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Research report

Neurogenic bladder dysfunction does not correlate with astrocyte and microglia activation produced by graded force in a contusion-induced spinal cord injury

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ABSTRACT

Rodent models for the study of neurogenic bladder dysfunction after spinal cord injury (SCI) are difficult to standardize, particularly when evaluating the specific contribution of the SCI to end-organ function. The purpose of this study was to evaluate the degree of bladder dysfunction associated with a highly reproducible, contusion-induced SCI in female rats. An infinite horizon impactor was used to create a contusion SCI with a magnitude of either 100 or 150 kDyne at the T8/T9 thoracic region of female Sprague-Dawley rats. Locomotor function, and the presence of astrocytes (positive regions for Glial Fibrillary Acidic Protein) and microglia (positive cells for the integrin CD11b) at the SCI site were determined at four weeks after SCI. Similarly, cystometric properties were characterized in urethane anesthetized rats at four weeks post-SCI. The significant increases in astrocyte and microglia in the T8/T9 region in all of the SCI animals did not correlate with locomotor impairment or bladder dysfunction. After performing the cystometric studies substantial differences were found in both SCI groups when compared to intact animals, specifically a high frequency of non-voiding contractions, different durations for intraluminal pressure-high frequency oscillations, intercontractile intervals, impaired micturition volumes, and estimated voiding efficiency. These results suggest that a contusion SCI can increase microglia and astrocyte activation without a strong association with bladder dysfunction. The present study will be important for precise considerations about correlating the intensity of an SCI with impairment outcomes at both locomotor or organ function levels.

1. Introduction

Sensory fibers innervating the urothelium, lamina propria and detrusor muscle in the urinary bladder communicate organ filling status, via lumbosacral afferent nerve terminals, with the central nervous system for central processing leading to relaxation of the urethra and subsequent contraction of the detrusor muscle allowing coordinated urination (Byrne et al., 1998; de Groat et al., 2015). Among patients with spinal cord injury (SCI), neurogenic bladder dysfunction (NBD) is one of the most devastating complications leading to urinary retention, urinary incontinence or bladder overactivity which drastically affects their quality of life (Collinger et al., 2013; Rabadi and Aston, 2016). Further complicating the clinical situation, the neurogenic bladder cannot empty efficiently as a result of constant contractions of the external urethral sphincter (EUS) that prevents efficient micturition, therefore leading to additional complications including urinary tract infections, bladder stones or kidney damage (Perkash, 2004; Sahai et al., 2011).

It has been found in humans and animals that the SCI level, as well as the intensity of the injury, generate a "neurogenic bladder" by preventing the normal function of descending axons (de Groat et al., 1998), interneurons (de Groat et al., 1998; de Groat and Yoshimura, 2012), afferent and efferent neural transmission (de Groat and Yoshimura, 2006; Tai et al., 2006), and attenuated bladder-brain communication due to local scar formation following a period of spinal shock (Cohn et al., 2016; Toyooka et al., 2011). Overall, the combination of neural disruption with spinal cord scar

Abbreviations: SCI, spinal cord injury; NDB, neurogenic bladder dysfunction; kDy, kDyne; BBB, Basso, Beattie and Bresnahan locomotor score; NVC, non-voiding contraction; IPHFO, intraluminal pressure high frequency oscillations; ICI, intercontractile interval; EVE, estimated voiding efficiency; CMG, cystometry/cystometric; LUT, lower urinary tract.

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formation creates a daunting clinical condition to be addressed after SCI. Some pharmacological treatments for NBD are available, but moderate effectiveness and prominent side effects frequently result in poor patient compliance (del Popolo et al., 2012; Taweel and Seyam, 2015).

It is clear that in order to carry out more efficient pre-clinical studies, and before evaluating new targets aimed at improving spinal neuroregeneration or efficient micturition after SCI, well characterized animal models of SCI-induced neurogenic bladder dysfunction are necessary. Thus, the main objective of this study was to describe the association between astrocyte scar formation, microglia activation, locomotor function, and neurogenic bladder dysfunction after a single semi-chronic (4 weeks) spinal injury of moderate or severe intensity. In this study a precise and highly reproducible contusion-induced SCI at the thoracic region of female rats was used. Intact animals with the same age and weight were used for comparison of histological and cystometric parameters.

2. Materials and methods

The procedures for SCI were approved by our institutional IACUC and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.), 2011). None of the intact or SCI animals became ill or died prior to the experimental endpoint. We made every effort to minimize the suffering and number of animals according to the requirements of our IACUC.

2.1. Impact SCI rat model

Female Sprague-Dawley rats weighing 250–300 g (Envigo, Houston, TX) were housed in a pathogen free environment with 12 h light/dark cycles, controlled temperature of 25 °C, and ad-libitum access to food and water in plastic cages containing corn cob bedding. Rats were anaesthetized with 2% isoflurane to perform a laminectomy at the T8/T9 level. An IH-0400 infinite horizon impactor (Precision Systems & Instrumentation, Fairfax Station, VA) was used to apply a dorsal-central impact with a force of either 100 kDyne (kDy) for a mild injury or 150kDy for a severe injured condition (N = 6 rats per group). Muscle layers and the fascia were sutured and the skin stapled. Buprenorphine (0.05 mg/kg) and carprofen (5 mg/kg) were subcutaneously applied twice a day for three days. Ampicillin (100 mg/kg) was intramuscularly applied for five days to prevent urinary tract infections. Urinary bladder expression was provided twice a day for a period of two weeks to all the of the SCI animals. Intact rats of same age and weight did not receive any of the described procedures.

2.2. Locomotor assessment

Hindlimb locomotor function was determined by evaluating video recordings of intact and SCI animals at 0, 3, 14 and 28 days after surgery. Locomotor changes were determined in accordance with the Basso, Beattie, and Bresnahan (BBB) score (Basso et al., 1995), which varies from 0 (no movement) to 21 (normal limb function) by a laboratory technician blinded to the study.

2.3. Cystometric evaluation

Both SCI and intact rats were anesthetized with urethane (1.2 g/kg, subcutaneously) to evaluate cystometric properties at four weeks after initiation of the study. The bladder was exposed through a midline abdominal incision to secure a suprapubic PE-50 catheter in the dome region for intravesical infusion of saline solution (0.9% NaCl) at a rate of 0.1 ml/min. The changes in bladder pressure and voiding events were recorded for a period of 60–90 min at a sampling rate of 60 Hz using a pressure and a force transducer, respectively (World Precision Instruments. Sarasota, FL). Cystometric data were acquired and analyzed using WinDaq software (DataQ Inst. Akron, OH). Changes in bladder pressure higher than 5 cm-H_2O, without allowing the release of saline, were considered as a non-voiding contraction (NVC) and indicative of NBD. The frequency of NVC (NVC/h), duration of intraluminal pressure high frequency oscillations (IPHFO, s), maximal voiding pressure (cm-H_2O), intercontractile interval (ICI; s), and micturition volume (ml) were determined as suggested in previous publications (Andersson et al., 2011). To determine voiding efficiency it is necessary to calculate the bladder residual volume after a voiding contraction, but unfortunately that was not performed during the experiments. Thus, an estimated voiding efficiency (EVE) was envisioned as a way to establish an approximation of how efficient bladder voiding contractions were. The EVE was determined by dividing the voided volume ($Void_{Vol}$) of a single micturition event by the calculated infused volume ($Calc_{Vol}$ = 0.1 ml/min × ICI) and multiplied by 100 (EVE = 100*($Void_{Vol}$/$Calc_{Vol}$).

2.4. Immunofluorescent procedures

After cystometric assessments, rats were transcardially perfused with an ice-cold phosphate-buffered solution, followed by freshly-made 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). The thoracic spinal region containing the T8/T9 SCI site was isolated and sequentially cryoprotected in 10 and 30% sucrose. Conventional immunofluorescent procedures were performed in parallel for four rats from each one of the three groups using longitudinal spinal cord sections (25 μm thick) previously embedded in optimal cutting temperature compound (Fisher Scientific, Waltham, MA). Microglia cells were identified by using a specific surface integrin CD11b monoclonal antibody at a 1:50 dilution (MCA275R; Bio-Rad, Raleigh, NC). The distribution of microtubule-associated protein 2 (MAP2) was determined with a polyclonal antibody (ab5392; abcam, Cambridge, MA) using a 1:500 dilution, and considered as a marker for microtubule assembly in neurons. The presence of astrocytes was determined with an antibody against glial fibrillary acidic protein (GFAP) using a specific monoclonal antibody at a dilution of 1:1,000 (MAB3402; Millipore, Billerica, MA). Nuclei were stained by applying gold antifade mountant reagent with DAPI (P36931; ThermoFisher, Grand Island, NY). Secondary antibodies were conjugated with either FITC- (535 nm) or TRITC- (620 nm), while DAPI signals were determined at 460 nm.

2.5. Image acquisition and processing

Stained spinal cord sections were imaged using a CCD camera (1392 × 1040 pixels) controlled by the NIS-Advanced Research software (Nikon, Brighton, MI), with 10X and 40X objectives, and constant exposure time for the excitation settings on all slides for a particular antibody. High resolution images (40X) were analyzed for determining stained zones with ImageJ software (National Institutes of Health; Bethesda, MD). Briefly, TIFF pictures from the sections incubated with antibodies against GFAP (seen in red), CD11b (also seen in red), and MAP2 (seen in green) were used to determine the "% of area" based on the amount of an specific area covered in each of the Red/Green/Blue channels for a particular channel in the picture, and expressed as the percentage for each one of the used antibodies. The total amount of the R/G/B signals was considered as 100%.

2.6. Data analysis

Data group analysis was performed using Prism6 (GraphPad Software, La Jolla CA). The percentages of area for CD11b, MAP2, and GFAP on each experimental group were further examined by