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

Seong-Soo Choi, M.D., Ph.D.,¹ Kyung-Don Hahm, M.D., Ph.D.,¹ Hong-Gi Min, M.D.,² Jeong-Gil Leem, M.D., Ph.D.¹

Department of Anesthesiology and Pain Medicine,¹ Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea
Department of Anesthesiology and Pain Medicine,² Seogwipo Medical Center, Jeju Special Self Governing Province, Seogwipo, Korea

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 quisquulate (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 · Quisquate · 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. The pain, usually described as burning, stabbing, or electrifying, greatly affects patient's quality of life. 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. Tissue levels of EAAs are increased in areas of traumatic or ischemic SCI. Excitatory amino acids in the damaged tissue produce their toxic actions via both ionotropic and metabotropic glutamate receptors. 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, have prompted efforts to clarify the role of the NMDA receptor in producing excitotoxic injury in the spinal cord.

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. 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. Yezierski...
et al.20,21) have reported that intraspinal injection of the AMPA-
metabotropic receptor agonist, quinqueulate (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 SCI20. 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 injury21,22, there are no reports demonstrating pathologi-
ical 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 morpholog-
ic changes in the dorsal horn of the spinal cord after NMDA
or QUIS injection in rats. In addition, the profiles of pain be-
haviors 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 con-
trol 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 beta-
dine, 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, Har-
vard Apparatus Inc., USA).

Intraspinal injection was conducted by previously published
methods with modification21. After making a midline inci-
sion, 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 Hamil-
on 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)
digested 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 mM21. 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 re-
turned to their home cages.

Behavioral tests

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

For the assessment of cold allodynia20, 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 fre-
cuency of paw withdrawal was expressed as a percentage (the
number of paw withdrawals divided by the total number of tri-
als, times 100). Animals showing signs of cold allodynia on
more than two consecutive test sessions were interpreted as pos-
itive allodynia to cold stimuli.

Response to mechanical stimuli was tested with calibrated von
Frey filaments as previously published methods20, stimulus intens-
ities ranging from 0.5 to 50 g were applied six times to the gla-
brous skin of each hindpaw. Filaments were applied to the point
of bending, at which time evidence of responsiveness or non-re-
sponsiveness was determined. During each test session, the fila-
ment 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 mea-
sured withdrawal thresholds were less than 5 g was 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 catego-
rized into four classes (I-IV) as described previously21: 1) Class
I : hair removal over contiguous portions of a dermatome; 2)
Class II : extensive hair removal combined with signs of dam-
age 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 $\chi^2$ 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-
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 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).

**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\textsuperscript{10,21}. When concentrations of EAAs increase dramatically, excessive receptor activation leads to prolonged periods of depolarization and initiates

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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.
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.

CONCLUSION

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 dyesthetic (aversive, but not painful) sensation.19 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.20 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 duration21. 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 times22. 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.

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