NGF) provided by target organs such as the urinary bladder. The expression of these factors dramatically increases initially post-SCI and gradually declines over the subsequent 4-6 weeks post-SCI (Vizzard, 2000). The question of whether a gradual decline of neurotrophic support is accompanied by neuronal atrophy following SCI warrants a systematic investigation of their temporal expression patterns over the acute and chronic stages of SCI.

The finding of PG neuron atrophy following SCI is intriguing and somewhat surprising considering the non-SCI models to study plasticity of these neurons. Partial obstruction of the urethra, which causes a tenfold increase in bladder muscle weight with concomitant increase in NGF leads to significant hypertrophy of PG neurons (Gabella et al., 1992; Steers et al., 1991). Contrary to this finding, bladder hypertrophy following a low thoracic SCI did not result in the enlargement of PG neurons (Krusz et al., 1995). Similarly, while an upper thoracic (T3) injury leads to significant bladder hypertrophy (Hunter et al., 2018), it does not result in PG neuron hypertrophy. Evidently, bladder hypertrophy is not the sole determining factor of the neuron size following SCI. While the exact mechanisms remain unclear, mechanistic studies in the future must also consider that the neurons in PG innervate nearly all organs in pelvic viscera in addition to the urinary bladder. Nonetheless, an important takeaway concept from studies of urethral obstruction is that removal of the obstruction reverses the neuronal hypertrophy. The bidirectional neuroplastic potential of PG neurons to increase and decrease in size is likely relevant for preventing or treating SCI-induced maladaptations via therapeutic interventions.

A final consideration for future research should be the considerable heterogeneity of the neurons in terms of their vulnerability to injury, neurotransmitter expression and sex differences, which is partly driven by the diversity of target organs. For instance, PG in male rats contain more sympathetic noradrenergic neurons compared to females, as the male reproductive organs are larger and require denser sympathetic innervation (Furness, 2015). Although the PG neurons can be broadly classified as sympathetic and parasympathetic (with norepinephrine and acetylcholine as primary neurotransmitters respectively), both classes share the expression of neuropeptide Y or vasoactive intestinal peptide, or both (Wanigasekara et al., 2003). Furthermore, a small population of neurons also exists that are neither adrenergic, nor cholinergic (Park et al., 2006). Taken together, the intricacy of neurotransmitter expression adds another layer of complexity to the analysis of PG neuron subpopulations, requiring particular consideration in future research.

5. Conclusion

Despite being a patient priority, the impact of SCI on pelvic organ function remains an under-explored research avenue (Wheeler et al., 2018). Given the contribution of PG in multitude of pelvic organ functions, the research investigating PG following SCI is underwhelming. While non-SCI studies suggest that the bladder size mandates the size of PG neurons, this does not hold true following SCI, indicating a greater order of complexity in this condition. Detailed investigation of PG neuron subpopulations paralleled with gene expression studies is needed to obtain a precise understanding of pelvic organ dysfunction following SCI. The timeline of plastic changes ensuing after SCI suggest a therapeutic window with implications for targeted therapeutic interventions.

Disclosure

None.

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