Changes of Bladder Activity and Glycine Levels in the Spinal Cord and Serum after Spinal Cord Injury in Rats

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ABSTRACT
We examined whether the glutamate and glycine levels in the central nervous system (CNS) were related to bladder activity and their serum levels in rats after spinal cord injury (SCI). Female rats were anesthetized with halothane, and the spinal cord was completely transected at the lower thoracic level. At 1 day to 8 weeks after SCI, bladder activity and the glutamate and glycine levels in the CNS and serum were measured. Urinary retention was observed in the acute period after SCI. Isovolumetric cystometry showed no bladder contractions at 1 and 3 days after SCI, but contractions were seen after 2 to 8 weeks. The glycine level was increased in the lumbosacral cord at 1 day after SCI, but it was decreased at 2 to 8 weeks. The serum glycine level was also increased at 1 week after SCI, and it was decreased gradually over 4 to 8 weeks. The glutamate level in each CNS region and serum of SCI rats did not differ from those of control rats and sham-operated rats at 1 day to 8 weeks after surgery. Therefore, the change of the glycine level in the lumbosacral cord may influence bladder contractions and its serum level after SCI.

Micturition is mediated by the spinobulbospinal reflex pathway, which consist of an afferent limb from the lumbosacral cord to the integration center in the rostral brainstem known as the pontine micturition center (PMC), and an efferent limb from the PMC back to the parasympathetic nucleus in the lumbosacral cord that controls detrusor muscle contractions (2, 15, 22). The efferent limb simultaneously inhibits the sympathetic and pudendal nuclei that modulate internal and external urethral sphincter contractions (30). After the spinal cord is injured, flaccidity and urinary retention with disappearance of bladder contractions occur during the acute period. However, a potential spinal micturition pathway subsequently becomes active and modulates bladder contractions during the chronic period (7, 8). But external urethral sphincter activity is not inhibited completely during bladder (detrusor) contractions after spinal cord injury (SCI), residual urine exists to some extent. The loss of coordinated external urethral sphincter activity is known as detrusor-sphincter dyssynergia (DSD), and it is one of the major characteristics of abnormal micturition in animals and humans with SCI (12).

In the central nervous system (CNS), various amino acids act as neurotransmitters. Glutamate is the major excitatory neurotransmitter (18), and glycine is the inhibitory neurotransmitter (1). The glutamate and glycine are considered to influence the activation of the micturition reflex pathway. In our previous study, intrathecal injection of glutamate increased the glycine level in the lumbosacral cord in rats, while intrathecal injection of glutamate receptor antagonist decreased both glutamate and glycine levels in the lumbosacral cord (29). When
the thoracic spinal cord was transected in rats, the glycine showed an acute increase in the lumbosacral cord and it was inhibited by the intrathecal injection of glutamate receptor antagonist (29). These results suggest a close relationship between glutamatergic neurons and glycineergic neurons in the lumbosacral cord.

Recently, there have been attempts to use the amino acid analysis as an indicator of central nervous diseases (3). The glutamate level increased in the cerebrospinal fluid (CSF) and serum of patients with large cerebral infaracts (4, 5, 6). Changes of amino acids levels in the CSF and serum have also been detected in patients with amyotrophic lateral sclerosis (ALS) (23).

In the present study, therefore, we examined whether the glutamate or glycine levels in the CNS had influence on bladder activity after SCI and whether the changes of their concentration in the CNS were reflected in their serum levels.

MATERIALS AND METHODS

Animal model. Female Sixty-three female Sprague-Dawley rats weighing between 250–300 g were divided into the following three groups: 1) an intact control group (n = 7), 2) a SCI group (n = 42), and 3) a sham-operated group (n = 14). Rats from the SCI group were anesthetized with 2% halothane (Takeda chemical institute, Osaka, Japan), and laminectomy was performed at the lower thoracic level. The dura was opened and the spinal cord was completely transected at the 9th or 10th thoracic cord under direct vision. Rats from the sham-operated group only underwent laminectomy. Each animal received subcutaneous injection of an antibacterial agent (ampicillin 100 mg/day; Mitaka Pharmacoeuticals, Tokyo, Japan) for 2 days after surgery to prevent infection. The protocol used in this study was approved by the Institute for Animal Experiment, Faculty of Medicine, University of the Ryukyus. Postoperative bladder management was handled by expressing the urine via manual manipulation of the bladder until 2 weeks after SCI, and body movement in response to pinching the tail was observed.

Isovolumetric cystometry. Isovolumetric cystometry was performed in intact control rats, sham-operated rats at 1 day after surgery, and CI rats at 1 and 3 days after SCI, as well as at 1, 2, 4, and 8 weeks (n = 7 each). Under halothane anesthesia, a polyethylene catheter (PE-50) was inserted into the bladder through the urethra and the residual urine volume was measured. The urethra was ligated over the catheter near the external urethral meatus to produce isovolumetric conditions in the bladder. The catheter was connected via a polyethylene tubing to an infusion pump and a pressure transducer via a three-way stopcock. The tubing was filled with physiological saline, and bladder pressure was displayed on a chart recorder (San-ei Electric, Nagano, Japan). Under the restricted condition using a NAIGAI-CFK-1P (NMS, Tokyo, Japan), they were allowed the stillness for about 30 min to recover from anesthesia. The bladder was filled with physiological saline to the residual urine volume plus 0.5–1.5 ml through the catheter, and isovolumetric cystometry was performed to over the threshold volume inducing isovolumetric rhythmic bladder contractions. After infusion was stopped, the isovolumetric cystometry was performed for at least 30 min.

Preparation of samples. Following cystometry, the lumbosacral cord and blood sample were harvested from SCI rats under halothane anesthesia at 1 and 3 days after surgery, as well as after 1, 2, 4, and 8 weeks (n = 7 each). The whole CNS and blood samples were also harvested from intact rats and sham-operated rats at 1 day and 8 weeks (n = 7 each) after surgery. The CNS was separated into the following five parts; the cerebrum from the cerebral cortex to the precollicular level, cerebellum, brainstem from the precollicular level to the medulla, cervicothoracic cord, and lumbosacral cord. These tissues were separately homogenized in cold 0.5 M hydrochloric acid (1.0 ml/0.1 g tissue: Kanto Chemical, Tokyo, Japan) and the homogenates were centrifuged at 15,000 rpm for 5 min at 2–4°C. The supernatant was passed through a Sep Cartridge IC-Ag column (Lida, Tokyo, Japan) for dechlorination, and it was centrifuged again at 7,500 rpm for 15 min at 2–4°C with an Ultrafree C3 THK filter (Millipore, Bedford, USA) for deproteinization. The blood samples were centrifuged at 3,000 rpm for 10 min at 2–4°C, and the serum thus obtained was deproteinized with an Ultrafree C3 THK filter. All samples were immediately stored at −80°C and were warmed to room temperature prior to assay.

Capillary electrophoresis for amino acid determination. Amino acid levels in each sample were detected by a capillary electrophoresis system (Hewlett-Packard 10CE, Hanover, Germany) equipped with a deuterium (1H) lamp. Samples were injected at 50 mbar for 4 seconds and were separated at a constant voltage of −30 kV at 30°C in a capil-
lary tube coated internally with polyimide fused silica (Yokogawa Analytical Systems, Tokyo, Japan). The tube had a total length of 80.5 cm (72 cm effective length) and an internal diameter of 75 μm. Some amino acids were to be detectable, and the glutamate and glycine which were the highest quantity in the excitatory or inhibitory amino acid neurotransmitters in the CNS were measured by the indirect UV detection method using a diode-array detector (signal at 350 nm and reference at 200 nm) (19).

Statistical analysis. Results are reported as the mean ± standard deviation (SD). Student's t-test for unpaired data was used for statistical analysis, and P < 0.05 was considered to indicate statistical significance.

RESULTS

In SCI rats, flaccidity and urinary retention were present from the time of SCI, and a full bladder was detected by palpation. Two days after surgery, pinching of the tail induced body movement as a spinal reflex in all SCI rats, and it gradually increased in intensity. Expressing urine by manual manipulation of the bladder was relatively easy until 3 to 4 days after surgery, but subsequently it often become difficult and the bladder was tense because of strong urethral sphincter contraction. Micturition evoked by tail pinching or an automatic spinal reflex micturition occurred from 1 to 2 weeks after surgery. After the appearance of spiral reflex micturition, manual manipulation became relatively easy again. On isovolumetric cystometry, the intravesical pressure was fairly stable at 1 and 3 days after SCI, but bladder contractions was recognized in 5 out of 7 SCI rats after 1 week and in all 7 rats after 2 weeks. The amplitude of bladder contractions was 43.0 ± 3.2 cmH₂O in control rats and 41.9 ± 4.2 cmH₂O in sham-operated rats after 1 day, and there was no significant change of the amplitude of bladder contractions in SCI rats after 2 weeks (40.9 ± 3.6 cmH₂O) to 8 weeks (32.0 ± 11.9 cmH₂O) when compared with that in sham-operated rats. The frequency of bladder contractions was stable after 2 weeks (1.35 ± 0.31 times/min) to 8 weeks (1.28 ± 0.18 times/min), although these frequencies were significantly increased when compared with that (0.55 ± 0.13 times/min) in sham-operated rats.

In all SCI rats, the lower thoracic cord was recognized to be transected completely when the CNS was removed. The glutamate and glycine levels of the control rats and sham-operated rats were not significantly different in each CNS region after 1 day and 8 weeks (Table 1). The glutamate level in each CNS region of SCI rats was also not significantly different compared with those in control and sham-operated rats after 1 day or 8 weeks. In contrast, the glycine level was significantly (P = 0.008) increased in the lumbar sacral cord of SCI rats after 1 day (1.53 ± 0.30 μmol/g) when compared with that (0.99 ± 0.27 μmol/g: baseline) in sham-operated rats after 1 day, although it returned to baseline level after 3 days (0.91 ± 0.16 μmol/g) and 1 week (0.91 ± 0.20

<table>
<thead>
<tr>
<th>Glutamate (μmol/g. tissue)</th>
<th>Cerebrum</th>
<th>Cerebellum</th>
<th>Brainstem</th>
<th>CTC</th>
<th>LSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.94 ± 0.29</td>
<td>3.59 ± 0.45</td>
<td>4.21 ± 0.70</td>
<td>4.25 ± 0.99</td>
<td>3.11 ± 0.25</td>
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<td>SCI sham 1 day</td>
<td>8.82 ± 0.65</td>
<td>3.76 ± 0.51</td>
<td>4.25 ± 0.64</td>
<td>4.07 ± 0.43</td>
<td>2.89 ± 0.24</td>
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<td>SCI sham 8 weeks</td>
<td>8.52 ± 0.64</td>
<td>3.67 ± 0.67</td>
<td>4.33 ± 0.93</td>
<td>4.12 ± 0.37</td>
<td>3.03 ± 0.27</td>
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<tr>
<td>SCI 1 day</td>
<td>8.75 ± 0.69</td>
<td>3.71 ± 0.54</td>
<td>4.26 ± 0.42</td>
<td>4.32 ± 0.38</td>
<td>3.51 ± 0.83</td>
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<tr>
<td>SCI 8 weeks</td>
<td>8.73 ± 1.40</td>
<td>3.98 ± 1.16</td>
<td>4.15 ± 0.89</td>
<td>4.20 ± 0.58</td>
<td>2.70 ± 0.83</td>
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<table>
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<tr>
<th>Glycine (μmol/g. tissue)</th>
<th>Cerebrum</th>
<th>Cerebellum</th>
<th>Brainstem</th>
<th>CTC</th>
<th>LSC</th>
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</thead>
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<tr>
<td>Control</td>
<td>0.34 ± 0.11</td>
<td>0.22 ± 0.14</td>
<td>1.18 ± 0.24</td>
<td>1.62 ± 0.81</td>
<td>1.03 ± 0.43</td>
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<tr>
<td>SCI sham 1 day</td>
<td>0.39 ± 0.13</td>
<td>0.23 ± 0.09</td>
<td>1.14 ± 0.31</td>
<td>1.36 ± 0.33</td>
<td>0.99 ± 0.27</td>
</tr>
<tr>
<td>SCI sham 8 weeks</td>
<td>0.35 ± 0.20</td>
<td>0.27 ± 0.14</td>
<td>0.95 ± 0.31</td>
<td>1.25 ± 0.37</td>
<td>0.86 ± 0.20</td>
</tr>
<tr>
<td>SCI 1 day</td>
<td>0.39 ± 0.13</td>
<td>0.24 ± 0.14</td>
<td>1.03 ± 0.74</td>
<td>1.37 ± 0.74</td>
<td>1.53 ± 0.30</td>
</tr>
<tr>
<td>SCI 8 weeks</td>
<td>0.42 ± 0.15</td>
<td>0.23 ± 0.09</td>
<td>1.00 ± 0.34</td>
<td>1.31 ± 0.23</td>
<td>0.36 ± 0.16</td>
</tr>
</tbody>
</table>

CTC: cervicothoracic cord, LSC: lumbar sacral cord
Values are mean ± S. D.
1): P = 0.043, 2): P = 0.010, 3): P = 0.008, 4): P < 0.001
μmol/g). Then, the glycine subsequently showed a significant decrease below baseline after 2 weeks (0.49 ± 0.08 μmol/g, P = 0.048), 4 weeks (0.38 ± 0.19 μmol/g, P = 0.001) and 8 weeks (0.36 ± 0.16 μmol/g, P = 0.001) (Fig. 1A).

The serum glutamate levels showed no significant difference between control rats, sham-operated rats, and SCI rats (Table 2). The serum glycine levels were also not significantly different between control rats (0.19 ± 0.03 mM) and sham-operated rats (0.19 ± 0.05 mM) after 1 day. In SCI rats, however, the serum glycine level was significantly increased after 1 week (0.23 ± 0.03 mM) when compared with that (0.19 ± 0.05 mM) in sham-operated rats. After 2 weeks, the serum glycine level returned to baseline, and it subsequently showed a significant

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**Fig. 1** Changes of the glycine level in the lumbosacral cord (A) and serum (B) after spinal cord injury. (A) The glycine level in the lumbosacral cord was significantly increased at 1 day, but decrease below baseline after 2 to 8 weeks when compared with that in sham-operated rats at 1 day after surgery (sham). (B) Serum glycine level was significantly increased at 1 week, but it was decreased after 4 to 8 weeks when compared with that in sham-operated rats at 1 day after surgery (sham). Values are mean ± SD. *: P < 0.05, **: P < 0.01, compared with that sham-operated rats at 1 day after surgery.
Table 2  Serum levels of glutamate and glycine in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutamate (nM)</th>
<th>Glycine (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.06 ± 0.02</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>SCI sham 1 day</td>
<td>0.06 ± 0.01</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>SCI sham 8 weeks</td>
<td>0.05 ± 0.03</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>SCI 1 day</td>
<td>0.04 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>SCI 8 weeks</td>
<td>0.05 ± 0.01</td>
<td>0.12 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± S. D.
1: P = 0.008, 2: P = 0.006

decrease below baseline after 4 weeks (0.14 ± 0.02 mM, P = 0.041) and 8 weeks (0.12 ± 0.04 mM, P = 0.029) in SCI rats (Fig. 1B).

DISCUSSION

The glycine is postsynaptic inhibitory neurotransmitter that chiefly resides within the segmental interneuron pool (14). These interneurons are closely associated with alpha motoneurons (10, 31, 32), so the glycine level is likely to be related to muscle tone. In intact rats, intrathecal injection of glycine or glycine agonist inhibits alpha motoneurons, while injection of strychnine (glycine receptor antagonist) increases muscle tone in SCI rats (27, 28). So, the acute flaccidity after SCI may be caused by the inhibition of somatic neurons due to a rapid increase of the glycine level.

There may be two reasons for the acute increase of glycine in the lumbosacral cord after SCI; one possibility is the extinction of inhibitory projections to glycineric neurons from the upper CNS, and the other possibility is the maintenance of stimulatory projections to glycineric neurons from glutamatergic neurons in the lumbosacral cord. In our previous study, intrathecal injection of glutamate increased the glycine level in the lumbosacral cord of intact rats, while intrathecal injection of MK-801 (glutamate receptor antagonist) decreased the glutamate and glycine level in the lumbosacral cord of intact rats or rats with acute SCI (29). These results suggest that glutamatergic neurons stimulate glycineric neurons, glycineric neurons inhibit glutamatergic neurons, and glutamatergic neurons also stimulate glutamatergic neurons in the spinal cord. However, the glutamate level in the lumbosacral cord did not change after SCI, although the glycine level showed an acute increase. These findings may be explained as follows; 1) continuous stimulation of glycineric neurons by glutamatergic neurons was more effective than inhibition of glutamatergic neurons by glycineric neurons, and 2) glutamatergic neuronal activity in the lumbosacral cord was maintained at a constant level by the balance between extinction of facilitatory and inhibitory projections from the upper CNS, and the increase and decrease of facilitatory or inhibitory projections to glutamatergic neurons in the lumbosacral cord.

The bladder has two pathways for the control of micturition which are the spinobulbospinal reflex pathway and the potential spinal reflex pathway (8). The potential reflex pathway mediated by spinal cord as a micturition center became active when the spinobulbospinal reflex pathway from the upper CNS was intercepted by SCI. The increase of glycine in the lumbosacral cord may suppress the potential spinal-to-bladder reflex pathway, thus causing urinary retention in the acute period. Failure to initiate relaxation of the urethral sphincter is often seen after SCI. All of the skeletal muscles are flaccid below the level of injury, but not the external urethral sphincter (12, 13). In the present study, expressing urine by manual manipulation of the bladder was relatively easy until 2 to 3 days after SCI, but then became more difficult because of urethral sphincter contraction. Shefchik et al. (25) reported that suppression of motoneurons supplying the external urethral sphincter during micturition may be partly due to direct glycineric inhibition of those motoneurons based on animal experiments using strychnine and immunohistochemistry. Sie et al. (26) also reported that terminals from the PMC made contact with glycine-immunoreactive dendrites and glycine- and gamma-aminobutyric acid (GABA)-immunoreactive dendrites in the sacral dorsal gray commissure, and that these interneurons might inhibit external urethral sphincter motoneurons during micturition. The present study also suggested that glycine may be one of the main neurotransmitters that inhibits external urethral sphincter motoneurons. On the other hand, external urethral sphincter contraction is thought to be mediated by glutamatergic projections to the external urethral sphincter motoneurons in both normal rats and SCI rats (33). Intrathecal injection of LY293558, a glutamate (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid: AMPA) receptor antagonists, completely blocks urethral contractions and partially inhibits bladder contractions in chronic SCI rats (21). In the present study, the glutamate levels in each CNS region (including the lumbosacral cord) did not change after SCI. Therefore, it seems that DSD is mediated by persistent glutamatergic projec-
tions and the lack of glycinergic or GABAergic projections to the external urethral sphincter motoneurons. Over the longer term, the glycine level in the lumbosacral cord decreased significantly and the spinal micturition reflex developed, suggesting that inhibitory glycinergic neuronal activity was decreased. The ratio of excitatory amino acids to inhibitory amino acids in the spinal cord is reported to be increased in the spastic state (4, 24). In the present study, the glutamate/glycine ratio (3.5/1.53 = 2.29) of the lumbosacral cord was decreased in the acute period than that (3.11/0.13 = 3.02) in control rats, while it was increased in the chronic period (2.70/0.36 = 7.50), suggesting that the glutamate/glycine ratio in the lumbosacral cord is also related to the spinal micturition reflex activity.

The glycine level decreased gradually in the lumbosacral cord at 3 days after SCI and the body movement in response to tail pinching occurred at that time, but automatic reflex micturition did not appear until 1 to 2 weeks after SCI. It seems likely that the delayed appearance of the spinal micturition reflex was caused by at least 4 factors: 1) the potential reflex pathway mediated by the lumbosacral cord as a micturition center is slow to become active; 2) the spinal micturition reflex is more easily inhibited by glycinergic neurons than the body movement reflex; 3) the spinal micturition reflex is also inhibited by opiate neurons such as enkephalin neurons, because intrathecal injection of naloxone (an opiate antagonist) induces bladder contraction of rabbits with acute spinal cord injury (11); and 4) chronic overdistention of the bladder by urinary retention may damage the bladder muscle and limit the functional recovery of voiding (9). However, although body movement in response to tail pinching occurred 2 days after SCI, about one week (1 to 2 weeks after SCI) was needed for the appearance of the micturition reflex in all SCI rats. Expressing urine through manual manipulation of the bladder was relatively easy until 2 to 3 days after SCI, but then became more difficult and the bladder was strongly tense because of strong urethral sphincter contraction. Therefore, the delayed appearance of the spinal micturition reflex may be mainly due to the 4th reason.

Some amino acid neurotransmitters have been linked to certain diseases of the CNS. In patients with ischemic cerebral infarction, it has been suggested that the glutamate level in the CSF within 24 to 48 hours of onset is related to infarct size and neurological deficits, and that the CSF level of glutamate is reflected by the serum level (4, 5). Chronic degenerative diseases such as Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis also show different patterns of serum glutamate or glycine levels (16, 17, 23). In the present study, it is uncertain why the change of glycine in the lumbosacral cord was slow to be reflected in the serum glycine level with a delay of a few weeks. However, our previous study showed that serum glycine levels decreased in patients with chronic SCI when compared with healthy controls (20). Therefore, the serum glycine level may be a useful marker of spinal glycinergic neuronal activity.

Acknowledgments

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REFERENCES


