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Laboratory Investigation

Comparison of the Spinal Neuropathic Pain Induced by Intraspinal Injection of N-Methyl-D-Aspartate and Quisquate in Rats

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Objective : Excitatory amino acids play important roles in the development of secondary pathology following spinal cord injury (SCI). This study was designed to evaluate morphological changes in the dorsal horn of the spinal cord and assess profiles of pain behaviors following intraspinal injection of N-methyl-D-aspartate (NMDA) or quisqualate (QUIS) in rats.
Methods : Forty male Sprague-Dawley rats were randomized into three groups : a sham, and two experimental groups receiving injections of 125 mM NMDA or QUIS into their spinal dorsal horn. Following injection, hypersensitivity to cold and mechanical stimuli, and excessive grooming behaviors were assessed serially for four weeks. At the end of survival periods, morphological changes in the spinal cord were evaluated.
Results : Cold allodynia was developed in both the NMDA and QUIS groups, which was significantly higher in the QUIS group than in the NMDA group. The mechanical threshold for the ipsilateral hind paw in both QUIS and NMDA groups was significantly lower than that in the control group. The number of groomers was significantly higher in the NMDA group than in the QUIS group. The size of the neck region of the spinal dorsal horn, but not the superficial layer, was significantly smaller in the NMDA and QUIS groups than in the control group.
Conclusion : Intraspinal injection of NMDA or QUIS can be used as an excitotoxic model of SCI for further research on spinal neuropathic pain.

Key Words : Neuropathic pain · NMDA · Quisqualate · Rat · Spinal cord injury.

INTRODUCTION

Although the loss of sensory and motor function is regarded as the most significant consequence of spinal cord injury (SCI), the condition of pain is still a major challenge for patients[20]. The pain, usually described as burning, stabbing, or electrifying, greatly affects patient's quality of life[16]. A better understanding of the pathophysiological and neurochemical responses to spinal injury is needed for the development of more effective treatments for pain induced from the spinal origin.

The mechanism of pain after SCI involves a cascade of events triggered by the spinal cord injury. The structural damage leads to reorganization of spinal and supraspinal circuits responsible for the integration and processing of sensory information. Furthermore, excitotoxic and inflammatory processes contribute to the cascade of secondary injury. Previous studies have provided evidence supporting the involvement of excitatory amino acids (EAAs) in the development of secondary pathology following spinal cord injury[5]. Tissue levels of EAAs are increased in areas of traumatic or ischemic SCI[10]. EAAs in the damaged tissue produce their toxic actions via both ionotropic and metabotropic glutamate receptors[7,14]. Previous studies documenting the involvement of the N-methyl D-aspartate (NMDA) ionotropic glutamate receptor and non-NMDA receptors in traumatic and ischemic brain injury[1,13], have prompted efforts to clarify the role of the NMDA receptor in producing excitotoxic injury in the spinal cord[3,11,21].

Activation of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor induces a cation influx that produces membrane depolarization. This, in turn, initiates activation of NMDA receptor, resulting in a massive influx of calcium into the intracellular space[1]. Prolonged activation of these receptors leads to increased intracellular concentrations of sodium, potassium, and calcium ions leading ultimately to cell death and hyperexcitability of postsynaptic neurons[19]. Yezierski

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et al.[20,21] have reported that intraspinal injection of the AMPA-metabotropic receptor agonist, quisqualate (QUIS), produces progressive pathological changes and pain-related behaviors resembling those described following SCI. In addition, inhibition of spinal AMPA receptors has shown to attenuate mechanical allodynia and neuronal hyperexcitability following SCI[6]. Therefore, AMPA receptors might exert an important regulatory role in the pathophysiological changes that following SCI. Although it is also well known that NMDA receptors play an important role in the development of chronic neuropathic pain following neural injury[2,4], there are no reports demonstrating pathological changes in the spinal dorsal horn or development of pain-related behaviors following intraspinal injection of NMDA.

Therefore, in the present study, we compared the morphological changes in the dorsal horn of the spinal cord after NMDA or QUIS injection in rats. In addition, the profiles of pain behaviors following intraspinal injection of NMDA and QUIS were evaluated.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee, and was conducted in accordance with NIH guidelines for the care and use of laboratory animals. Adult male Sprague-Dawley rats weighing 180-200 g were used. The rats were housed at a constant humidity and temperature with a 12-hour light/dark cycle and free access to food and water.

Intraspinal injections

Rats were randomized into three groups : a sham operated control group (n=10), a group receiving 125 mM NMDA (n=15), and a group receiving 125 mM QUIS (n=15). Anesthetized rats injected intraperitoneally with zoletil (12.5 mg) and xylazine (3 mg) were immobilized with a hip bar. Spontaneous respiration was maintained, and the rats were shaved, scrubbed with betadine, and wiped with 70% alcohol. Core body temperature was monitored using a rectal probe and was maintained at 37.0°C±0.5°C using a heating pad (Homeothermic Blanket System, Harvard Apparatus Inc., USA).

Intraspinal injection was conducted by previously published methods with modification[9,21]. After making a midline incision, the spinous process and vertebral laminae of L1 were removed. For intraspinal injections, we used a 34-gauge beveled NanoFil needle (WPI, Sarasota, FL, USA) attached to a Hamilton syringe (volume, 5 μL) mounted on a micromanipulator. Intraspinal injections were made into the left side of the spinal dorsal horn between the dorsal vein and dorsal root entry zone at a depth of 1000 μm below the spinal cord surface. NMDA (Sigma, St. Louis, MO, USA) and QUIS (Sigma, St. Louis, MO, USA) were diluted with sterile saline. Animals in the NMDA and QUIS groups were injected with 0.6 μL of 125 mM NMDA or 125 mM QUIS, respectively, over a 20-second interval (three tracks of 0.2 μL separated by 0.3 mm parallel to the long axis of the cord). Although excitotoxic damage was observed in the animals treated with NMDA and QUIS concentrations ranging from 1 to 10 mM, pain-like behaviors did not occur with the same regularity and/or time course as in animals injected with a concentration of 125 mM[9]. For this reason, the present study describes results obtained with a concentration of 125 mM NMDA and QUIS. The animals in the control group received the same operation without injections. After injections, muscles were sutured, the skin was closed, and the animals were returned to their home cages.

Behavioral tests

All animals were evaluated 3 days prior to intraspinal injections in order to establish baseline responses to mechanical and cold stimuli. Post-injection testing started 7 days after intraspinal injection and continued for 4 weeks. All the tests were done by one examiner blinded to injection strategies. Due to the nature of responses required for behavioral evaluations, animals experiencing signs of post-injection motor dysfunction (e.g., hindlimb paresis and/or paralysis) were excluded from behavioral evaluations.

For the assessment of cold allodynia[9], the rat was placed under a transparent plastic dome on a metal mesh floor, and acetone was applied to the plantar surface of the hind paw. An acetone bubble that formed at the end of a piece of small polyethylene tubing connected to a syringe was brought into contact with the heel. Acetone was applied five times to each paw at intervals of 5 minutes. A prompt foot withdrawal response to the acetone application was interpreted as a sign of cold allodynia. The frequency of paw withdrawal was expressed as a percentage (the number of paw withdrawals divided by the total number of trials, times 100). Animals showing signs of cold allodynia on more than two consecutive test sessions were interpreted as positive allodynia to cold stimuli.

Response to mechanical stimuli was tested with calibrated von Frey filaments as previously published methods[9], stimulus intensities ranging from 0.5 to 50 g were applied six times to the glabrous skin of each hindpaw. Filaments were applied to the point of bending, at which time evidence of responsiveness or non-responsiveness was determined. During each test session, the filament producing a threshold response (i.e., 50%) in each animal was determined for the left hind paw. Positive responses included withdrawal, licking, and/or vocalizations. Animals whose measured withdrawal thresholds were less than 5 g at every testing were interpreted as positive allodynia to mechanical stimuli.

After intraspinal injection, the animals were inspected weekly for signs of excessive grooming (i.e., removal of hair, superficial skin damage) for 4 weeks. Excessive grooming behavior was a progressive condition, and the severity of grooming was categorized into four classes (I-IV) as described previously[11] : 1) Class I : hair removal over contiguous portions of a dermatome; 2) Class II : extensive hair removal combined with signs of damage to the superficial layers of skin; 3) Class III : hair removal and damage to dermal layers of skin; and 4) Class IV : subcutaneous tissue damage.

Tissue processing and analysis of histological damage

At the end of the 4-week survival period, animals were anesthetized with zoletil (12.5 mg) and xylazine (3 mg), injected intraperitoneally, and perfused transcardially with 4% buffered paraformaldehyde. Spinal segments containing microinjection sites were sectioned at 10 μm after paraffin imbedding. Sections were stained with 0.1% cresyl violet solution, mounted with permanent mounting medium, and examined with a light microscope. Damaged areas were reconstructed with an overhead projector and camera lucida, and the size of spinal gray matter in two regions-superficial (lamina I and II) and neck (lamina III to V)-was measured by examining three serial transverse sections through the epicenter of injection sites using an image analysis system (Image J software, Universal Imaging Corp., USA). This analysis was conducted by an examiner blinded to the behavioral results and injection protocols. Tissue blocks that were mechanically damaged during histological processing were excluded from this analysis.

Data analysis

Responses to mechanical and cold stimuli, and sizes of spinal gray matter were expressed as means±standard errors of the mean (SEM). Significant differences were evaluated by analysis of variance followed by Turkey-Kramer multiple comparisons. The number of animals that developed cold allodynia, mechanical allodynia, and/or excessive grooming among the three treatment groups was compared using the χ^2 test. The severity of grooming was analyzed using the non-parametric Kruskal-Wallis test for multiple comparisons followed by the Mann-Whitney U-test to compare individual groups. A p-value less than 0.05 was considered significant.

RESULTS

Behavioral profiles following intraspinal injection of NMDA and QUIS

Intraspinal injection of NMDA or QUIS resulted in mechanical and cold allodynia, as well as excessive grooming behaviors similar to those described in neuropathic pain models. One rat in the QUIS group showed motor weakness second week after injection and was excluded from this experiment. During the observation period, cold allodynia developed in 40% (6/15) of animals in the NMDA group, 86% (12/14) of those in the QUIS group, and 10% (1/10) of those in the control group. The number of animals that developed cold allodynia was significantly higher in the QUIS group than in the NMDA group (p<0.05) (Fig. 1A). There was no significant difference in the percent-re-

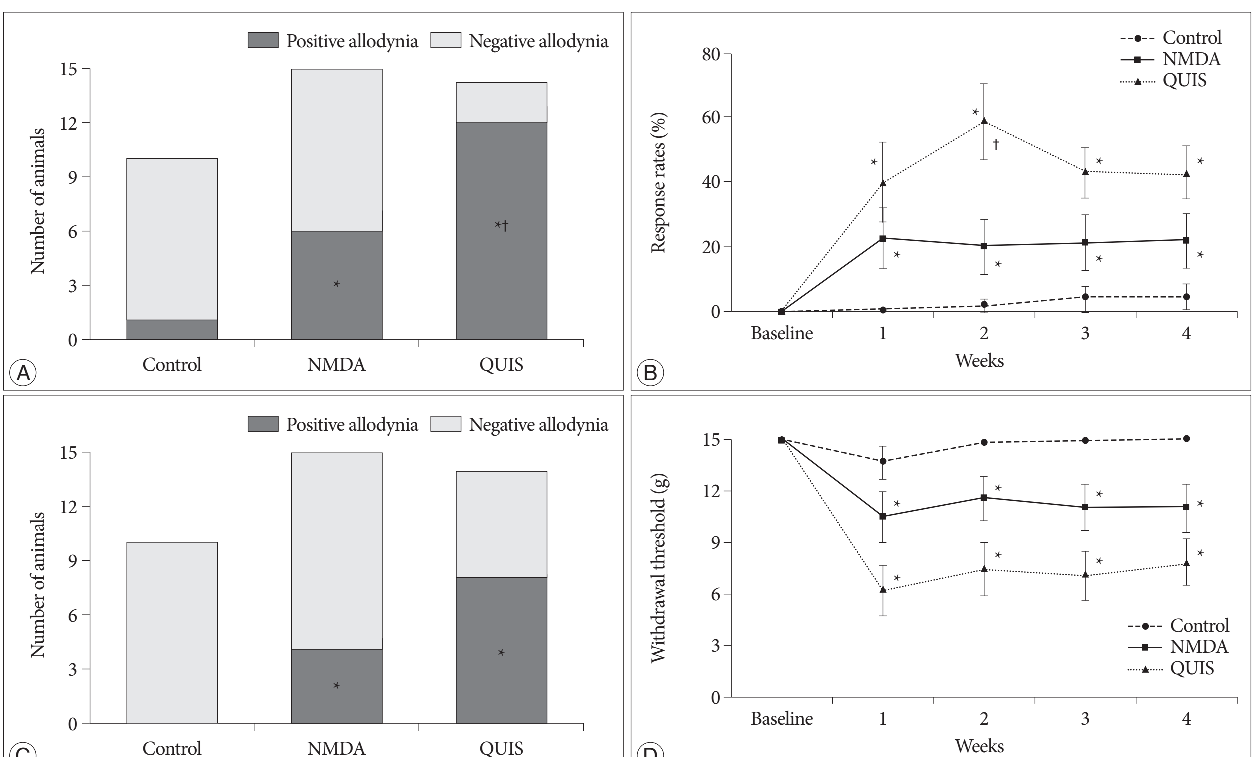

Fig. 1. The effects of intraspinal NMDA or QUIS injections or sham surgery on the responses to cold (A and B) and mechanical (C and D) stimuli delivered to the hind paws. Baseline responses were measured 3 days prior to receiving intraspinal injections/sham surgery. Cold and mechanical allodynia developed after intraspinal injection of NMDA or QUIS. Positive responses were defined as sustained signs of cold allodynia and/or measured withdrawal threshold of less than 5 g (mechanical allodynia). Data are expressed as the number of animals or mean±SEM (*p<0.05 compared with the control group; $^{\dagger}p$<0.05 compared with the NMDA group). NMDA : N-methyl D-aspartate, QUIS : quisqualate, SEM : standard errors of the mean.

sponse rates between the NMDA and QUIS groups in the first, third, or fourth week after intraspinal injection, but in the second week, response rates were higher in the QUIS group than in the NMDA group ($p<0.05$) (Fig. 1B).

Mechanical allodynia developed in 27% (4/15) of animals in the NMDA group and 57% (8/14) of those in the QUIS group, a difference that was not significant; no animals in the control group developed mechanical allodynia (Fig. 1C). The withdrawal thresholds for the ipsilateral hind paw in both QUIS and NMDA groups were significantly lower than those for the control group after intraspinal injection ($p<0.05$), but there was no significant difference between NMDA and QUIS groups throughout the observation period (Fig. 1D).

Excessive grooming behavior was initiated at second week following intraspinal NMDA or QUIS injections. The behavior was observed in 73% (11/15) of animals in the NMDA group, 36% (5/14) of those in the QUIS group, and none of those in the control group. The number of groomers was significantly higher in the NMDA group than in the QUIS group ($p<0.05$) (Fig. 2A), but the severity of grooming was not significantly different between NMDA and QUIS groups during any of the observation periods (Fig. 2B).

Morphological changes following intraspinal injection of NMDA and QUIS

Dilation of the central canal, ipsilateral neuronal loss of lamina, and intraspinal cavities developed following intraspinal injection of NMDA or QUIS (Fig. 3). The size of superficial and neck regions of the spinal dorsal horn were measured based on the histological reconstructions from spinal cords of seven animals per group, a reduction in sample size necessitated by mechanical damage to some tissue blocks during processing. The neck size of the dorsal horn of the NMDA and QUIS group, but not the superficial region, was significantly smaller than that of the control group ($p<0.05$). But, there was no significant difference between the NMDA and QUIS groups (Fig. 4).

DISCUSSION

Animals intraspinally injected with QUIS are used as a model for studying the development and maintenance of central neuropathic pain-like behaviors after SCI, based on the demonstrated involvement of elevated EAAs in damaged tissue[10,21]. When concentrations of EAAs increase dramatically, excessive receptor activation leads to prolonged periods of depolarization and initiates

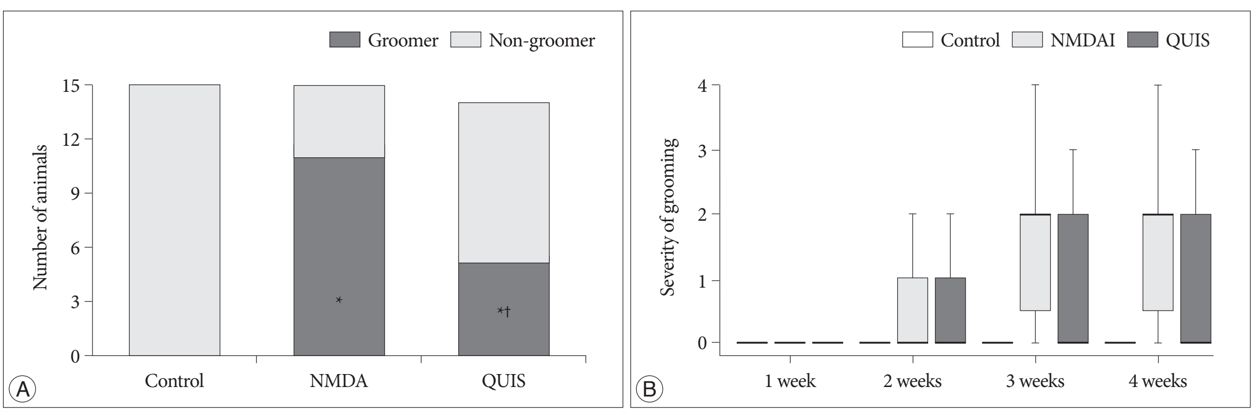

Fig. 2. Excessive grooming behaviors developed in animals that received intraspinal NMDA or QUIS injections. The number of groomers is significantly higher in the NMDA group than in the QUIS group (A), but there is no significant difference in the severity of grooming between the two groups (B). Data in A are expressed as number of animals; in B, boxes show interquartile ranges and bars denote 10th and 90th percentiles (*$p<0.05$ compared with the control group; †$p<0.05$ compared with the NMDA group). NMDA : N-methyl D-aspartate, QUIS : quisqualate.

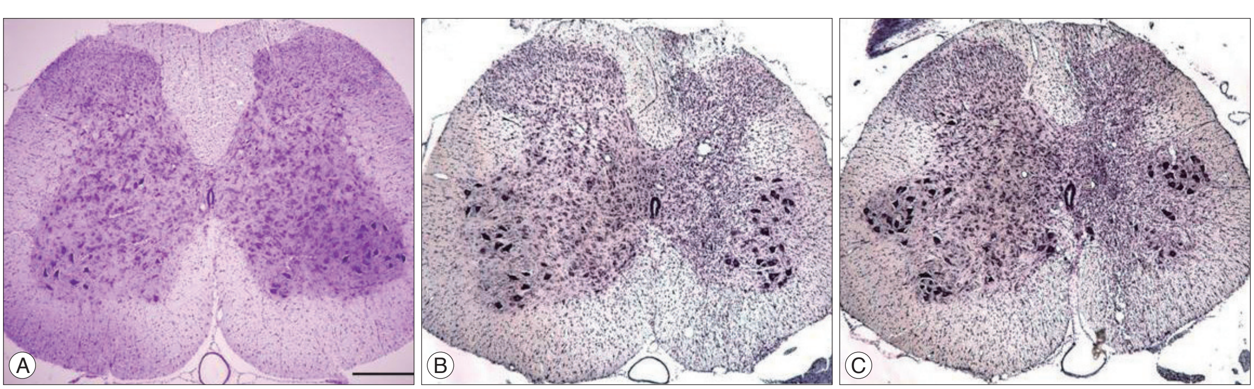

Fig. 3. Representative cresyl violet-stained spinal cord sections of sham-operated control rats (A), NMDA-injected rats (B), and QUIS-injected rats (C). Dilation of the central canal, ipsilateral neuronal loss of lamina III and V, and intraspinal cavities are seen in B and C. Scale bar in A (inset) equals 500 μm. NMDA : N-methyl D-aspartate, QUIS : quisqualate.

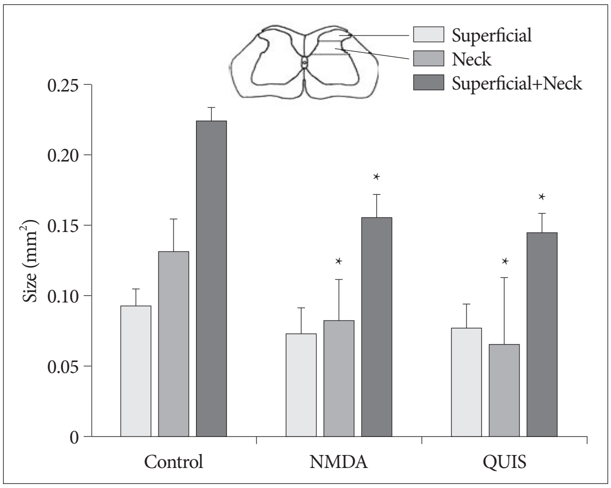

Fig. 4. The estimated size of the dorsal horn of the spinal gray matter is shown. The results are based on histological reconstructions from spinal cords of seven animals per group. Inset : schematic representation of the spinal cord showing the superficial (lamina I and II) and neck (lamina III and V) regions. Neuronal loss is observed in the neck region of the spinal dorsal horn following intraspinal injection of NMDA or QUIS. Values are mean±SEM (*p<0.05 compared with the control group). NMDA : N-methyl D-aspartate, QUIS : quisqualate, SEM : standard errors of the mean.

a cellular cascade that ultimately results in neuronal death. Neuronal death is associated with stroke, hypoxia-ischemia, and traumatic brain injury[3)], suggesting that increased levels of EAAs play an important role in producing pathological changes associated with ischemic or traumatic SCI[17,22)].

In models of evoked nociception following SCI, attempts to detect allodynia or hyperalgesia usually involve observation of nocifensive behaviors, such as flexor withdrawal of a stimulated limb or tail. In the present study, intraspinal injections of NMDA or QUIS resulted in cold and mechanical allodynia. One interesting finding of the current study is that cold and mechanical allodynia were more frequently observed in the QUIS group than in the NMDA group. A possible explanation for this finding is that the reflex arc responsible for the hind paw withdrawal response was more severely compromised in the NMDA group than in the QUIS group. Because NMDA receptors are present on the endothelial cells of the gray matter vasculature, damage to blood vessels in the gray matter can induce damage to the adjacent white matter[12)]. Siddall et al.[15)] reported that the incidence of allodynia was higher in rats with mild lesions and lower in rats with more extensive ones. At present, the exact source(s) of this discrepancy is (are) unclear. Additional studies will be needed to elucidate the effects of NMDA on lesion size and location following intraspinal injection.

In most animal models used to study chronic neuropathic pain, excessive grooming is regarded as an indication of dysesthesia and pain[18)]. Excessive grooming behavior reflects the presence of abnormal sensations rather than anesthesia (no sensation). However, overgrooming could occur in response to pain or a paresthetic (e.g., itching or tingling) or a dysesthetic (aversive, but not painful) sensation.[8)] In the current study, excessive grooming behavior developed in 73% of animals in the NMDA group, 35% of those in the QUIS group and none of those in the control group. Yezierski et al.[21)] reported excessive grooming behavior in the excitotoxic SCI model using QUIS, suggesting at-level pain and hypersensitivity to mechanical and thermal stimuli in the hindlimbs and indicating low-level pain. They were unable to conclude which features of a lesion were critical for producing mechanical hypersensitivity in the hindlimb and could not correlate the severity of cord damage with the magnitude of the mechanical hypersensitivity.

In addition, we observed central canal dilation, neuronal loss of lamina that spared the superficial lamina, and intraspinal cavities following intraspinal injection of NMDA or QUIS. It has previously been shown that morphologic changes in the spinal cord following QUIS injection are directly related to the injected volume of QUIS and survival duration[21)]. Previously, it has been reported that injection volumes of 1.2 μL resulted in more extensive neuronal loss than 0.6 μL, and spinal cavitation was generally larger in animals with longer survival times[22)]. In this study, we injected three tracks of 0.2 μL of NMDA or QUIS to avoid animal loss due to post-injection motor dysfunction. Because we positioned injection sites in the middle of the gray matter between laminae III and V, it can be assumed that pathological changes were confined to the neck of the spinal dorsal horn. There was no significant difference between NMDA and QUIS groups in the severity of neuronal loss, measured by the size of the dorsal horn and general characteristics of the spinal cord in the present study.

CONCLUSION

Intraspinal injections of NMDA or QUIS resulted in pathological changes in the spinal dorsal horn and evoked pain-related behaviors. Evoked pain behaviors-cold and mechanical allodynia-were more frequently observed in the QUIS group than in the NMDA group, whereas excessive grooming behavior was more frequent in the NMDA group. These results suggest that intraspinal injection of NMDA or QUIS can be used as an excitatory model of SCI for further research on spinal neuropathic pain.

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