Altered Glutamate Receptor Function during Recovery of Bladder Detrusor-External Urethral Sphincter Coordination in a Rat Model of Spinal Cord Injury

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ABSTRACT
Coordination of the bladder detrusor and the external urethral sphincter is a supraspinally controlled reflex that is essential for efficient micturition. This coordination is permanently lost after spinal cord transection but can recover chronically after incomplete spinal cord injury (SCI). As glutamatergic transmission plays a key role in all levels of detrusor-external urethral sphincter coordination, we examined the role of potential alterations in glutamatergic control in its recovery after SCI. Rats were subjected to standardized incomplete contusion injury. Detrusor-external urethral sphincter coordination was studied during early (subacute) and chronic time points. Sensitivity of coordinated activation of the external urethral sphincter in response to bladder distension to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (kainate antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium (NBQX)) and the N-methyl-D-aspartate (NMDA) antagonist R(−)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP) was determined by intrathecal administration at the L6 spinal cord level during urodynamic recordings. We found that while detrusor contractions recovered at 5 days after SCI, coordinated activation of the external urethral sphincter was significantly impaired at 5 days and recovered only by 8 weeks. There was no difference in sensitivity of detrusor-external urethral sphincter coordination to NBQX at the subacute or chronic time points. However, external urethral sphincter response to bladder distension was sensitive to a 50% lower dose of CPP at 5 days compared with uninjured rats or chronic recovered SCI rats. Thus, alterations in NMDA receptor function appeared to be involved in recovery of detrusor-external urethral sphincter coordination after incomplete SCI.

Somatic and visceral functions are chronically impaired following SCI, but some potential for recovery exists, especially for the large number of patients with incomplete injuries (Bracken et al., 1990). Development of pharmacological strategies for treatment of SCI is impaired by lack of knowledge of basic mechanisms underlying recovery of functions that can occur after SCI. Recovery of somatic reflexes, and limb function in particular, is difficult to study because of complex integration of segmental, intersegmental, and supraspinal influences controlling these functions (Dietz et al., 1999). Visceral functions may be controlled by relatively simpler reflexive circuits that are well described (Gabella, 1995). We have recently focused on one of the visceral functions, lower urinary tract function, after experimental SCI (Pikov et al., 1998; Pikov and Wrathall, 2001). This has considerable clinical importance as lower urinary tract dysfunction significantly impairs well being of the SCI patient (Selzman and Hampel, 1993). The control pathways involved have been extensively studied in normal and spinal cord-transected animals (Tiseo and Yaksh, 1990; Morrison, 1997; de Groat et al., 1998). Urine storage and voiding are the main lower urinary tract functions and require the coordinated activity of the bladder detrusor and the EUS (Mersdorf et al., 1993). Detrusor areflexia ensues acutely after trauma, but with time, contractions reappear even after complete transection (Tiseo and Yaksh, 1990), indicating that spinal circuits alone are capable of automatic bladder control (de Groat et al., 1995). Detrusor-EUS coordination is mediated via a spinobulbo-spinal reflex (Holstege et al., 1986; de Groat, 1990) that is abolished after a complete transection (Kruse et al., 1993; Pikov et al., 1998). After incomplete SCI, a partial...
return of the reflex can be seen by 2 weeks postinjury (Pikov et al., 1998). The extent of chronic recovery of detrusor-EUS coordination depends on the amount of preserved supraspinal connections to lumbosacral spinal cord areas involved in control of the detrusor and EUS (Pikov and Wrathall, 2001).

Glutamate receptors are utilized in spinal circuits controlling the detrusor and EUS (Matsumoto et al., 1995a, b; Iwabuchi, 1997). Chronically, the pattern of glutamate receptor subunit mRNA expression in EUS-projecting motoneurons of SCI animals with recovered detrusor-EUS coordination is normal, whereas in more severely injured animals showing minimal recovery there is an elevated mRNA level of specific glutamate receptor subunits (Pikov and Wrathall, 2001). This suggested that glutamate receptor function might be altered after SCI and during the recovery of lower urinary tract function after injury.

In the present study, the coordinated EUS response to detrusor contractions was evaluated at 5 days postinjury, a time when adequate reflexive stimulation was provided by the reemerging detrusor contractions but the EUS response was impaired. It was also studied at 8 weeks, a chronic time point when recovery has occurred. The role of possible alterations in glutamatergic control of detrusor-EUS coordination after SCI was assessed using specific NMDA and AMPA/kainate receptor antagonists.

Materials and Methods

Experimental Groups. Adult female Sprague-Dawley rats (Zivic-Miller Laboratories, Zelienople, PA) weighing 270 to 350 g were anesthetized with chloral hydrate (360 mg/kg i.p.). Laminctomy was performed at T8, and SCI was produced with the Multi-center Animal Spinal Cord Injury Study injury device (Gruner, 1992), using the 10 g weight dropped from a height of 12.5 mm onto exposed dura (n = 37). SCI animals were randomly assigned for subacute (n = 19) or chronic (n = 18) survival. A group of control animals had laminctomies but were left uninjured (n = 15). Additional groups of subacutely injured animals (n = 7) and uninjured controls (n = 3) were generated for studies of histopathology of the injury site and immunohistochemistry of the lumbosacral micturition centers (see methods below).

Animal Care. Rats were housed in the Department of Comparative Medicine Animal Facility in a room with controlled humidity, temperature, a 12-h light/dark cycle, and had free access to food and water. This facility is supervised by a licensed veterinarian, meets all National Institutes of Health guidelines for the care of laboratory rodents, and is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The Georgetown University Institutional Animal Care and Use Committee approved all procedures used in this study. Postoperatively, rats were housed in pairs (to reduce stress from isolation) and kept at 22–25°C, on highly absorbent bedding. Bladders were manually expressed twice daily for 2 weeks after SCI. Under these conditions, mortality of spinal cord injured rats was 0%, and no evidence of infection, pressure sores, or self-mutilation was seen.

Behavioral Tests of Hindlimb Functional Deficits. All rats were tested blindly for functional deficits on days 1 and 7 and weekly thereafter through 8 weeks after injury. The CBS was used as a measure of overall hindlimb function deficit (Gale et al., 1985). To calculate the CBS, animals were evaluated with a battery of reflex tests, including toe spread and placing, withdrawal in response to different types of stimulation, righting, and hot plate noxious response. Rats were also tested for coordination between forelimbs and hindlimbs during walking, swimming, and standing on an inclined plane. The CBS ranges from 0 to 100 with 0 indicating no functional deficit and 100 indicating abnormal responses on all of the tests. Rats were also evaluated by the BBB open field locomotion scale (Basso et al., 1995), in which a completely paralyzed rat scores 0, and a rat with normal locomotion scores 21.

Assessment of Lower Urinary Tract Function. Spontaneous lower urinary tract function was assessed by measuring the volume of urine manually expressed during the first 2 weeks after SCI. The time point when the amount of manually expressed urine began to decrease was used as an indicator of recovery of spontaneous voiding as described previously (Pikov and Wrathall, 2001). Lower urinary tract function at 5 days (subacutely) and 8 weeks (chronically) after SCI was experimentally examined using a urodynamic procedure that allows a rapid collection of data over a large number of voiding cycles (Maggi et al., 1986; Pikov and Wrathall, 2001). For bladder IV recording, a transurethral bladder catheter (PE-50 tubing; Becton Dickinson, Parsippany, NJ) was inserted and connected to a pressure transducer. During the bladder detrusor contractions, fluid was released by flowing around the catheter in the urethra. For EUS electromyography, two wire electrodes were placed percutaneously in the sphincter area of the urethra. The signals from the pressure transducer and wire electrodes were preamplified, sampled at 1 kHz, and acquired on-line using BioBench 1.0 software (National Instruments, Austin, TX). Intravésical catheter and wire electrodes were inserted while animals were anesthetized with chloral hydrate (360 mg/kg i.p.). Animals were then placed in a body-shaped cloth glove and allowed to recover from anesthesia for 2 h. Threshold IVP (at the initiation of contraction) and maximal IVP during voiding as well as EUS activity during bladder filling and voiding were measured for each voiding cycle over a 20-min period in each of the animals. The rate of ESA was calculated from 60 Hz filtered electromyographic data using a custom-written peak detection macro for Excel (Microsoft Corporation, Redmond, WA), which counts the number of peaks above the defined threshold at 100-ms intervals. More details about the procedure can be found elsewhere (Pikov and Wrathall, 2001). Following the urodynamic experiment, the animal was reanesthetized with chloral hydrate and perfused with saline followed by 4% paraformaldehyde. The bladder was removed, drained of residual liquid, blot-dried, and weighed.

Intrathecal Drug Administration. The drugs used were NBQX and CPP, which were obtained from Sigma Chemical Co. (St. Louis, MO). The uninjured, subacute, and chronic SCI animals were randomly assigned for treatment with either NBQX (n = 8, 10, and 9, respectively) or CPP (n = 7, 9, and 9, respectively).

The intrathecal catheterization was done as previously described (Storkson et al., 1996). Animals were anesthetized with chloral hydrate (360 mg/kg i.p.). While lifting the ventral iliac spines, a 20-gauge short bevelled guide cannula containing a PE-10 catheter (Becton Dickinson, Parsippany, NJ) was inserted tangentially between the L5 and L6 vertebrae (level of the cauda equina) and advanced 3 to 5 mm rostrally. The cannula was then retracted, and the catheter was advanced slowly in the subarachnoid space so that the tip was over the L6 level of spinal cord. Leakage of fluid through the outer tip of the catheter and ease of advance confirmed the proper placement of the catheter in the subarachnoid space. The catheter was fixed in place by suturing it to superficial muscles. The outer tip of the catheter was sealed until the time of injection. The drugs were applied in a 5-μl volume followed by a 5-μl flush with saline at a rate of 1.4 μl/min using a syringe pump (Harvard 22; Harvard Apparatus, Inc., Holliston, MA). Increasing doses of NBQX (2.6, 7.9, 26, 79, and 260 nmol) or CPP (4, 12, 40, 120, and 400 nmol) were given sequentially at 50-min intervals, and the urodynamic recordings began after a 10-min delay subsequent to each application to allow drug diffusion into spinal cord tissue. Averaged urodynamic parameters were quantified from three to five micturition cycles during the 20-min period of evaluation.

Statistical Analysis. All data were subjected to statistical analysis using SigmaStat 2.0 program (SPSS Inc., Chicago, IL). Student’s t test or one-way analysis of variance were followed by post hoc tests.
Results

SCI and Hindlimb Functional Recovery. The initial injury, as indicated by the compression rate of the SCI impact, was similar in all SCI animals in this study (0.40 ± 0.01 m/s). Hindlimb behavioral recovery was evaluated by CBS and BBB scores (Fig. 1). There was a severe functional impairment at 5 days after SCI in the subacute group, and at 7 days in the chronic group (about 20–30% of normal function by either test). Behavioral scores improved over time and reached a plateau by 21 to 28 days postinjury. The pattern of hindlimb functional recovery was similar to that in previous studies (Basso et al., 1995; Pikov and Wrathall, 2001).

Recovery of Bladder Function. Initiation of the recovery of spontaneous bladder detrusor contractions in SCI animals was detected by a decrease in the amount of manually expressed urine (Pikov and Wrathall, 2001). No difference was observed between subacute and chronic groups; the average was at 2.1 ± 0.4 days. At 5 days postinjury (subacutely), the amount of expressed urine was significantly lower (2.0 ± 0.2 ml) than the maximal values seen at 2 days (2.6 ± 0.2 ml). The bladder weight in subacute (0.23 ± 0.02 g) and chronic (0.22 ± 0.03 g) SCI groups was significantly higher compared with uninjured animals (0.11 ± 0.01 g), with no difference between the weights at the subacute and chronic time points. Measurement of IVP during urodynamic experiments (Fig. 2A, upper panels) showed a reduction in contraction amplitude from that of uninjured rats at the subacute time and some recovery chronically. Quantification of dIVP during detrusor contractions (Fig. 2B, upper panel) revealed a 30% decrease subacutely compared with uninjured animals. Chronic dIVP values were intermediate and not statistically different from either uninjured or subacute values. Qualitatively, the increases and decreases of IVP during detrusor contractions occurred more slowly in subacute and chronic SCI animals compared with uninjured animals. Chronically, in addition to large voiding contractions, multiple small nonvoiding contractions appeared throughout the filling and voiding phases of the micturition cycle (Fig. 2A, upper right panel).

Effect of Intrathecal Glutamate Receptor Antagonists on Detrusor-EUS Coordination. The competitive NMDA and non-NMDA receptor antagonists CPP and NBQX were given in increasing doses intrathecally at the L6 spinal cord segment while the urodynamic procedure was performed in the awake restrained animals. IVP and ESA parameters were measured to evaluate the effect of drugs on detrusor and EUS activity (Fig. 3). To study the effect of drugs on detrusor-EUS coordination independently from their action on the detrusor itself, a range of doses was chosen that did not have a significant effect on detrusor contraction amplitude (Fig. 3, A and B, upper panels). Detrusor-EUS coordination, measured by dESA, was somewhat decreased in uninjured animals after treatment with low doses of either drug and was dramatically inhibited by high doses (Fig. 3, A and B, lower panels). Mean values of dIVP and dESA were calculated for all SCI groups at all doses (Fig. 4). Neither of the glutamate receptor antagonists inhibited dIVP at the doses used (Fig. 4, upper panels), and analysis of variance post hoc tests revealed that dIVP values were statistically decreased only at the highest dose of CPP (400 nmol = 2.6 log[nMol]) for uninjured and chronic animals. In contrast, dESA was decreased with each drug in a dose-dependent manner (Fig. 4, lower panels), with a maximal (60–70%) dESA inhibition seen at the highest doses used. The inhibition was significant (Newman-Keuls post hoc test) for each individual dose beginning from the second until the fourth (of five doses used) in all of the experimental groups, except for the subacute group treated with CPP in which only the first and second doses produced significant decreases in dESA. This indicates that the chosen doses covered the spectrum from the response floor to the response ceiling in uninjured and most of the experimental groups.

ID_{50} values for individual animals were used to calculate the average ID_{50} values for NBQX- and CPP-treated uninjured and SCI groups (Fig. 5). No difference in ID_{50} was found among NBQX-treated injury or control groups, indicating that the sensitivity of detrusor-EUS coordination to NBQX was not changed as a result of SCI or subsequent

![Fig. 1. Recovery of hindlimb function after SCI. The function was assessed using behavioral tests: the BBB open field locomotion score and CBS. Animals in the subacute group where tested at 5 days postinjury, and chronically surviving animals were tested weekly until 8 weeks postinjury.](image-url)
functional recovery. The effect of CPP, however, was not similar among the groups. SCI animals in the subacute group had a 50% lower ID_{50} for CPP compared with uninjured animals. However, at 8 weeks after injury, the sensitivity of detrusor-EUS coordination to CPP returned to the preinjury value.

**Discussion**

Partial recovery of hindlimb somatic sensorimotor functions following incomplete SCI has been previously described (Gale et al., 1985; Basso et al., 1995). Recently, we demonstrated that a visceral function, detrusor-EUS coordination,
is also capable of chronic recovery, which depends upon the severity of SCI (Pikov and Wrathall, 2001). Because detrusor-EUS coordination is mediated supraspinally (Holstege et al., 1986; de Groat, 1990; Pikov and Wrathall, 2001), its recovery after SCI is a suitable experimental model for studying mechanisms of recovery of supraspinal control. In the present study, aspects of the time course of this recovery were examined. The initial return of detrusor contractions was seen at 2 days, and by 5 days (subacutely) their amplitude became similar to that at 8 weeks (chronically) postinjury. Detrusor-EUS coordination was decreased to 50% subacutely, recovering to normal values chronically after SCI.

Considerable knowledge about the normal pharmacology of the reflex circuitry controlling this micturition (Tiseo and Yaksh, 1990; Morrison, 1997; de Groat et al., 1998) enabled us to examine functional characteristics relevant to the observed recovery.

Using pharmacological antagonism of glutamate receptors, we demonstrated that at the subacute time, in addition to decreased baseline (predrug) EUS response, there was a significant increase in the sensitivity of the EUS reflex to NMDA receptor antagonism. Chronically, the sensitivity recovered to a preinjury level. Sensitivity of EUS function to AMPA/kainate receptor antagonism was unchanged acutely or chronically after SCI. Our finding of changes only in NMDA receptor function is consistent with evidence suggesting that NMDA and non-NMDA receptor functions are part of parallel but functionally separate synaptic circuits that are important in micturition (Yoshiyama et al., 1995).

The altered sensitivity to CPP that we observed during recovery of lower urinary tract function after SCI could be due to altered expression and/or function of NMDA receptors involved in the reflex and/or subacutely altered glutamatergic neurotransmission. Decreased glutamatergic neurotransmission due to decreased neurotransmitter levels, although theoretically possible, seems unlikely. Previous investigators have shown that glutamate and aspartate levels are either unchanged or increased acutely distal to the injury site after SCI (McAdoo et al., 1999). A decrease in the number of glutamatergic synapses involved in the reflex is also possible and potentially able to affect the baseline EUS response, but would not be expected to alter the sensitivity of response to CPP for two reasons. First, decreased receptor number would cause a reduction of EUS response similarly at all of the doses tested due to the linear correlation between receptor number and drug binding (Bylund and Yamamura, 1990), leaving the ID_{50} unchanged. We assume here [based on the empirical value of NMDA receptor density in spinal cord (Sun and Faden, 1994)] that the number of functional NMDA receptors in spinal cord is much lower than the number of CPP molecules applied. Second, by separately grouping the uninjured animals with low and high baseline EUS responses, we found no difference in their mean ID_{50} values for CPP antagonism (V. Pikov, unpublished observation).

An intriguing hypothesis to explain our results is an alteration in NMDA receptor subunit composition during recovery of lower urinary tract function. CPP has different affinities to NMDA receptors, depending upon the NR2 subunit present as part of the receptor complex. NMDA receptors in the rat spinal cord consist of NR2A, NR2B, and NR2D subunits (Luque et al., 1994; Virgo et al., 2000) and are characterized by different CPP affinity to a receptor containing these sub-
units, ranking in the order of NR2A > NR2B > NR2D, whereas the affinity to glutamate is in the opposite order (Monaghan et al., 1998). Thus, increased sensitivity to CPP could be due to a shift in the ratio of NR2 subunits toward either a higher proportion of NR2A or a smaller proportion of NR2D in lumbosacral cord at 5 days after SCI. Further studies will be needed to provide direct evidence to support or refute this, or an alternative, hypotheses.

It is interesting to contrast the effect of glutamate receptor antagonists on the EUS with a lack of effect on bladder contractility even at the doses that abolished most EUS activity. This finding is in accordance with previous studies that found differential effects of NMDA and non-NMDA receptor antagonists on bladder and urethral activities in uninjured as well as spinally transected rats (Yoshiyama et al., 1995; Nishizawa et al., 1999). Bladder activity can, however, be significantly inhibited by larger doses of antagonists (V. Pikov, unpublished results), indicating a possible difference in sensitivity threshold for glutamate receptor blockade between these somatic (EUS) and parasympathetic (bladder) spinal reflexes.

NMDA receptors are involved in different forms of spinal cord plasticity (Ma and Woolf, 1995; Urban and Gebhart, 1998). After complete spinal cord transaction, NMDA receptor binding is decreased at 1 to 2 weeks (Krenz and Weaver, 1998) but not at 1 day (Sun and Faden, 1994). NMDA receptors underlie polysynaptic neurotransmission of somatic (Turski et al., 1990) and visceral reflexes (Mills et al., 1988; Sundaram and Sapru, 1991). Therefore, alteration in NMDA receptor function might mediate the decrease in reflexive function seen at 6 days after SCI (Thompson et al., 1992). Consistent with this, sensitivity of a spinal reflex to NMDA was shown to be decreased between 1 day and 2 weeks after spinal transection (Maiorov et al., 1997).

The experiments in this study were carried out on awake restrained animals. Therefore, measures to prevent any painful sensations by the animals were undertaken. We preadapted them to the restraint system and paid attention to breathing pattern and lack of vocalizations or struggle, to assure that they were not in distress. To minimize the duration of restraint for the animals, we decided not to utilize random order drug doses, which would have required a prolonged waiting time between the doses to assure complete washout of the previous dose. Instead, we used the increasing dose paradigm when there was the possibility that some amount of the previous dose was still present but was small compared with the next higher dose. Our estimations of ID50 must be considered in this context and may represent slightly different values than would have been obtained by use of a random drug dose protocol.

In general, the results of our study support the importance of NMDA receptors in functional recovery after spinal cord injury. Similar roles for the NMDA receptors were seen in other parts of the central nervous system. For example, neglect symptoms produced by unilateral frontal cortex ablation demonstrate recovery over 3 or more weeks after surgery that is associated with altered (increased) striatal NMDA receptor function as assayed by receptor binding studies (Vargo and Marshall, 1996). In contrast, no alterations are seen in ligand binding for AMPA or dopamine receptors in the striatum. NMDA receptors in primate somatosensory cortex appear to play an important role in the cortical reorganization that allows recovery of responsiveness for 4 weeks after transection of the median nerve (Garraghty and Muja, 1996). Blockade of NMDA receptor function during that period largely prevents recovery of cortical responsiveness. Similarly, NMDA receptor function is important in the development of vestibular compensation after unilateral labyrinthectomy (Hirate et al., 2000).

In summary, we have characterized aspects of the recovery of bladder-EUS coordination after incomplete SCI and identified the occurrence of a significant alteration in NMDA receptor function during recovery. This system may provide a useful model in which the cellular and molecular mechanisms involved in recovery of a specific and clinically important function can be probed. Furthermore, with increased understanding of the underlying mechanisms involved, it may be possible to develop pharmacological strategies to speed and/or enhance the degree of recovery of lower urinary tract function after incomplete SCI.

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References


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