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Comparison of the effects of complete and incomplete spinal cord injury on lower urinary tract function as evaluated in unanesthetized rats

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Abstract

In rats, phasic external urethral sphincter (EUS) activity (bursting) is postulated to be crucial for efficient voiding. This has been reported to be lost after spinal cord transection (txSCI), contributing to impaired function. However, anesthesia may confound evaluating EUS activity. We therefore evaluated urodynamic parameters in unanesthetized, restrained rats and compared the effects of txSCI to that of a clinically relevant, incomplete, contusive injury (iSCI) on lower urinary tract function. Adult female rats were subjected to txSCI or standardized iSCI at the T8 vertebral level. As expected, all injured rats were initially unable to void but developed a reflex bladder with time, with iSCI rats recovering more rapidly than txSCI rats. LUT function was evaluated urodynamically at 2 and 6 weeks after injury. In response to infusion of saline into the bladder, controls consistently exhibited coordinated contraction of the bladder and activation of the EUS *in a phasic pattern* and had a high voiding efficiency ($86.4\% \pm 2.5\%$). Voiding efficiency of iSCI rats was reduced to approximately 57% and txSCI rats to approximately 32%. However, two different patterns of EUS activity during voiding were present in *both* txSCI and iSCI groups at both time points: (1) rats with phasic EUS activity, similar to controls and (2) those that only exhibited tonic EUS activity during voiding. The former had more normal voiding efficiencies. Thus, phasic EUS activity and the improved voiding efficiency associated with it, can occur and be detected in unanesthetized rats after both incomplete and complete SCI.

Keywords

Micturition; Bladder; External urethral sphincter

Introduction

Bladder dysfunction has consistently been ranked as one of the top concerns among paraplegics and quadriplegics, usually of higher importance than the loss of locomotion (Anderson 2004;

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Benevento and Sipski 2002). Normal lower urinary tract (LUT) function in the rat requires coordinated contractions of the urinary bladder smooth muscle with intermittent contractions of the external urethral sphincter (EUS) striated muscle in 4–6 Hz frequency bursts (Holstege et al 1986; Kakizaki et al 1997). Bladder-EUS coordination is regulated by spinal circuits along with supraspinal input from the rostral brainstem, particularly the pontine micturition center, or Barrington's nucleus. Efficient voiding also involves the integration of sympathetic, parasympathetic and somatic pathways within the lumbosacral cord (Kruse et al 1990; Vizzard et al 1995; Marson 1997).

Most studies examining LUT function after SCI have used completely transected animals. Initially after thoracic spinal cord transection (txSCI), the bladder is areflexic, causing urinary retention and bladder enlargement. Hyperreflexic bladder contractions reappear after 7–10 days; voiding is possible but inefficient (Chancellor et al 1994; Yoshiyama 1999). Bladder hyperreflexia, characterized by a number of non-voiding cycles before a voiding contraction, is hypothesized to be due to increased afferent signaling induced by the distended bladder, evidenced by hypertrophy in bladder afferent neurons and expansion in their terminal distribution in the spinal cord (Kruse et al 1995; Vizzard 1999, 2000; Zvarova 2004). Impaired supraspinal control disrupts bladder-EUS coordination (Kruse et al 1993; de Groat 1995) and induces dyssynergia. In the rat, micturition-associated phasic EUS activity, characterized by alternating bursts of EUS activity and relaxation (silent periods), is lost after SCI and has been reported to be replaced by tonic, dyssynergic activity (Kruse et al 1994; Chancellor 1994; Yoshiyama et al 2000). While human patients do not show the same phasic pattern of EUS relaxation during voiding, the necessary EUS relaxation is lost in both humans and rats, resulting in inefficient voiding (Blaivas et al 1981; Kruse et al 1993; Dolber et al 2007).

However, most patients suffer from incomplete injuries (Bracken et al 1990), so it is critical to also study LUT function after clinically relevant incomplete, contusive injury (iSCI). The few experimental studies on iSCI have reported that the coordinated activation of the EUS during voiding bladder contractions is initially lost but demonstrates partial recovery 8 weeks after injury (Pikov and Wrathall 2001). The higher EUS EMG activity at the time of bladder contractions was positively correlated with the amount of spared white matter at the injury epicenter and chronic serotonin immunoreactivity levels in the lumbosacral cord, suggesting that spared descending tracts lead to better LUT function (Pikov et al 1998; Pikov and Wrathall 2001). However, these studies did not examine the *pattern* of the increased EUS activity, in terms of whether the EUS activation occurred in a normal phasic bursting pattern, or was merely tonic. Further, voiding efficiency was not examined.

To test the hypothesis that iSCI rats would have better overall LUT function than txSCI rats and show improvement (partial recovery) over time due to spared descending tracts, we performed a comprehensive study on LUT function after both complete and incomplete SCI, examining in detail both bladder activity and the pattern of EUS EMG activity measured during urodynamic assessments. Because recent reports have highlighted the potential effects of anesthesia on masking or influencing the EUS bursting pattern, especially after injury (Cheng and de Groat 2004), we evaluated LUT function in unanesthetized but restrained rats.

Materials and Methods

Animals

A total of 53 adult female Sprague-Dawley rats (200–250 g, Harlan, Indianapolis, IN) were used. They were housed 2 to 3 per cage and kept on a 12-hour light-dark cycle, with food and water provided ad libitum. The experimental protocol was approved by the Georgetown University Animal Care and Use Committee.

Spinal cord injury and post-surgical care

Rats were anesthetized with chloral hydrate (360 mg/kg, i.p.) and a laminectomy was made at T8 to expose the dura. A standardized mild, incomplete contusive SCI (iSCI) was produced using the MASCIS injury device (Gruner 1992) with a 10-g weight dropped from a height of 12.5 mm onto the exposed dura (n = 16). To produce a complete transection (txSCI), a dural hook was inserted under the dura and gently elevated while completely severing the spinal cord, and a sterile piece of Gelfoam was placed between the severed ends of spinal cord (n = 18). The overlying muscle and skin over the wound was then sutured. Laminectomy-only rats served as age-matched controls (n = 16).

After surgery, animals were placed on a heating pad until they awoke. Bladders of injured animals were manually expressed twice daily (early morning and evening) by gentle external crede, with the volume of expressed urine recorded, until spontaneous micturition was re-established. This provided an estimation of the amount of time required for re-emergence of spontaneous micturition. Rats were given easy access to food and water and were placed in cages with highly absorbent bedding. Fluid intake was not controlled as it has been shown that this was not a factor in the amount of time required for the development of reflex bladder (Chancellor et al 1994). Oral antibiotics (sulfamethoxazole and trimethoprim oral suspension, 4mg/1mg, Hi-Tech Phamacal Co., Inc, Amityville, NY) were given prophylactically to prevent urinary tract and/or bladder infections. All of the laminectomy controls survived for the specified post-surgery period but two contused rats and three transected rats died prematurely (7–48 days after injury) and were replaced to achieve the stated sample size.

Behavioral assessment of SCI

All animals were tested for hind limb function and open field locomotion 1 day after injury, and weekly thereafter blindly by 2 trained observers. Recovery of overall hind limb function was estimated using the Combined Behavioral Score (CBS; Gale et al 1985). This rates overall sensorimotor function based on a series of tests including open field locomotion, hindlimb withdrawal reflexes in response to extension, pain, and pressure, placing, toe spread, righting, an incline plane test, and swimming. Scores ranged from 0 (normal) to 100 (no normal function in any of the tests).

Spontaneous locomotion was assessed using the Basso, Beattie, and Bresnahan (BBB) open field locomotor test (Basso et al 1995). The BBB scale ranged from 0 (no observable hind limb movement) to 21 (completely normal open field locomotor function).

Urodynamic assessment of LUT function

Two or six weeks (wks) after injury, or laminectomy alone, LUT function was analyzed in awake rats urodynamically. For the insertion of intravesical bladder catheters and EMG electrodes, rats were briefly anesthetized with isoflurane (3% induction; 1.5% maintenance in O₂). The bladder was exposed by a midline abdominal incision. A polyethylene catheter (PE-50) was heated to create a collar and passed through a small incision at the apex of the bladder dome and secured with cotton thread. The catheter was connected to an infusion pump for continuous perfusion with room temperature saline (0.1 ml/min) during cystometry. The signal from the pressure transducer was amplified (BRIDGE Amp, AD Instruments, Colorado Springs, CO), sampled at 1 kHz MacLab/8, AD Instruments, Colorado Springs, CO) and acquired on the computer using Powerlab Chart v4.2 (AD Instruments, Colorado Springs, CO).

To record EUS electromyograms (EMG), two fine, insulated silver wire electrodes (0.005 inch diameter; Sigmund Cohn, Mount Vernon, NY) were placed bilaterally via a percutaneous approach alongside the urethra. Specifically, the hooked wire electrode was positioned at the tip of a 30 gauge needle and was inserted into or alongside the EUS and then withdrawn leaving

the wires embedded in the muscle. The EMG activity was preamplified (Grass P15 A.C. Preamplifier), filtered through a 60 Hz filter, sampled at 1 kHz and acquired online simultaneously with the intravesical pressure.

Rats were then placed into a Ballman cage (Natsume Seisakusho, Tokyo, Japan) and allowed to fully recover from isoflurane anesthesia as indicated by normal reflex (eye-blink, fore limb pinch test) responses. The Ballman cage allows evaluation of LUT function in awake, restrained conditions. In addition, the elevated platform of the Ballman cage allows for easy visualization of voiding. Voided saline was collected and measured as voided volume (VV). Residual saline (RV) was also collected by withdrawal through the intravesical catheter and manual expression of the bladder. Voiding efficiency (VE) was estimated as a percentage: $VE = [VV / (VV + RV)] * 100$.

Cystometric variables analyzed were contraction amplitude and duration, intercontraction interval, voiding pressure and # non-voiding cycles. Values of each parameter were averaged over 10 voiding cycles. Voiding pressure was measured as an increase from baseline pressure. The number of non-voiding cycles (with amplitude > 10 mm Hg) was represented as the number of non-voiding cycles per 10 minutes of recording. The presence or absence of phasic EUS activity was analyzed in the EUS EMG.

Influence of light chloral hydrate anesthesia on LUT function

To compare urodynamically measured LUT function in unanesthetized rats with that after light chloral hydrate anesthesia, as previously used in studies of iSCI (Pikov et al 1998; Pikov and Wrathall 2001; Pikov and Wrathall 2002), urodynamic assessments were performed on several additional uninjured rats (n = 3). The animals were first examined under awake, restrained conditions as described above. After baseline bladder and EUS traces were obtained, chloral hydrate was administered at 180 mg/kg, i.p. (50% of the full anesthetic dose). LUT function was assessed again at 15 minutes after administration, and a third time, at 2.5 hours after administration. Reflex tests (forelimb pinch reflex, eye-blink reflex) were administered to determine the plane of anesthesia at the different time-points. In addition, any spontaneous movements of the animals were noted.

Spinal cord histopathology

At the end of experiments, rats were anesthetized with 8% chloral hydrate and perfused first with 0.9% saline followed by 4% paraformaldehyde. After perfusion the bladder was removed, emptied, blotted dry and its weight recorded.

The spinal cord was removed and post-fixed in 4% paraformaldehyde for 1 hour then cryoprotected in a sucrose gradient before being frozen. In order to histologically quantify the extent of incomplete injury in those animals that underwent iSCI and to confirm a complete transection in txSCI animals, a 1.5 cm segment of spinal cord centered at the epicenter of the injury was sectioned at 14 μ m and thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were then stained with Eriochrome-cyanine RC to stain myelinated white matter (blue) and counterstained with hematoxylin (cell nuclei) and eosin/phloxine (cytoplasm) to visualize cells and gray matter neuropil. The injury epicenter was defined as the section with the least amount of spared white matter. Digitized images were taken of the stained sections using a Zeiss Axiophot microscopy camera. The pictures were analyzed objectively for white matter using Zeiss Axiovision software to calculate the area of eriochrome-stained white matter.

Immunohistochemistry

To confirm that txSCI completely disrupted supraspinal connections and that iSCI rats had remaining but decreased innervation by descending pathways, we performed immunohistochemistry for serotonin (5HT), as a marker for descending pathways. We examined innervation of the dorsolateral nucleus (DL) at lumbar level 6 (L6) as this nucleus innervates the EUS. The lumbar enlargement of the spinal cord was sectioned at 14 μ m and thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). L6 and the location of the DL was determined using cresyl violet-stained slides. Immunohistochemistry was then performed on adjacent sections to study the innervation by 5HT. Tissue from all groups of rats were performed simultaneously for unbiased comparison of immunohistochemical staining.

Sections were fixed with 10% buffered formalin and washed with high salt buffer (HSB). Blocking was performed using 5% BSA in HSB. After washing, sections were incubated overnight at room temperature with primary antibody to 5HT [Immunostar, Hudson, WI]: 1:20,000. They were then washed and incubated with secondary antibody (Alexafluor 488; 1:1,000; Invitrogen, Carlsbad, CA) for 90 minutes. Sections were washed and coverslipped with Vectashield containing the nuclear stain DAPI (Vector, Burlingame, CA).

Sections labeled with anti-5HT were viewed and photographed using a Zeiss Axiocam camera. For each animal, pictures were taken of the left and right DL at 40x magnification from 3 sections of each slide. Immunoreactivity was analyzed using Scion Image software to measure fluorescence against a black background within the field of view. Fluorescent images were converted to gray scale for the purposes of densitometry. Quantification of immunoreactivity was given as the number of 5HT-positive pixels. The mean number of pixels over the 6 sections from each animal was calculated.

Statistical Analyses

Data reported are means $\pm$ standard error for the values of all animals in the group. One-way ANOVA with Tukey's post hoc comparison test was used for all comparisons except for the analyses for hind limb behavior and expressed urine volume. For these parameters, comparisons were made using two-way ANOVA with repeated measures. Statistical evaluation of the data was performed using the statistical program Graphpad Prism. $P < 0.05$ was selected for statistical significance.

Results

Hind limb Function

Behavior was evaluated at 1 day after spinal cord injury, and weekly thereafter, using two standard behavioral tests, the BBB (Basso et al 1995) and the CBS (Gale et al 1985). Within each injury group, there were no statistical differences in hind limb function between the 2 week rats and the 6 week rats, thus data for rats from the two time-points were grouped together. Both iSCI and txSCI rats showed almost complete paralysis of the hind limbs (Fig. 1A) as well as a large number of sensorimotor functional deficits (Fig. 1B) at 1 day after SCI. By 1 week after injury, there was a significant difference in the hind limb motor function between the two injury groups (Figs. 1A, 1B). By 6 weeks after SCI, iSCI rats had attained weight-bearing locomotion with some coordination between the fore- and hind-limbs, which requires supraspinal control, whereas the txSCI were not capable of weight-bearing steps.

Lesion site histopathology

Tissue sections from the injury epicenter were used to evaluate the amount of white matter sparing in iSCI rats and to confirm complete transections in txSCI rats (Fig. 2). The lesion

epicenter was defined as the section of the spinal cord that had the least amount of spared white matter. Corresponding sections in uninjured controls were also examined for white matter area.

In the uninjured animal (Fig. 2A), gray matter (pink) was surrounded by a wide rim of white matter (blue). In the iSCI rats (Fig. 2B, C), the rim of white matter was considerably smaller and normal gray matter was replaced by a central lesion zone. By 2 weeks after iSCI (Fig. 2B), the diameter at the epicenter was reduced and there was typically only a narrow peripheral rim of spared white matter mostly in the ventral and ventrolateral areas. The same was seen at 6 weeks after injury (Fig. 2C). On the other hand, there was no white matter at the injury epicenter in any of the txSCI rats (Fig. 2E, F). The pink stain was reflective of the gel foam that was placed into the gap made by the transection. The area of spared white matter was quantified in the iSCI rats and compared to that at T8 for uninjured laminectomy control tissue embedded in the same block. By 2 weeks after SCI, there was more than a 30% loss of white matter at the injury epicenter in the iSCI rats; no significant additional loss was seen at 6 weeks after injury (Fig. 2D).

Emergence of reflex bladder

Immediately following SCI, rats from both injury groups were unable to spontaneously void, instead requiring manual expression of their bladders (crede) twice daily until reflex bladder contractions re-emerged. By measuring and recording the amount of urine that was manually expressed, the time required for spontaneous micturition to be re-established can be estimated (Pikov and Wrathall 2001). There were no significant differences between 2 week and 6 week rats within each injury group, thus 2 and 6 week rats were grouped together. All injured rats exhibited an increase in the volume over the first few days after SCI, reflecting an increase of bladder size. The volume decreased as spontaneous voiding began (Fig. 3A). When comparing transected rats with contused rats, significantly more urine was expressed from the former than the latter ($p=0.0009$). In addition, txSCI rats required a longer time to develop a reflex bladder so that crede could be terminated (Fig. 3A; $p<0.0001$).

Bladder weight

At the time of sacrifice, bladders were removed, blotted dry, and weighed. There was no significant difference between the 2 week iSCI rats and the 6 week iSCI rats, indicating that the alterations that occur in the bladder had stabilized by 2 weeks after iSCI, and thus the rats from the 2 time-points were grouped together. While iSCI rats appeared to have heavier bladders than uninjured animals, the difference was not statistically significant (Fig. 3C). However, the txSCI rats had significantly heavier bladders than both uninjured and iSCI rats (50.8% and 81.0% heavier, respectively; Fig. 3C).

Urodynamic Assessment of LUT function in unanesthetized, restrained rats

Normal, uninjured rats exhibited regular voiding bladder contractions (intercontraction interval [ICI] = 100.7 ± 8.3 secs) that had large amplitudes (contraction amplitude = 31.4 ± 2.9 mmHg) and were coincident with increased EUS activity (Fig. 4A). Within a voiding cycle, when the bladder pressure reached the micturition threshold, the pressure increased rapidly, and at the same time, the EUS activity began to exhibit a bursting pattern, with long and distinct silent periods (EUS relaxation) interspersed between active spikes of EMG activity (Fig. 4B; voiding cycle marked by * in Fig. 4A). At the termination of voiding, phasic EUS activity stopped and a tonic level of activity resumed. During voiding cycles in an uninjured animal, fluid was emitted as a stream with a high voiding efficiency (Table 1). However, a caveat of recording from awake animals is that although they are tightly restrained, they are still able to move enough to exhibit movement artifacts in both bladder and EUS traces. To preserve the integrity of the traces, the movement artifacts were not removed electronically.

As hypothesized, some aspects of LUT function in iSCI rats were more normal than in txSCI rats, such as contraction duration and voiding pressure (Table 1). However, while voiding efficiency had a tendency to be higher in iSCI rats, it was not significantly different from that in txSCI rats (Table 1). Surprisingly, instead of having EUS activity patterns that were different between complete transection (txSCI) and incomplete contusion (iSCI) injury groups, we found that there were two distinct patterns of EUS activity within *both* transection and contusion injury groups that led to distinct functional outcomes at both time-points: Phasic activity or only Tonic activity.

Phasic rats—This subset of rats resembled normal, uninjured rats in that they exhibited phasic EUS activity with periods of EUS relaxation (silent periods) during voiding bladder contractions that were interspersed with periodic active spikes, which led to stream-like voiding and high voiding efficiency that was not significantly different from uninjured rats. They displayed several non-voiding contractions before each genuine voiding contraction, and each increase in bladder pressure was coincident with an increase in EUS activity (Fig. 4C, G). Within the voiding period, bursting EUS activity was observed in both txSCI-Phasic (Fig. 4H) and iSCI-Phasic rats (Fig. 4D). However, the interburst “silent” interval between phasic EUS bursts was significantly shorter in the injured animals than in the uninjured (Table 2). Voiding efficiency in Phasic rats was *not* significantly different from that in uninjured animals but was higher than Tonic rats in both iSCI and txSCI groups (Table 2).

Tonic rats—This subset of rats did *not* exhibit any EUS relaxation and only had tonic EUS activity during voiding contractions, which led to slow drop-by-drop leaking by overflow incontinence and low voiding efficiency that was significantly lower than normal, uninjured rats. The two subsets of rats were observed in both txSCI and iSCI groups at both subacute and chronic time-points. During the urodynamic assessments, Tonic rats, both iSCI (Fig. 4E) and txSCI (Fig. 4I), had frequent bladder contractions (bladder hyperactivity). Although most contractions were “voiding” contractions, only a few drops were expelled in each cycle, resulting in significantly lower voiding efficiencies (Table 2). The intercontraction intervals in Tonic rats showed a tendency towards being shorter than those in uninjured rats (Table 2). Voiding pressure was higher in the Tonic rats than in the uninjured animals. Further, only tonic EMG activity was seen in the EUS, even during voiding (Fig. 4F, J), demonstrating bladder-EUS dyssynergia. Thus “voiding” in the case of these animals may be viewed as leaking of urine when intravesical pressure exceeded dyssynergic urethral pressure rather than a coordinated voiding event.

In addition, while iSCI rats as a group tended to develop a reflex bladder more rapidly than txSCI rats, in post-hoc tests where injured rats were grouped by the presence (Phasic) or absence (Tonic) of EUS relaxation during voiding in the urodynamics assessment, we found that Tonic rats retained a significantly larger volume of urine prior to developing spontaneous micturition and took a significantly longer time to develop reflex bladder (Fig. 3B; $p < 0.0001$).

To determine if the rostral-caudal extent of the tapering lesion was different between Phasic and Tonic rats, the area of the lesion was measured at 4 mm rostral and 4 mm caudal to the injury epicenter. There were no significant differences between Tonic and Phasic rats in terms of the lesion area (rostral: $0.46 \pm 0.03 \text{ mm}^2$ versus $0.35 \pm 0.05 \text{ mm}^2$; caudal: $0.52 \pm 0.07 \text{ mm}^2$ versus $0.41 \pm 0.06 \text{ mm}^2$), total cross-sectional area of the cord (rostral: $3.63 \pm 0.11 \text{ mm}^2$ versus $3.30 \pm 0.12 \text{ mm}^2$; caudal: $3.96 \pm 0.35 \text{ mm}^2$ versus $3.71 \pm 0.11 \text{ mm}^2$) or the percent of the whole cord occupied by the lesion area (rostral: $12.66\% \pm 1.25$ versus $10.51\% \pm 1.18$; caudal: $13.38\% \pm 2.35$ versus $11.03\% \pm 1.44$) at either 4 mm rostral or 4 mm caudal to the injury epicenter.

Effects of light chloral hydrate on bladder and EUS activity

Uninjured, unanesthetized rats ($n = 3$) that exhibited regular voiding contractions which were coincident with phasic EUS bursting activity (Fig. 5A, B) and a high voiding efficiency ($89.13 \pm 8.36\%$), showed reduced voiding efficiency after light chloral hydrate anesthesia. Fifteen minutes after chloral hydrate administration, voiding efficiency had dropped to $28.67 \pm 8.67\%$ and phasic EUS activity had disappeared with only tonic EUS activity remaining during voiding contractions (Fig. 5C, D). Even 2.5 hours after chloral hydrate administration, when both the forelimb pinch reflex and the eye-blink reflex were normal and spontaneous movement re-appeared, phasic EUS activity could still be masked (Fig. 5E, F) and voiding efficiency was still significantly lower than normal ($44.79 \pm 12.98\%$).

Reduced 5HT innervation of the dorsolateral nucleus

In the normal, uninjured rat, there is abundant innervation by descending serotonergic pathways to the lumbosacral cord, especially at the dorsolateral nucleus (DL), which innervates the EUS (Fig. 6A). After transection, 5HT immunoreactivity was completely absent from the lumbosacral spinal cord (Fig. 6b4), thus confirming a complete transection of supraspinal connections.

Two weeks after iSCI, there was a significant decrease in 5HT immunoreactivity at the DL (Fig. 6b2,3), as reflected by the quantification of immunoreactive pixels (Fig. 6C, D). 5HT immunoreactivity between iSCI-Phasic (Fig. 6b2) and iSCI-Tonic (Fig. 6b3) rats was compared to determine if the amount of serotonergic innervation at the DL could account for the difference in EUS activity patterns. We found that at 2 weeks after iSCI, while both Tonic and Phasic rats had less 5HT immunoreactivity than uninjured controls, they were not significantly different from each other (Fig. 6C).

Discussion

We compared the effects of incomplete contusion (iSCI) and complete transection injury (txSCI), on lower urinary tract (LUT) function in unanesthetized rats. Several aspects of LUT function were less impaired and/or recovered more rapidly in iSCI rats compared to txSCI rats. After iSCI, reflex bladder developed more rapidly, some urodynamic parameters were closer to normal than after txSCI (contraction duration and voiding pressure) and bladder weights were not greater than those of uninjured rats. In contrast, txSCI rats exhibited slower reflex bladder development, higher voiding pressures, longer contraction durations and heavier bladders than uninjured controls. However, some rats in both injury groups had EUS activity patterns that were surprisingly normal, in terms of phasic activation during bladder contractions and high voiding efficiencies.

Phasic EUS activity is thought to be necessary for efficient voiding in rats. It consists of silent periods, when the urethra is relaxed, permitting urine to be expelled, and active bursting periods, when the urethra is closed (Mersdorf et al 1993; Kakizaki et al 1997; Cheng and de Groat 2004). Suppression of phasic EUS activity by the neuromuscular junction blocker alpha-bungarotoxin produces tonic EUS activity during voiding (similar to what we observed in Tonic rats) and dramatically decreases voiding efficiency in uninjured rats (Yoshiyama et al 2000). Previous data showing the elimination of EUS bursting activity after a complete transection (Kruse et al 1993; Kruse and de Groat 1993; Pikov et al 1998) was recently challenged by a study showing that phasic EUS activity can be observed in chronically transected rats, if the urodynamics procedure is performed with no or extremely light anesthesia (Cheng and de Groat 2004). Such results appeared to contradict earlier data suggesting that phasic EUS bursting has to be mediated supraspinally (Mersdorf et al 1993; Kakizaki et al 1997).

The few studies that have examined urodynamics after iSCI have used rats under light chloral hydrate anesthesia (Pikov et al 1998; Pikov and Wrathall 2001; Pikov et al 2002), possibly adversely affecting the results. Indeed, as part of the current study, we found that even very light chloral hydrate anesthesia can inhibit phasic EUS activity as well as reduce voiding efficiency. Further, in the previous studies of iSCI, voiding efficiency was not calculated nor was the detailed pattern of EUS activity examined. Instead, increased EUS activity (higher EMG) at the time of bladder contractions was taken as a measure of bladder-EUS coordination. Such an increase was invariably observed in normal rats and also seen with time after iSCI, suggesting the recovery of LUT function in chronic iSCI rats (Pikov et al 1998; Pikov and Wrathall 2001; Pikov and Wrathall 2002; Emch and Wrathall 2006). In our recordings of unanesthetized rats, we showed for the first time that two weeks after injury, 5 out of 8 iSCI rats exhibited phasic EUS bursting that was associated with high voiding efficiency. However, the number of animals that had phasic EUS activity and near-normal voiding efficiency did not subsequently increase with time after iSCI, thus showing no evidence of further recovery beyond two weeks.

We also confirmed the recent report (Cheng and de Groat 2004) that txSCI rats were capable of producing phasic EUS bursting during voiding. In that study, phasic EUS activity re-appeared in txSCI rats, but only after 48 hours after urethane administration. We extended the results to show that txSCI rats only transiently anesthetized with isoflurane (15 mins) were able to exhibit phasic EUS bursting activity by 20 minutes later, as soon as voiding contractions could be detected. Further, we showed in the first direct comparison, that a subset of txSCI rats could perform as well as a subset of iSCI rats. This was surprising for two main reasons: (1) we hypothesized that iSCI rats would exhibit better LUT function due to spared supraspinal connections and (2) complete transections were considered to produce uniform SCI and were not expected to result in such a divergence in functional recovery.

Since 5HT in the lumbosacral cord was not significantly different between iSCI-Phasic rats and iSCI-Tonic rats, and since even txSCI rats, with no supraspinal connections, were able to produce phasic EUS activity associated with higher voiding efficiencies, mechanisms that can mediate bursting after SCI must be located intrinsically within the spinal cord below the injury site. Spinal pattern generators implicated in locomotion (Stelzner and Cullen 1991; Cazalets et al 1992; Beato et al 1997) and the ejaculation reflex (Truitt and Coolen 2002; Coolen et al 2004) have been identified within the upper lumbar region. Similar pattern generators may be involved in mediating phasic EUS activity. In a recent study (Chang et al 2006), normal bursting was eliminated in rats chronically after transections at L3–4 and L6–S1, but could sometimes be detected in rats after T8–9 transections. They concluded that bursting is dependent on supraspinal control in intact rats and eliminated after a complete lumbar transection, but the circuitry between T8–9 and L4 is preserved after a thoracic transection so that phasic activity can re-emerge chronically. It is possible that there are different EUS activity patterns in Phasic and Tonic rats because the spinal pattern generator for phasic EUS activity was damaged or disturbed differently in the two functional groups. While our results showed that there were no significant differences in overtly lesioned area between Phasic and Tonic rats in areas distal from the injury site, suggesting that length of lesion was not involved, there may be more subtle differences not detected in this analysis that could influence the pattern of EUS activity chronically after injury.

Our results showing that the return of phasic EUS activity during voiding can occur independent of the preservation of supraspinal connections is different from the recovery of locomotor, anorectal and erectile function after iSCI (Sarunashi et al 1996; Holmes et al 2005), where recovery was dependent upon the presence and plasticity of supraspinal connections. Both studies showed marked deficits in function accompanied by significantly decreased 5HT immunoreactivity in the lumbosacral spinal cord 1 week after injury. By 4–6

weeks after injury, 5HT immunoreactivity at the lumbosacral cord had returned to normal levels along with significant functional recovery. In contrast, our results showed that phasic EUS activity can re-emerge after injury regardless of the amount of spared supraspinal connections, even in the absence of supraspinal innervation, pointing to the involvement of spinal mechanisms for the recovery of this specific aspect of LUT function.

Results from our urodynamic assessment of unanaesthetized restrained female rats should be considered in light of a recent study which examined micturition in male rats after iSCI using different methods (Nout et al 2005): telemetrically monitoring pressure waves in the bulb of the corpus spongiosum of the penis (CSP) and urine output in the home-cages. Both studies performed similarly severe iSCI with the same injury device and observed similar hind limb behavioral deficits and histopathology as well as a similar time-point for the reappearance of spontaneous micturition, 4–5 days after iSCI. In addition, both we and Nout *et al* (2005) observed recovery of effective micturition by 2 weeks after iSCI. Further, in 3 of their rats in which the details of the pressure waveforms in the CSP during urination were analyzed, pulsatile urine flow was correlated with multiple pressure waves (frequency = 9 Hz). These were similar to those of normal rats and to what would be expected from the phasic pattern of EUS activation that we observed in 5 of 8 iSCI rats at 2 weeks after injury, except that phasic EUS activity occurs at a lower frequency (4–6Hz). If such pressure waveforms were found consistently in iSCI males, it would suggest gender differences in recovery of micturition.

One could speculate that varying hormonal levels during the estrus cycle could influence the re-emergence of phasic EUS activity after SCI in females, as has been reported for female rats in a bladder inflammation model, with rats in proestrus and estrus exhibiting more severe bladder hyperreflexia than rats in metestrus and diestrus (Johnson and Berkley 2002; Dmitrieva and Berkley 2005). In addition, both estrogen and progesterone have been shown to be neuroprotective after spinal cord trauma. Male rats treated with exogenous progesterone after spinal cord contusion exhibited better locomotor outcomes and more sparing of white matter at the injury epicenter (Thomas et al 1999) whereas acutely injured male rats treated with estrogen reduced inflammation and myelin loss (Sribnick et al 2005). We can speculate that the stage of estrus at the time of SCI could affect the response of the LUT spinal pattern generator, explaining how some rats in both iSCI and txSCI groups recover phasic EUS activity.

In conclusion, it appears that local circuitry in the distal spinal cord, probably involving spinal pattern generators, can play a key role in the recovery of LUT function after SCI. Since this appears to be true in both incomplete and complete spinal cord injury, development of pharmacological interventions that can re-introduce appropriate periods of EUS relaxation (e.g. Dolber et al 2007) after SCI will likely benefit a wide range of SCI patients.

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Figure 1.

Hind limb function after iSCI and txSCI.

Rats with incomplete contusion injury (iSCI) and those with complete transection injury (txSCI) were significantly different in their recovery of hind limb function. However, within each injury group, there were no differences in hind limb function between the 2 week rats and the 6 week rats, thus rats from the two time-points were grouped together. Rats were evaluated 1 day after injury, then weekly thereafter with (A) the BBB scale for open-field hindlimb locomotor function (Basso et al 1995) and (B) the CBS score for overall sensorimotor function (Gale et al 1985). At 1 day post-operation (DPO1), rats from both groups demonstrated almost complete paralysis and areflexia of the hindlimbs and were indistinguishable from each other.

However, starting from DPO7, iSCI rats consistently had better hindlimb function than txSCI rats. Behavioral scores reached a plateau by DPO21, with no further improvement after that time in either group. There were no differences between the 2wk and 6wk animals within either injury type. N numbers are shown in Table 1. Data shown as mean $\pm$ SEM; where no error bars are shown, the SEM was smaller than the symbols. *** $p < 0.001$ versus iSCI rats.

Figure 2.

Histopathology after SCI and quantification of spared white matter area.

The amount of spared white matter at the lesion epicenter was significantly reduced after iSCI and completely eliminated after txSCI. Sections from the injury epicenter and corresponding sections from uninjured (laminectomy control) rats were stained with hematoxylin (pink), eriochrome (blue) and eosin to distinguish between white and grey matter (A–C; E, F). The area of white matter at the injury epicenter was measured (D); txSCI rats had no white matter at the injury epicenter with the pink stain reflecting the gel foam that was inserted into the gap made by the transection. iSCI rats had a significantly reduced amount of white matter area both subacutely and chronically but there was no significant difference between the 2 time-points. Data shown as mean $\pm$ SEM. *** $p < 0.001$ versus uninjured. Scale bar = 0.5mm.

Figure 3.

Expressed urine volumes and bladder weights after SCI.

Initially after injury, rats lost the ability to spontaneously void, and the bladder was manually expressed twice daily. The expressed volume was recorded to determine the amount of urine retained as well as the time required for reflex bladder to completely develop. (A) Significantly more urine was retained by txSCI rats and they took a longer time to fully develop reflex bladder than did iSCI rats ($p < 0.0001$ by 2-way ANOVA with repeated measures). (B) In addition, regardless of injury type, Phasic rats retained significantly less urine and required less time to develop a reflex bladder than Tonic rats ($p < 0.0001$ by 2-way ANOVA with repeated measures). At the time of sacrifice, bladders were dissected and weighed. While iSCI rats showed a trend towards having heavier bladders, they were not significantly different from uninjured rats (C). In contrast, txSCI rats had significantly heavier bladders than both uninjured and iSCI rats.

*** $p < 0.001$ versus uninjured; ### $p < 0.001$ versus iSCI rats.

Figure 4.

Patterns of bladder and EUS activity during urodynamics.

Two distinct patterns of EUS activity emerged after *both* txSCI and iSCI: 1) Phasic EUS activity (Phasic) during voiding contractions with clear silent periods interspersed with active spikes. 2) Tonic EUS activity (Tonic) during bladder contractions with no silent periods. Both Phasic and Tonic rats were present after iSCI and txSCI, at 2 and 6 weeks after injury (see Table 2 for numbers of rats in each group). In the uninjured rats, regular bladder voiding contractions were observed: Voiding occurs in a stream-like manner in each of the voiding contractions with a concomitant increase in EUS activity (A). In the enlarged 5 second trace (B), a clear bursting pattern with silent and active periods could be seen in the uninjured rats. Similar patterns in EUS activity were observed in Phasic rats after both iSCI (iSCI-Phasic; D) and txSCI (txSCI-Phasic; H): EUS activity at the time of voiding bladder contractions was phasic with active spikes interspersed with silent periods. In contrast, Tonic rats, both iSCI-Tonic (F) and txSCI-Tonic (J) exhibited only tonic EUS activity during bladder contractions. However, Phasic iSCI and txSCI rats (C, G) exhibited non-voiding contractions which were absent in normal, uninjured rats before voiding contractions. The Tonic rats had extremely frequent

voiding contractions, indicating bladder hyperreflexia (E, I). Scale bar for 10 minute trace = 100 seconds. Scale bar for 10 second trace = 1 second. * marks voiding contractions that are enlarged and shown in the 5 second trace. ☆ marks an example of a movement artifact that led to a spike in both the bladder and EUS traces. Arrows indicate silent periods.

Figure 5.

Bladder and EUS activity can be inhibited with light chloral hydrate anesthesia.

Bladder and EUS activity can be affected by chloral hydrate administration. LUT function was first assessed in unanesthetized but restrained, uninjured rats (A, B). Chloral hydrate was then administered (180 mg/kg, i.p.; 50% of full anesthetic dose). LUT function was assessed again 10 minutes after chloral hydrate (C, D), then again 2.5 hours (E, F) after administration. Phasic EUS activity was clearly seen in the unanesthetized rat during voiding bladder contractions (B). Voiding pressure was increased (C, E) and only tonic EUS activity was observed (D, F) after chloral hydrate administration. Even 2.5 hours after chloral hydrate administration, when both the forelimb pinch reflex and the eye-blink reflex were normal and spontaneous movement re-appeared, phasic EUS activity was still absent. Scale bar for 5 minute trace = 1 minute. Scale bar for 5 second trace = 1 second. * marks voiding contractions that are enlarged and shown in the 5 second trace. ☆ marks an example of a movement artifact that led to a spike in both the bladder and EUS traces.

Figure 6.

Effects of iSCI and txSCI on supraspinal innervation of the dorsolateral nucleus (DL) by serotonin (5HT).

Immunohistochemistry was performed using serotonin (5HT) as a marker for descending pathways at the DL nucleus two weeks after SCI or laminectomy. There was abundant 5HT immunoreactivity in the normal, uninjured rat (b1). 5HT immunoreactivity was completely absent in the transected rats (b2). Quantitative analyses of immunoreactive pixels showed reduced immunoreactivity for 5HT at the DL after iSCI (b3, b4; C). Furthermore, within the iSCI rats, there was no significant difference in 5HT immunoreactivity at the DL between the rats that exhibited phasic EUS bursting (iSCI-Phasic; b2; n = 5) and those that exhibited only tonic EUS bursting (iSCI-Tonic; b3; n = 3) during voiding contractions. Data shown as mean $\pm$ SEM. *** $p < 0.001$ versus uninjured. Scale bar for immunohistochemistry = 50 μ m.

Table 1
Bladder and EUS activity during urodynamics – Comparison among uninjured, iSCI and txSCI rats.

	Contraction amplitude (mmHg) ¹	Contraction Duration (secs) ¹	Intercontraction interval (secs) ¹	Voiding pressure (mmHg) ¹	# Non-voiding cycles ²	% Voiding efficiency ³
Uninjured (n = 16)	31.4 ± 2.9	16.0 ± 1.2	100.7 ± 8.3	32.9 ± 2.5	0	86.4 ± 2.5
iSCI (2 wks) (n = 8)	35.3 ± 1.4	24.9 ± 2.5 ^{##}	211.3 ± 86.1	37.9 ± 1.7	11.5 ± 2.3 ^{***}	56.7 ± 9.1 ^{**}
iSCI (6 wks) (n = 8)	35.4 ± 3.8	19.0 ± 1.6 ^{##}	76.9 ± 28.9	41.1 ± 1.8 [*]	11.13 ± 2.5 ^{***}	56.6 ± 9.6 ^{**}
txSCI (2 wks) (n = 8)	40.4 ± 2.7	34.0 ± 3.2 ^{***}	101.3 ± 13.1	47.8 ± 2.2 [*]	5.9 ± 1.3 ^{***}	32.9 ± 8.0 ^{**}
txSCI (6 wks) (n = 10)	30.9 ± 2.0	38.9 ± 3.2 ^{***}	221.8 ± 67.3	44.1 ± 2.9 [*]	6.9 ± 1.0 ^{***}	31.4 ± 8.7 ^{**}

Uninjured (laminectomy control) rats were examined at both 2 weeks and 6 weeks after laminectomy. Results were found to be statistically similar between the two groups and thus were combined. Data for all groups shown as mean±SEM.

* p<0.05,

** p<0.01,

p<0.001 versus Uninjured.

^{##} p<0.01 versus txSCI (6 wks).

¹ The mean of 10 cycles was calculated for each rat, with the final result given as the average of all animals in an experimental group.

² The number of non-voiding cycles within a 10 minute period.

³ Voiding efficiency was determined using the voided volume and residual volume from the last voiding cycle).

Table 2
Patterns of bladder and EUS activity during urodynamics in Phasic and Tonic rats after iSCI and txSCI.

	% Silent period / Bursting Period	Contraction amplitude (mmHg)	Contraction duration (secs)	Intercontraction interval (secs)	Voiding pressure (mmHg)	% Voiding efficiency
Uninjured (n = 16)						
Phasic (16 / 16)	70.2 ± 1.9	31.4 ± 2.9	16.0 ± 1.2	100.7 ± 8.3	32.9 ± 2.5	86.4 ± 2.5
iSCI (2 wks) (n = 8)						
Phasic (5 / 8)	45.0 ± 11.6 [*]	36.7 ± 1.7	25.5 ± 3.4	306.1 ± 121.4	36.4 ± 2.0	71.5 ± 6.8
Tonic (3 / 8)	---	31.8 ± 1.2	23.6 ± 2.6	53.4 ± 11.5	41.2 ± 3.1 [*]	32.0 ± 11.9 ^{***}
iSCI (6 wks) (n = 8)						
Phasic (1 / 8)	76.5	59.8	17.0	276.2	36.7	90.9
Tonic (7 / 8)	---	32.0 ± 1.8	19.3 ± 1.8	48.4 ± 5.8	41.7 ± 1.9 [*]	51.6 ± 9.5 ^{***}
txSCI (2 wks) (n = 8)						
Phasic (2 / 8)	62.2; 33.5	38.6; 40.9	22.9; 25.5	99.7; 163.7	35.1; 47.8	33.3; 71.6
Tonic (6 / 8)	---	41.0 ± 4.2	39.2 ± 2.9 ^{***}	91.2 ± 13.0	49.6 ± 2.5 [*]	26.4 ± 7.9 ^{***}
txSCI (6 wks) (n = 10)						

	% Silent period / Bursting Period	Contraction amplitude (mmHg)	Contraction duration (secs)	Intercontraction interval (secs)	Voiding pressure (mmHg)	% Voiding efficiency
Phasic (4 / 10)	44.8 ± 5.2 *	34.6 ± 2.2	33.3 ± 3.9 ***	398.9 ± 122.0	36.0 ± 3.1	60.0 ± 9.2
Tonic (6 / 10)	----	28.5 ± 2.6	42.7 ± 4.3 ***	103.7 ± 25.5	49.6 ± 2.5 *	12.3 ± 3.7 ***

Uninjured (laminectomy control) rats were examined at both 2 weeks and 6 weeks after laminectomy. Results were found to be statistically similar between the two laminectomy control groups and thus were combined. Data for all groups shown as mean ± SEM, except when n < 3, in which case individual values are reported. Ratios following Phasic/Tonic subgroups represent the number of rats within each injury group that resulted in exhibiting phasic or tonic EUS EMG activity during voiding contractions. Only groups with n > 3 were used for statistical comparisons.

* p<0.05,

*** p<0.001 versus Uninjured.