Trigeminal Expression of N-Methyl-D-Aspartate Receptor Subunit 1 and Behavior Responses to Experimental Tooth Movement in Rats

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ABSTRACT

Objective: To test the hypothesis that peripheral N-methyl-D-aspartate (NMDA) receptors play a role in pain induced by experimental tooth movement.

Materials and Methods: Male Sprague-Dawley rats weighing between 200 g and 300 g were used in this study. Expression of NMDA receptors subunit 1 (NMDAR1) in the mandibular portion of the trigeminal ganglion (TG) was determined by Western blotting 4 hours and 1, 2, 3, 5, 7, and 14 days after tooth movement. Changes in the time taken by the rats on nocifensive behavior then effects of NMDA receptor antagonist MK-801 and force magnitude on these changes in behavior and NMDAR1 expression were evaluated.

Results: Experimental tooth movement led to a statistically significant increase in NMDAR1 expression at protein level from day 1 to 7 after force application initiating tooth movement. Time spent on nocifensive behavior dramatically increased from day 1 to 7. The rhythm in NMDAR1 expression in the TG and behavioral activities correlated well with the initial orthodontic pain responses. The magnitude of the nocifensive behavior and NMDAR1 expression were both force magnitude dependent and could be reduced by peripheral NMDA receptor antagonist MK-801.

Conclusions: The hypothesis is accepted. Peripheral NMDA receptors are modulated by experimental tooth movement and involved in the development of tooth movement pain. (Angle Orthod. 2009;79:951–957.)

KEY WORDS: NMDA Receptor; Experimental tooth movement; Trigeminal ganglion; Pain; Face grooming

INTRODUCTION

The N-methyl-D-aspartate receptor (NMDAR) is one of the glutamate receptors, a hetero-ligomeric complex of NMDA receptor subunits NR1, NR2 A-D, and NR3 A-B. The pharmacologic and physiologic characteristics of the NMDARs are determined by their subunit composition.1 Co-expression studies indicate that formation of functional NMDAR channels requires a combination of NR1 (NMDAR1), an essential channel-forming subunit, and at least one of the NR2 subunits.2 Considerable evidence has shown that NMDA receptors mainly contribute to the development and maintenance of central sensitization, which is considered an important component of inflammatory pain.3,4 Less well understood is the role of periphery NMDARs in the pain mechanism. Functional contribution of peripheral NMDARs in inflammatory pain has been investigated,5 and NMDARs undergo different transcriptional regulation. Inflammation pain induced by complete Freund’s adjuvant (CFA), for instance, demonstrates a significant increase in the number of axons labeled for NMDA receptors.6 Decreases in numbers

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of NMDAR1-labeled neurons were, however, reported in other research.\textsuperscript{7,8} Despite the accumulating data discussing the function of NMDA receptors in other pain models, especially in the central nervous system, the role of peripheral NMDA receptors in pain induced by tooth movement is still unclear.

The present study is aimed at validating the hypothesis that peripheral NMDA receptors play a contributory role in the development of tooth movement pain by documenting the changes in time spent on nociceptive-like behavioral responses and in NMDA receptor subunit 1 (NMDAR1 or NR1) expression in the trigeminal ganglion (TG) at the protein level in rats. Effects of NMDA receptor antagonist MK-801 on these changes were also investigated.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing between 200 g and 300 g were used in the study. They were housed in standard clear plastic cages with soft bedding and had free access to food and water ad libitum in a thermoregulated room at 25°C ± 2°C with a 12-hour light-dark cycle, lights on at 0600 hours and off at 1800 hours for at least 5 days prior to commencement of the experiment.

The study was approved by the ethical board of Sichuan University. Guidelines for investigations of pain in animals, given by The International Association for the Study of Pain, were followed,\textsuperscript{9} and steps were taken to minimize both the animal numbers and discomfort.

Appliance for Experimental Tooth Movement

A fixed, Ni-Ti alloy closed-coil spring appliance was constructed for mesial movement of the left and right mandibular first molar as described by Nakamura et al.\textsuperscript{10} After an intraperitoneal injection of sodium pentobarbital at a dose of 40 mg/kg body weight, a 40-g force was applied on the experimental animals. The sham-treated rats received the same procedures as the experimental rats, but the springs in their mouths were not activated.

Behavior Testing

Face-grooming directed to the irritated area has been shown to be a nociceptive response to orofacial pain\textsuperscript{11,12} and experimental tooth movement pain in rats.\textsuperscript{13} Our preliminary study demonstrated that the directed face-grooming behavior of wiping the mouth was the most robust behavioral response. Therefore, we quantified and presented our findings associated with the directed face-grooming behavior of mouth wiping.

The face-grooming activity was monitored in a transparent plastic cage (30 × 30 × 30 cm) in a room with a 45-dB background noise between 0900 hours and 1200 hours. The behavior was videotaped for 10 minutes each time,\textsuperscript{11} starting 15 minutes after placement in the cage. Each animal was videotaped three times at 20-minute intervals. Videotaped behavior was analyzed offline by an investigator blind to the information of the rats.

Part A: Assessment of Behavioral Responses After Initiation of Experimental Tooth Movement

Forty-nine rats were videotaped on days 1, 3, 5, 7, and 14 after force application. Our preliminary study has demonstrated that the behavioral responses and expression of NR1 in sham-treated rats were not changed a lot along with the progress. Therefore, we designed one sham group to reduce the experiment’s cost and minimize the animal numbers, according to the guidelines for pain research\textsuperscript{9} (Table 1, Part A).

Part B: Assessment of Behavioral Responses and Expression of NMDAR1 After Experimental Tooth Movement Induced by Different Forces

A force of 20 g, 40 g, and 80 g was applied to rats for 1 day after experimental tooth movement. Following behavior observation, they were killed under deep anesthesia, and ipsilateral TGs were collected for Western blotting. The procedure for observation was the same as used in part A. (See Table 1, Part B.)

Part C: Effect of NMDA Receptor Antagonist MK-801 on Behavioral Responses and NMDAR1 Expression

An experimental group (n = 8) received an injection of noncompetitive NMDA receptor antagonist MK-801 sodium (Sigma, St Louis, MO) at an interval of 8 hours, 0.3 mg/kg, dissolved in 0.9% saline in a volume of 100 μL into the periodontal ligament of the two lower incisors and first molars for 1 day after experimental tooth movement began. The dosage of MK-801 used has proved effective in previous studies.\textsuperscript{14} The control group (saline, n = 8) received 100 μL normal saline locally like the MK-801 injection. After behavioral evaluation, the expression level of NMDAR1 was studied by immunohistochemistry (IHC) in TG. The procedure of the IHC is described below. (See Table 1, Part C.)

The animals were lightly anesthetized by inhalation of halothane to allow the injection with a 26-gauge needle. The face-grooming activities were videotaped according to the procedure described earlier, 20 minutes after the final injection.
Table 1. Illustration of Experimental Design, it Demonstrates the Groups, Animal Numbers and the Time Frame in Each Section.

<table>
<thead>
<tr>
<th>Part</th>
<th>Rat Group</th>
<th>Animal Total Number</th>
<th>Animal Number at Each Time Point</th>
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<tr>
<td>A</td>
<td>Untreated</td>
<td>12</td>
<td>12</td>
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<tr>
<td>(Behavior)</td>
<td>Experimental (40-g force)</td>
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<td>5</td>
</tr>
<tr>
<td>B</td>
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<td>8</td>
</tr>
<tr>
<td>(Behavior + WB)</td>
<td>80-g (Behavior + WB)</td>
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<td>8</td>
</tr>
<tr>
<td>C</td>
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<td>8</td>
<td>8</td>
</tr>
<tr>
<td>(Behavior, MK-801)</td>
<td>MK-801 + force</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>Sham-treated</td>
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<td>8</td>
</tr>
<tr>
<td>(WB)</td>
<td>Experimental (40-g force)</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
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<td>8</td>
<td>8</td>
</tr>
<tr>
<td>(IHC)*</td>
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<td>8</td>
</tr>
<tr>
<td></td>
<td>MK-801 + force</td>
<td>8</td>
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</tbody>
</table>

* WB indicates Western blotting; IHC, immunohistochemical.

Part D: Western Blotting

Under deep anesthesia, the caudal one-third of the left and right trigeminal ganglia was collected together since this portion has been identified as the mandibular division. Following the determination of the total protein, tissue lysates were fractionated by 15% SDS–PAGE and transferred onto nitrocellulose (NC) membrane, then incubated with primary antibody (polyclonal rabbit antisera against NMDAR1, Abcam, 1:500) and secondary antibody (Zhongshan Biotech Co, Beijing, China; 1:5000). β-Tubulin (Zhongshan Biotech) was used as an internal control protein. Band intensity was assessed using ImageQuant 5.2 software, (Amersham Biosciences, Piscataway, NJ) and the protein level of NMDAR1 was normalized to that of β-ubulin in the same sample. Data were expressed as mean ratio ± standard error of the mean (SEM). (See Table 1, Part D.)

Part E: NMDAR1 Immunohistochemical Staining in TG

Under deep anesthesia, the rats were perfused transcaldially with cold 1% paraformaldehyde in PBS for 1 minute and then with cold 4% paraformaldehyde in PBS for an additional 20 minutes. Finally, TGs were removed and stored in liquid nitrogen. TGs were serially cut at a thickness of 5 μm with a cryostat along the axis. Eight sections were randomly selected among these serial sections for immunohistochemical staining in each specimen according to the procedure described by Alavi et al. The sections were first incubated with rabbit anti-NMDAR1 polyclonal antiserum (Abcam, Cambridge, UK), diluted 1:250 for 3.5 hours at 37°C, then with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Zhongshan Biotech) at a concentration of 1:100 in PBS. To confirm specificity of labeling using the anti-NMDAR1 antibody, normal goat serum and PBS were substituted for the primary antibody. Finally, three fields were selected in each section, and the integrated optical density (IOD) measured by Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD) was used for the statistical analysis (Table 1, Part E).

Statistical Analysis

Statistical analysis was done using SPSS version 11.5 (SPSS Inc, Chicago, IL). One-way analysis of variance (ANOVA; least significant difference [LSD] or Student-Newman-Keuls [SNK]) or Student’s t-test was used for the analysis. The level of statistical significance was P < .05. Data were displayed as mean values ± SEM.

RESULTS

Time Course of the Changes in NMDAR1 Expression Following Experimental Tooth Movement at the Protein Level

A band of NR1 subunit was shown near to 120 KD. We found a gradual increase of NR1 expression that reached a statistically significant value on days 1, 2, 3, 5, and 7, peaking on day 7 (one-way ANOVA, LSD; P < .01). (See Figure 1.)

Directed Face-Grooming Behavior After Experimental Tooth Movement

Mouth wiping behavior could be regulated by experimental tooth movement and significantly increased on days 1, 3, 5, and 7, but not on day 14 when compared with the sham-treated group (one-way ANOVA, LSD; P < .01). It seemed to reach a maximum on days 1 and 3. (See Figure 2.)
Figure 1. The time course of changes in expression of NMDAR1 following experimental tooth movement in TG. Following experimental tooth movement, the expression of NMDAR1 was increased to statistically significant levels from 1 to 7 days at the protein level. ** $P < .01$; *** $P < .001$; **** $P < .0001$. Error bars represent standard error of the mean (SEM).

Figure 2. Nocifensive behavioral responses after experimental tooth movement. Experimental tooth movement significantly increased the time spent on mouth wiping on days 1, 3, 5, and 7, and the increase was almost in harmony with the initial orthodontic pain responses clinically. ** $P < .01$. Error bars represent standard error of the mean (SEM).

The Effects of Increasing Forces on Behavior Responses and Expression of NMDAR1 After Experimental Tooth Movement

The changes of expression of NMDAR1 in TG increased along with the increased force. A significant difference was observed among the three groups (one-way ANOVA, SNK; $P < .05$). The difference of behavioral responses was statistically significant between the 20-g and 40-g/80-g groups (one-way ANOVA, SNK; $P < .05$). Although not statistically significant, a difference was observed between the 40-g and 80-g groups, with the heavier force group spending a longer time on the nocifensive behavior. (See Figure 3.)

Effects of NMDA Receptor Antagonist MK-801 on Behavioral Responses and NMDAR1 Expression

Peripheral administration of MK-801 produced a marked statistically significant (Student’s $t$-test: $P < .0001$) reduction in behavioral responses on day 1 after experimental tooth movement. The immunohistochemical study indicated that the immunoreactive cells were mainly expressed in small- and medium-sized cells and some in large cells in the mandibular territory of the TGs. The increased immunostaining could be reduced by local injection of MK-801. A statistically significant difference was found between the experimental and control group (one-way ANOVA, SNK; $P < .05$). (See Figure 4.)
DISCUSSION

It has been previously demonstrated that orthodontic pain is correlated with an increase in inflammatory mediators such as prostaglandins, substance P (SP),17 and calcitonin gene-related peptide (CGRP).18 Gianopoulou et al17 found that the central NMDA receptors play a contributory role in the development and maintenance of experimental tooth movement pain in rats. The role of periphery NMDA receptors is, however, still unclear.

It has been recognized that tooth movement would lead to periodontal damage and inflammatory changes,19 thus we did not cover this issue. Considerable evidence has demonstrated that NMDA receptors are involved in the central or peripheral mechanism of inflammatory pain in other models.2,3,8 Similarly, the changes of NMDAR1 in our study may be correlated with the periodontal inflammation induced by experimental tooth movement.

Previous studies have demonstrated that the number of NR1-immunostained neurons is reduced by peripheral inflammation.7,20 However, along with the overall regression of inflammation following injection of CFA, a significant increase in the proportion of NR1-labeled unmyelinated axions6 and of the percentage of NR1-labeled DRG neurons21 was found. In the present study, experimental tooth movement resulted in a significant increase in the expression of NR1 in TG. Our findings are in agreement with the latter research. The discrepancy may be related to different noxious stimuli in different models. In previous studies, noxious chemicals such as CFA were used at different sites with different concentrations.

The patterns of face-grooming activity provoked by local irritation or by noxious stimulation could be distinguished from that related to the maintenance of the pelage, thermoregulation, or social signaling. In this case, the grooming activity increases significantly, especially at the painful body area,11,12 and it appears to be aimed at removing the cause of pain. Directed face grooming has been proved to be a reliable sign of orofacial pain in freely-moving rodents.11–13

In the present study, the time course of NMDAR1 regulation and the pain and discomfort in patients undergoing orthodontic treatment is similar. Clinically, initial orthodontic pain and discomfort starts from day 1 to 2 continuing through days 3 to 7.22 The rhythmic change of NMDAR1 also correlates well with the directed face-grooming activity. Therefore, the increase in NMDAR1 in TG is probably associated with this pain sensation. Our research focused on the initial stage of orthodontic pain, so the timeframe was mainly set between days 1 and 7. However, the omission of other time points should be considered in further research.

The possible role of periphery NMDA receptors in orthodontic pain is further verified by local application of MK-801. The increased nocifensive behavior was attenuated by peripheral injection of MK-801 and the same with the NMDAR1 expression. This finding suggests that MK-801 prevents activation of nociceptors such that their input does not reach the trigeminal ganglion. It has been previously demonstrated that activation of peripheral NMDARs resulted in pain and mechanical allodynia or hyperalgesia,23,24 and that these nociceptive behaviors could be blocked by peripherally applied NMDA receptor antagonists.25 Despite the presence of other inflammatory mediators in the periodontal tissues, blockade of NMDA receptors reverses the wiping behavior. This indicates that peripheral NMDA receptors contribute to the nociceptive behavior.
induced by experimental tooth movement and suggests a possible functional interaction between NMDA receptors and various inflammatory mediators. Immunohistochemistry reveals that NMDAR1 is present mainly in small- to medium-sized neurons in the mandibular territory of TGs, which also suggests a contributory role of NMDAR1 in the orthodontic pain mechanism.

Furthermore, both the expression of NMDAR1 and directed face-grooming behavior are modulated in a force magnitude-dependent manner. Similar behavioral responses were observed after applying capsaicin or formalin into the orofacial area of rats. C-fos expression after experimental tooth movement has also been found to be linked to the force magnitude. A possible explanation is that heavier force will cause greater inflammation leading to more glutamate release and NMDAR activation and behavior changes accordingly. As NMDAR1 has been identified as the essential channel-forming subunit of the NMDA receptors, our findings suggested that the development and maintenance of pain induced by tooth movement may be partly due to the functional plasticity of NMDA receptors that occurs in the TG neurons.

CONCLUSIONS

- Peripheral NMDA receptors are modulated by experimental tooth movement and play a role in the development of orthodontic pain.
- Targeting peripheral NMDA receptors should be effective and may avoid the side effects that occur following exposure of the central nervous system to NMDAR antagonists.

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REFERENCES


