Gene Knockdown of the N-Methyl-d-Aspartate Receptor NR1 Subunit with Subcutaneous Small Interfering RNA Reduces Inflammation-induced Nociception in Rats

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ABSTRACT
Background: Spinal N-methyl-d-aspartate receptors have been demonstrated to play an important role in the facilitation and maintenance of nociception. To avoid adverse effects of blocking N-methyl-d-aspartate receptors in the central nervous system, blocking N-methyl-d-aspartate receptor in peripheral nervous system is an ideal alternative. Transfection of small interfering RNAs (siRNAs) into cells has been revealed to provide potent silencing of specific genes. In this study, the authors examined the effect of subcutaneous injection of siRNA targeting the NR1 subunit of the N-methyl-d-aspartate receptor on silencing NR1 gene expression and subsequently abolishing inflammatory nociception in rats.

Methods: Male Sprague-Dawley rats received intradermal injection of NR1 siRNA and underwent injection of formalin or complete Freund’s adjuvant. The flinch response and mechanical hypersensitivity by von Frey filaments were assessed. Then the messenger RNA and protein of NR1 in skin and dorsal root ganglion were analyzed.

Results: The results revealed that subcutaneous injection of 1 nmol NR1 siRNA effectively diminished the nociception induced by formalin and complete Freund’s adjuvant stimuli and attenuated the level of NR1 messenger RNA and protein in skin and ipsilateral dorsal root ganglion. The antinociceptive effect and the inhibition of NR1 expression persisted for about 7 days after administration of NR1 siRNA.

Conclusions: The data of this study suggest that NR1 siRNA has potential therapeutic value in the treatment of inflammatory pain induced or maintained by peripheral nociceptor activity and support the potential application of this method to the study of nociceptive processes and target the validation of pain-associated genes.

What We Already Know about This Topic
• Sensory nerves express receptors for N-methyl-d-aspartate (NMDA), and peripheral release of glutamate can stimulate these receptors to induce sensitization and pain

What This Article Tells Us That Is New
• In rats, intradermal injection of small interfering RNA to a subunit of NMDA receptors was incorporated into sensory nerves, reducing their expression of NMDA receptors and their responses to sensitizing stimuli

S PINAL glutamate receptors including N-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazolone-4-propionic acid, and kainate variants have been demonstrated to play important roles in the facilitation and maintenance of nociception.1–5 Most of the currently available glutamate receptors antagonists cross the blood–brain barrier and produce undesirable side effects by blocking glutamate receptors in the central nervous system. The development of peripheral glutamate antagonists that do not interfere with central glutamate processing could provide novel approaches to the treatment of pain of peripheral origin. There is substantial evidence that NMDA receptors are located on sensory axons in the skin.6 Intraplantar injection of formalin results in nociceptive behaviors, which are attenuated after local injection of the NMDA antagonist MK-801.7 Complete Freund’s ad-
Cajal rat (CFA) is another inflammation-producing chemical used to produce long-lasting pain responses that mimic clinical pain in humans. The injection of CFA into the rat hind paw has been shown to produce mechanical hyperalgesia accompanied by long-lasting inflammation in the injected hind paw. A previous study has demonstrated that NMDA receptors play an important role in mediating the development of mechanical hyperalgesia after intraplantar CFA injection.

Several genes encoding NMDA receptor subunits have been identified. These genes fall into three categories: the NMDA receptor 1 (NR1), NMDA receptor 2 (NR2), and the NMDA receptor 3 subunits. The NMDA receptor is an ion channel and cannot function in the absence of the NR1 subunit. NR1 is ubiquitously expressed in the brain and spinal cord, and NR2 subunits are regionally localized. The level of ionotropic glutamate receptors, particularly NR1, in a number of sensory axons increases during inflammation, which contributes to peripheral sensitization in inflammation.

RNA interference is an evolutionarily conserved, posttranscriptional gene silencing mechanism mediated by small double-stranded RNA molecules, namely small interfering RNA (siRNA). Since its discovery in 1998, RNA interference has been developed as a powerful technique for functional investigation of protein function and target validation. siRNA mediates messenger RNA (mRNA) cleavage by forming an mRNA-degrading sequence-specific endonuclease complex, also referred to as the RNA-induced silencing complex, which effectively down-regulates the synthesis of the encoded protein. siRNA can transiently inactivate a single gene and, therefore, can inactivate receptor functions in a more specific and selective manner than receptor antagonists. Although intrathecal delivery of synthetic siRNAs has successfully achieved silence of molecular targets in various models of neurologic disease, including pain, no study has demonstrated an antinociceptive effect by subcutaneous delivery of siRNA to target specific receptors that mediate nociception in vivo. In this study, we examined the effect of gene silencing and antinociception in a rat model of formalin- and CFA-induced nociception after subcutaneous injection of synthetic siRNA targeting the NR1 subunit of the NMDA receptor.

Materials and Methods

Animals
Sprague-Dawley rats (n = 151), weighing 250–350 g, were housed 2 per cage. The cages were placed in the laboratory 24 h before testing for acclimatization. The rats were fed a standard laboratory diet and tap water ad libitum and kept at 23°C ± 1°C with a 12-h light–dark cycle. All animal protocols followed the guidelines for pain research and were approved by the Institutional Review Board of the I-Shou University, Kaohsiung, Taiwan.

Synthesis of siRNA
The rat NR1 subunit sequence was obtained from GenBank (accession number U11418; 2957 base pairs). siRNAs targeting rat NR1 were synthesized using a Silencer siRNA Construction kit (Ambion, Austin, TX). The target sequence of sense and antisense strands of NR1-1, NR1-2, and NR1-3 begins at nucleotide 278, 512, and 957. A mismatched NR1-1 siRNA (MM-NR1-1) without significant homology to any known rat gene sequence was obtained from Ambion. The siRNA sequences are as follows:

NR1-1: 5'-ACCAGGCCAAUAGGCAATT-3' and 3'-TTUGGUCGGGUUAAUUCAGCU-5'
NR1-2: 5'-UGUCCAAUCUACUGACAAUU-3' and 3'-UUACGGAUAGAGACUGCUU-5'
NR1-3: 5'-UGGCAAGAAUGAGUCAGCCUU-3' and 3'-UUACGCGUUUACUGACGCG-5'
MM-NR1-1: 5'-ACCAGGCGCAAACAGGACATT-3' and 3'-TTUGGUCGGGUUUGACGCUU-5'

Polymer Conjugate Synthesis
Polyethyleneimine (100 mM) was purchased from Fermentas Inc. (Glen Burnie, MD) and was used without further purification. The relative amount of RNA to carrier was 2 μl of 100 mM polyethyleneimine solution per nanomole of RNA. RNA-polymer complexes or polyethyleneimine alone was diluted with 5% dextrose in water to a total volume of 100 μl and allowed to form for 10 min at room temperature before injection.

Intradermal Injection of siRNA
The rats were randomly assigned to different groups: (1) test of different sequences: three different sequences of NR1-1, NR1-2, NR1-3 siRNA (1 nmol, n = 6 in each group). The vehicle group (2 μl polyethyleneimine, n = 6) served as the control group. (2) Test of doses: three doses of NR1-1 siRNA (0.5, 1, and 2 nmol, n = 6 in each group). Rats injected subcutaneously with 100 μl saline, 2 μl polyethyleneimine, or 1 nmol MM-NR1-1 siRNA (n = 6 in each group except n = 8 in 1 nmol MM-NR1-1 siRNA group and 1 nmol NR1-1 siRNA group) were included as control groups. All treatments were administered 3 days before formalin injection. The rats were further evaluated by rotarod performance before the administration of formalin in the control groups and 1 nmol NR1-1 siRNA group. To exclude the possible systemic effects of local injection of NR1-1 siRNA, animals (n = 6) received 1 nmol NR1-1 siRNA in one paw, followed by a formalin assay in the contralateral paw 3 days later. Skin tissue of six rats in each group included in the above test of sequence and doses were dissected immediately after the formalin test in each group for use in real-time polymerase chain reaction (PCR), western blotting of NR1. Rat skin tissues from 1 nmol MM-NR1-1 siRNA and 1 nmol NR1-1 siRNA groups (n = 2 each group) were dissected for immunohistochemical staining of NR1. (3) Time course tests: 3-, 7-, 14-, and 21-day recovery groups (injection of 1 nmol NR1-1
siRNA 3, 7, 14, and 21 days before formalin testing, n = 6 rats in each group). The vehicle groups (2 μl polyethyleneimine, n = 6 at each time period) served as the control. Skin tissues of six rats in each group were dissected immediately after the formalin test in each group for use in real-time PCR and western blotting of NR1. In 3-day groups of NR1-1 and polyethyleneimine, skin tissues were further used for real-time PCR analysis of NR2A, B, C, D, and interferon-α. (4) CFA test groups: rats received subcutaneous injection of CFA 2 days after subcutaneous injection of 1 nmol NR1-1 siRNA, 1 nmol MM-NR1 siRNA, 100 μl saline, or 2 μl polyethyleneimine (n = 5 in each group). Before any treatment, the baseline data of 50% withdrawal threshold were recorded. Mechanical hyperalgesia assay was performed 1 day after injection of CFA. To exclude the systemic effects of siRNA, another group of rats (n = 5) received injection of CFA on the contralateral paw 2 days after intradermal injection of 1 nmol NR1-1 siRNA and measurement of mechanical hyperalgesia 1 day after injection of CFA. Skin tissue was dissected immediately after the CFA test in each group for use in real-time PCR of NR1. (5) Expression of NR1 in dorsal root ganglion (DRG): eight rats were injected subcutaneously with 1 nmol NR1-1 siRNA in the left paw. Left and right L5 DRGs of rats were dissected 3 days later for use in immunohistochemical staining of NR1 (n = 4) and use in real-time PCR (n = 4).

Behavioral Tests
All behavioral testing was conducted in each treatment group by a blinded observer. To evaluate motor coordination, each rat was first trained on the morning before the test to remain on the rod was measured. A cutoff of 60 s was set at 40 r/min. The length of time (in seconds) that the rat was first trained on the morning before the test to remain on a rotarod revolving at 12 r/min. The rats were then placed on a rotarod by a blinded observer. To evaluate motor coordination, each rat was observed, and the number of paw flinches was counted during the first 5 min, and then for 1 min in every 5 min until 60 min after formalin injection.

CFA-induced inflammation was accomplished by injecting 0.1 ml CFA (Sigma, St. Louis, MO) into the subcutaneous space in the center of the plantar hind paw after animals had been anesthetized (2–3 min) with 2.5% isoflurane. Before the mechanical hyperalgesia assay, all animals were habituated to the testing environment for 2 days. To test for mechanical sensitivity, animals were put in a plastic box (11 × 13 × 24 cm) on an elevated metal mesh floor and allowed 30 min for habituation. Mechanical paw withdrawal thresholds were determined using the methods described by Chaplin et al. The hind paw was pressed with one of a series of von Frey hairs with logarithmically incremental stiffness (0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g; Stoelting, Wood Dale, IL) presented perpendicular to the plantar surface for 4–5 s for each hair. The 50% withdrawal threshold was determined using the up–down method by Dixon. The animals were tested 1 day before injection of treatment agent for baseline data and then 1 day after injection of CFA.

RNA Isolation and Real-time PCR
RNA was isolated and purified from skin and DRG using the total RNA Mini Kit (Tissue; Geneaid Biotech Ltd., Sijih City, Taiwan). Complementary DNA synthesis was performed by reverse transcription of each sample using random hexamer primers and the high-capacity complementary DNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA). Real-time PCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems Inc.) with SYBR Green detection in a two-step reaction. The following PCR program was used: stage 1, 50°C, 3 min; stage 2, 95°C, 10 min; stage 3, 50 cycles, each consisting of 15 s at 95°C; and 45 s at 60°C. The program ended at 25°C. β-Actin was used as a reference gene. It has been shown that the subunits NR1 and NR2 but not NR2D of the NMDA receptor participate in the development of formalin-induced nociception; therefore, we analyzed the mRNA level of NR1 and NR2 subunits of the NMDA receptor. To exclude the induction of the interferon response, we also analyzed the mRNA level of interferon-α. PCR primers for NR1 and NR2 subunits of the NMDA receptor and interferon-α were used as given in table 1. For real-time PCR, 12.5 μl of 2× SYBR Green PCR Master Mix (ABI, Foster City, CA) and 1.0 μl (10 μmol) of the desired primer mixture were added to the cDNA templates to reach a final volume of 25 μl. The PCR setup was singleplex, that is, the target and reference genes were detected in separate tubes. A “no-template” control was used for each primer pair consisting of water (sterile and ultraviolet cross-linked).

Results of the PCR analysis were calculated as threshold cycle (CT) values and were used to determine the amount of target gene mRNA in relation to the amount of reference gene mRNA. ΔC_T indicated the difference between the number of cycles necessary to detect the PCR products for NR1, each NR2 subunit, interferon-α, and the reference gene. ΔΔC_T was the difference between the ΔC_T of the control group (polyethyleneimine, saline alone group, or contralateral DRGs) and the ΔC_T values of the other injection groups or ipsilateral DRGs. Data were expressed as 2^-ΔΔC_T to give an estimate of the amount of target mRNA present in the tissue relative to the control group.

Western Blots
Total proteins from skin tissue were prepared by the addition of 1:20 dilution of T-PER Tissue Protein Extraction Reagent (PIERCE, Rockford, IL) (25 mM bicarbonate and 150 mM sodium chloride [pH 7.6]) containing protease inhibitors (100 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 80 μM aprotinin, crystalline, 5 mM bestatin, 1.5 mM...
E-64, protease inhibitor, 2 μM leupeptin, and 1 mM pepstatin A). The tissue was homogenized with a homogenizer. After being placed on ice for 30 min, the homogenate was centrifuged at 12,000 r/min, for 30 min at 4°C. The supernatant was collected and assayed for protein content using the Quant-iT Protein Assay kit (Invitrogen, Carlsbad, CA) and stored at -20°C until further use. Total protein (30 μg) was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel using sample buffer, running buffer, and molecular weight standards, as suggested by the manufacturer. After electrophoresis, the proteins were transferred to a polyvinylidine fluoride membrane and blocked with 5% nonfat dry milk.

The primary antibody (1:2,000 dilution of rabbit polyclonal antiguamotamin receptor NR1; Sigma) was added and allowed to incubate overnight at 4°C in fresh blocking buffer. The membranes were washed for 30 min in washing buffer at room temperature before the secondary antibody (1:5,000 dilution of horseradish peroxidase-coupled goat antirabbit immunoglobulin G; Chemicon, Billerica, MA) was added and allowed to incubate for 1.5 h at room temperature in blocking buffer. The membranes were washed in washing buffer for another 30 min, and the antibodies were then detected using Western Blot Chemiluminescence Reagent Plus (Millipore, Billerica, MA). For densitometric analyses, blots were scanned and quantified with Image-Pro Plus analysis software (MediaCybernetics, Silver Spring, MD), and the results were expressed as the ratio of NR1 immunoreactivity to β-tubulin immunoreactivity.

**Immunohistochemistry of Skin and DRG**

The rats were anesthetized with pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with 0.1 M phosphate-buffered saline solution (400 ml) followed by perfusion of 4% paraformaldehyde in phosphate-buffered saline solution. After 2 h, skin or DRG tissue was dissected and postfixed in 4% formalin in phosphate-buffered saline for 4 h. After postfixation, the tissue was cryoprotected with 30% sucrose overnight. Frozen sections (10 mm) were blocked with Image-iT Fix signal Enhancer (Invitrogen) for 1 h at room temperature, followed by overnight incubation at 4°C with rabbit polyclonal antiguamotamin receptor NR1 (1:100; Sigma). After overnight incubation, the sections were incubated for 1.5 h at room temperature in conjugated goat anti-rabbit immunoglobulin G fluorescein (1:2,000 dilution; Chemicon). Image analysis was performed with a Nikon eclipse E800 fluorescence microscope (Nikon instech company, Kawasaki, Japan) coupled to a cool digital camera (Diagnostic instruments Inc., Sterling Heights, MI) and an image-analyzing system (advanced spot software; Diagnostic instruments Inc.).

**Image Analysis**

All tissues were analyzed by someone who was blinded to experimental conditions. In skin sections, four NR1 antibody-stained sections were selected from each rat of two rats in each group to analyze the ratio of average of NR1-positive intensity to an average intensity of the whole epidermal layer. In DRG sections, three NR1 antibody-stained sections were selected from the left and right L5 DRGs of four rats sequentially to analyze fluorescence intensity. Average NR1 fluorescence intensity was measured for every NR1-positive neuron using Image-Pro Plus analysis software (MediaCybernetics, Silver Spring, MD) and then normalized against the fluorescence intensity of the entire image of the same section to obtain a measure of relative fluorescence for each neuron. Signals were analyzed under 200× magnification.

**Statistical Analysis**

All data are expressed as the mean ± SD or median (upper and lower quartiles). To assess the formalin-induced flinching response, the main effects of different time points and treatments and interaction of treatment × control effect were analyzed by two-way ANOVA. Then, one-way

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**Table 1. Primers Used for Real-time Polymerase Chain Reaction**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>NR1</td>
<td>Forward</td>
<td>5′-GCC ACT CCC GCA GCA AT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CCC CTG CCA TGT TCT CAA AA-3′</td>
</tr>
<tr>
<td>NR2A</td>
<td>Forward</td>
<td>5′-TCC ACT CAA GGA ATC TTG TGA GAT AT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ACT TGC CCA TGT GTA TTT ATT TGT TT-3′</td>
</tr>
<tr>
<td>NR2B</td>
<td>Forward</td>
<td>5′-AAC CCT CGT GGC CAG CA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGT GGA CAG ATG CGG GAA-3′</td>
</tr>
<tr>
<td>NR2C</td>
<td>Forward</td>
<td>5′-GCC CCA GTT TTG GAC CTT AGT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CCT GTG ACC ACC GCA AGA G-3′</td>
</tr>
<tr>
<td>NR2D</td>
<td>Forward</td>
<td>5′-AGG GTT TCT GCA TTT ATC TTC TGA A-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TGA CCA ATC ATG CCA TTC CA-3′</td>
</tr>
<tr>
<td>Interferon-α</td>
<td>Forward</td>
<td>5′-CTT GGC TGT TTG CCC CAT T-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CGT GAC AGT AGC TGC GGT TCC-3′</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>5′-GGT GGA CAG ATG CGG GAA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CAC GCC TGG TCA GGA TCT TC-3′</td>
</tr>
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</table>

The NR1, NR2A-D subunit, interferon-α, and β-actin primer sequences were derived from the National Center for Biotechnology Information (Bethesda, MD) nucleotide sequence (accession numbers U11418, AF001423, NM_012574, NM_012575, NM_022797, NM_203410, and NM_031144, respectively).
ANOVA was applied to compare group differences after NR1 siRNA treatment at each time point. All tests were followed by Student–Newman–Keuls test as the multiple comparison analysis. Data of NR1 mRNA and western blot in the time course study, in the DRG, and in the analysis of NR2 subunits and interferon-α for confirmation of specificity were analyzed by the two-tailed Student t test. The data from the CFA test were analyzed using the paired Student t test. The data from the rotarod test and mRNA and western blot of NR1 subunit in each group, except in the time course study, were analyzed using one-way ANOVA with Dunnett post hoc test to determine significant differences between groups. Group differences in ratio of mean fluorescence intensity of NR1 immunostaining were compared with the Mann–Whitney rank-sum test. A P value of less than 0.05 was considered statistically significant. Analysis was performed with SPSS software (version 14.0; SPSS Inc., Chicago, IL).

Results

Effects of Knockdown of the NR1 Subunit on Motor Coordination and CFA Stimuli

The rotarod test measures the ability of a rat to maintain balance and motor coordination. Motor coordination was not affected in rats after intradermal administration of NR1-1 siRNA, MM-NR1-1 siRNA when compared with rats in the vehicle- (polyethylenimine) or saline-treated groups (fig. 1A). We further examined the antinociceptive effect of NR1-1 siRNA after CFA stimuli. Compared with the baseline value in each group, a significant decrease in 50% withdrawal threshold of paw withdrawal from a painful CFA stimulus was noted in all groups with the exception of rats that received intradermal injection of NR1-1 siRNA (fig. 1B).

Injection of NR1 siRNA Reduces Formalin-induced Pain Response

Subcutaneous injections of 1% formalin produce two phases of nociceptive behavioral patterns. The first phase (acute phase) begins immediately after formalin injection and lasts 3–5 min, followed by a period of 10–15 min when the animals display little nociceptive behavior. The second phase (tonic phase) starts approximately 15–20 min after formalin injection and lasts for 20–40 min.

An initial analysis of two-way ANOVA showed that the treatment effect, the time effect, and the interaction of time × treatment were all significantly different (all P < 0.05). Then, analysis of one-way ANOVA revealed that NR1-1 siRNAs attenuated first phase and second phase behaviors. The number of flinches were decreased at 5 min and during the period of 20–40 min in rats that received intradermal injection of 1 nmol NR1-1 and NR1-2 siRNA compared with rats that received intradermal injection of 2 μl polyethylenimine or 1 nmol NR1-3 siRNA (fig. 2A). In the dose–effect study, the number of flinches was decreased at 5 min and during the period of 20–45 min in rats after administration of 1 nmol and 2 nmol but not 0.5 nmol NR1-1 siRNA compared with rats that received intradermal injection of 2 μl polyethylenimine or 1 nmol MM-NR1-1 siRNA or 100 μl saline (fig. 2B). No significant antinociceptive effect was noted in the rats that received an intradermal injection of formalin in the paw contralateral to the paw injected with NR1-1 siRNA (fig. 2B). Thus, the possibility that the antinociceptive effect of siRNA was due to a system effect was excluded. The results indicate, therefore, that the decrease in flinches induced by formalin after injection of 1 or 2 nmol NR1-1 siRNA was due to a local effect. Because there were no significant differences in the number of flinches or subsequent protein levels demonstrated by western blot between the two doses of 1 nmol and 2 nmol NR1-1 siRNAs, we used the 1-nmol dose in the subsequent time course study. In time course study, the formalin test performed on the third and seventh day after injection of 1 nmol NR1-1 siRNA revealed a significantly lower number of flinches at 5 min and during the period of 25–40 min during formalin painful stimulation (fig. 2C). There was also an additional antinociceptive effect (decrease in the number of flinches) at 20 and 45 min during formalin painful stimulation in rats examined on the seventh day after injection of 1 nmol NR1-1 siRNA (fig. 2C). The antinociceptive effect correlated with the diminished levels of NR1 mRNA expression and NR1 product shown on real-time PCR and western blot.

Fig. 1. (A) Knockdown of subcutaneous NR1 subunit does not affect motor coordination as measured by the rotarod test. No significant difference was noted among the four groups (n = 6 each group). (B) Knockdown of subcutaneous NR1 subunit 3 days after injection of NR1-1 siRNA increases withdrawal threshold in the complete Freund’s adjuvant (CFA)-induced inflamed hind paw (n = 5 each group). S = saline; PEI = polyethylenimine; MM = mismatched NR1-1 siRNA; Contra = injection of CFA in the paw contralateral to the siRNA-injecting paw; CFA 1 D = measurement of withdrawal threshold 1 day after injection of CFA. *P < 0.05 compared with baseline data. Values are mean ± SD.

NR1 siRNA Reduces Inflammatory Nociception

Fig. 1. (A) Knockdown of subcutaneous NR1 subunit does not affect motor coordination as measured by the rotarod test. No significant difference was noted among the four groups (n = 6 each group). (B) Knockdown of subcutaneous NR1 subunit 3 days after injection of NR1-1 siRNA increases withdrawal threshold in the complete Freund’s adjuvant (CFA)-induced inflamed hind paw (n = 5 each group). S = saline; PEI = polyethylenimine; MM = mismatched NR1-1 siRNA; Contra = injection of CFA in the paw contralateral to the siRNA-injecting paw; CFA 1 D = measurement of withdrawal threshold 1 day after injection of CFA. *P < 0.05 compared with baseline data. Values are mean ± SD.
Injection of siRNA Silences the NR1 Gene

To examine the effect of siRNA on gene expression, we used real-time PCR and western blot for analysis of mRNA and protein expression of NMDA receptor and interferon-α (figs. 3A–E, and 4A–C). The mRNA and protein levels of NR1 were significantly lower in the NR1-1 siRNA group and in the NR1-2 siRNA group than in the polyethyleneimine group (figs. 3A and 4A). The decrease in mRNA and protein level in the NR1-1 siRNA group (≈80%) was greater than that in the NR1-2 siRNA group (≈60%) (figs. 3A and 4A). Thus, we used NR1-1 siRNA in the subsequent dose–response and time course studies. In the dose–effect study, the mRNA and protein levels were significantly lower in the 1- and 2-nmol NR1-1 siRNA groups than in the saline, polyethyleneimine, and MM-NR1-1 siRNA groups (figs. 3B and 4B). However, similar decreases in mRNA and protein level were found between the 1- and 2-nmol NR1-1 siRNA groups (figs. 3B and 4B). We used the 1-nmol dose in the subsequent time course and CFA studies. In the time course study, significant decreases in NR1 mRNA and NR1 protein were noted 3 and 7 days after injection of 1 nmol NR1-1 siRNA. Partial recovery of mRNA and protein level was found on day 14, and full recovery of mRNA and protein level was noted 21 days after injection of 1 nmol NR1-1 siRNA (figs. 3C and 4C). The skin tissue taken from rats injected with 1 nmol NR1-1 siRNA after CFA test revealed significantly lower mRNA than the tissue of rats that received an injection of saline, polyethyleneimine alone, or MM-NR1-1 siRNA (fig. 3D). The effect of gene knockdown by NR1-1 siRNA was specific because injection of NR1-1 MM-siRNA had no antinociceptive effect on formalin- and CFA-induced nociceptive behavior (figs. 1B and 2B) and did not change the level of NR1 mRNA or protein (figs. 3B, 3D, and 4B). NR1-1 siRNA treatment did not affect the mRNA level of the formalin-induced nociception-related subunits NR1, NR2A, NR2B, and NR2C or the level of unrelated subunits NR2D (fig. 3E). No significant changes in the mRNA level of interferon-α were noted (fig. 3E). Furthermore, the formalin assay revealed that injection of both NR1-1 siRNA and NR1-2 siRNA provided antinociceptive effects (fig. 2A), and real-time PCR showed that each of these two siRNAs simultaneously decreased the levels of NR1 mRNA and protein (figs. 3A and 4A).

NR1 siRNA Decreases NR1 Immunohistochemical Stain in Skin Tissue and DRG

Significantly lower NR1 immunoreactivity at the dermal–epidermal junction was detected in skin dissected 3 days after subcutaneous injection of 1 nmol NR1-1 siRNA than skin dissected 3 days after subcutaneous injection of 1 nmol MM-NR1-1 siRNA (fig. 5A–C). To test whether the NR1 siRNAs were retrograde transported to DRG and subsequently silenced the expression of NR1 in DRG, we performed immunohistochemical staining and measurement of mRNA of NR1 in L5 DRG. In L5 DRG ipsilateral to a NR1-1 siRNA-treated hind paw, the fluorescence intensity of NR1-positive neuron and mRNA of NR1 were significantly reduced when compared with contralateral L5 DRG (fig. 6A–F). The NR1 stain intensities showed a significant shift from a higher intensity distribution (fig.
6C) in contralateral DRG sections to a lower intensity distribution (fig. 6D) in ipsilateral DRG sections.

Discussion

In this study, we have developed a new therapeutic approach for treating inflammatory pain by subcutaneously injecting siRNA designed to target the NR1 subunit of the NMDA receptor. Glutamate has been implicated as a potential mediator of pain-related neuroplasticity, especially in the spinal cord and at peripheral sites.1,12,24–26 A number of studies have shown that certain NMDA receptor antagonists are useful analgesics in the treatment of chronic27,28 and acute pain.29 However, these antagonists have numerous side effects and, therefore, have limited practical use. The side effects are believed to be due to the antagonizing effect of the NMDA receptor in the central nervous system. The side effects can be avoided by antagonizing the subunits of the NMDA receptor that are locally distributed in pain pathways, such as by knockdown of NR 2B in the dorsal horn.18

In this study, we provide a way to avoid the side effects due to antagonizing NMDA receptors in the central nervous system by lowering the number of NR1 subunits of NMDA receptors in subcutaneous tissue. To the best of our knowledge, this is the first report of successful in vivo application of an siRNA-based management of pain targeting subcutaneous receptors.

It seems likely that the antinociceptive effects of NR1 siRNA occur as a result of silencing the gene encoding NMDA receptors located on primary afferent axons, postganglionic sympathetic efferents, Schwann cells, and keratinocytes in the skin that has been demonstrated to play a role in modulating peripheral nociceptive transmission.30

In addition, the silence of NMDA receptors on primary afferent axons seems as a result of the degrading mRNA of NR1 siRNA Reduces Inflammatory Nociception
NR1 in DRG. In support of this hypothesis, the dynamic transportation in and out of cell membrane of functional NMDA receptors assembled in the endoplasmic reticulum has been reported. \(^3\) Plasmid DNA expressing a fluorescent reporter protein complexed with polyethylenimine and plasmid DNA expressing short hairpin RNA complexed with cationized gelatin injected subcutaneously seemed to undergo rapid retrograde transport to L4 and L5 DRGs and express the reporter protein or inhibit the expression of target gene. \(^3\) Behavioral studies have demonstrated that activation of glutamate receptors by subcutaneous injection of glutamate, \(^6\) NMDA, \(\alpha\)-amino-3-hydroxy-5-methylisoxazolone-4-propionic acid or kainite \(^3\) into rat hind toes results in nociceptive behaviors such as mechanical hyperalgesia and mechanical allodynia, and these behaviors are attenuated by local injection of appropriate antagonists. \(^3\) In our study, the systemic effect of siRNA was excluded because the antinociceptive behaviors elicited by formalin- and CFA-induced no-

**Fig. 4.** Representative western blots of N-methyl-D-aspartate receptor NR1 subunit after injection of NR1 small interfering RNA (siRNA). (A) NR1-1 and NR1-2 siRNA significantly inhibited the expression of NR1 protein. Lane representing PEI: 3 days after intradermal injection of 2 \(\mu\)l polyethylenimine (PEI) as the control. Lanes representing NR1-1, 1-2, 1-3: 3 days after intradermal injection of 1 nmol NR1-1, 1-2, or 1-3 siRNA. (B) NR1-1 siRNA at doses of 1 nmol and 2 nmol significantly inhibited the expression of NR1 protein. Lanes representing saline, PEI, MM, 0.5 nmol, 1 nmol, 2 nmol: 3 days after intradermal injection of 100 \(\mu\)l saline, 2 \(\mu\)l PEI, 1 nmol mismatched NR1-1 siRNA, 0.5 nmol, 1 nmol, or 2 nmol NR1-1 siRNA. (C) Significant inhibition of NR1 protein was noted 3 and 7 days after injection of 1 nmol NR1-1 siRNA. Lanes representing PEI or siRNA at 3, 7, 14, and 21 days: 3, 7, 14, and 21 days after intradermal injection of 2 \(\mu\)l of PEI as the control or 1 nmol NR1-1 siRNA. Six rats were used for every time point. *\(P < 0.05\) vs. control groups (n = 6 each group). Values are mean ± SD.
ception were not noted after subcutaneous administration of NR1 siRNA in the contralateral hind paw. The decrease of mRNA and protein expression of NR1 in skin and DRG after subcutaneous injection of NR1 siRNA supports the retrograde transport of NR1 siRNA to DRG and the subsequent inhibition of NR1 expression in DRG neuron and axon in the skin.

The formalin test is the most frequently used method for assessing the efficacy of antihypersensitivity elicited by NMDA receptor antagonists. The immediate response to intraplantar formalin reflects the activation of primary afferent nociceptors. The later response, phase 2, reflects a continuing stimulation of peripheral nociceptors and central sensitization triggered by the phase 1 input from the periphery. Afferent input evokes the release of excitatory amino acids and peptides that lead to the initiation of a state of facilitation, mediated in part by the activation of NMDA subtype glutamate receptors. As shown in this study, treatment with NR1-1 siRNA not only inhibited the phase 2 response but also inhibited the response at the fifth minute in phase 1. We hypothesize that depressed expression of NMDA receptors by local pretreatment with NR1 siRNA reduces the activity during phases 1 and 2 in NMDA receptor-containing nociceptor axons, leading to a reduction in the release of excitatory amino acids and peptides in the skin, which further depresses the central sensitization of dorsal horn neurons.

It has been reported that the proportion of the NR1 subunit in NMDA receptor-labeled unmyelinated axons increases after CFA-induced inflammation and that the blockade of peripheral NMDA receptors can reverse mechanical hyperalgesia 2 days after CFA injection. These results suggest that there may be a continuous release of glutamate in amounts sufficient to maintain the sensitization of nociceptors under persistent inflammatory conditions. Even a low concentration of glutamate may be effective in nociceptor activation because of the upregulation of NMDA receptors on the peripheral ends of unmyelinated axons after inflammation. Thus, we propose that the reduction in CFA-induced inflammatory pain in this study was due to the silencing of the NR1 subunit expression by siRNA.

Similar studies have used genetic manipulation techniques to determine the importance of NMDA receptors in pain hypersensitivity. South et al. reported that a conditional deletion of the NR1 subunit produced by localized lumbar spinal injection of an adenovirus vector expressing Cre recombinase into floxed NR1 mice was able to reduce intraplantar formalin-induced hypersensitivity by 70%. The Cre/loxP system is useful for tissue-specific deletion of genes that can only be partially deleted from specific tissue, because complete knockout would be lethal. The disadvantage of this technique is that, in the transgenic Cre mouse, the inactivation of the targeted gene occurs only in cells expressing Cre recombinase and the same gene will remain active in all cells and tissues that do not contain Cre. Bursztajn et al. showed that genetically engineered male mice that had their normal NR1 gene knocked out expressed a modified NR1 gene at either normal level (NR1+/+,, wild type) or at a low level (NR1+/−, knockdown). In each mouse, peripheral nerve injury was induced by transection of the L5 spinal nerve. The NR1+/− mice displayed decreased mechanical allodynia in comparison with their wild-type

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**Fig. 5.** Representative immunofluorescence staining of skin tissue 3 days after injection of 1 nmol mismatched (MM) NR1-1 small interfering RNA (siRNA) (A) and skin tissue 3 days after injection of 1 nmol NR1-1 siRNA (B). The arrows show NR1 immunofluorescence at dermal-epidermal junction (bar = 50 μm). Epider = epidermis; Der = dermis. (C) Significant decrease level of NR1 fluorescence intensity was shown in the NR1-1 siRNA-treated skin compared with MM-NR1-1 siRNA-treated skin. *P < 0.05 compared with injection of mismatched NR1-1 siRNA. MM-siRNA: mismatched NR1-1 siRNA, siRNA: NR1-1 siRNA. Values are median (upper and lower quartiles).
counterparts. Use of genetically modified mice, however, raises an important issue in relation to compensatory mechanisms that may be triggered in development, and thus, their use may complicate interpretation of results. The advantage of knocking down the peripheral cutaneous NR1 subunit, as described in this study, is that even the entire NR1 gene is deleted, resulting in reduced pain response without causing lethal or other side effects. Complete suppression of NMDA receptor function in the central nervous system by classic NMDA receptor antagonists or viral vectors produces unfavorable effects including alterations in behavior precluding unbiased behavioral testing.

Fig. 6. Representative immunofluorescence staining and pixel distribution of NR1 immunoreactivity in dorsal root ganglion (DRG) 3 days after injection of 1 nmol NR1-1 small interfering RNA (siRNA). (A) NR1 immunofluorescence staining in DRGs contralateral to injection of NR1-1 siRNA; (B) NR1 immunofluorescence staining in DRGs ipsilateral to injection of NR1-1 siRNA. The arrows in (A) show NR1-positive neuron. The arrows in (B) show diminished fluorescence of NR1-positive neurons after intradermal injection of NR1-1 siRNA (bar = 100 μm in A and B). (C and D) Representative pixel distributions of NR1 immunoreactivity in individual DRG neuron contralateral (C) and ipsilateral (D) to NR1-1 siRNA injection. The NR1 stain intensities showed a significant shift from a higher intensity distribution in contralateral DRG sections to a lower intensity distribution in ipsilateral DRG sections. (E) Relative fluorescence was calculated by dividing the average intensity of each NR1-positive neuron by the intensity of whole image of the same section. Significant diminished fluorescence intensity of NR1 was noted in DRG ipsilateral to intradermal injection of NR1-1 siRNA. Values are median (upper and lower quartiles). (F) Significant inhibition of NR1 mRNA was noted in DRG ipsilateral to intradermal injection of NR1-1 siRNA. *P < 0.05 compared with contralateral DRG. Ipsi = ipsilateral; Cont = contralateral. Values are mean ± SD.
For delivery of naked siRNAs into subcutaneous tissue in vivo, intradermal injections of lymphocyte function-associated antigen-1-targeted fusion proteins using a gene gun have been used. However, such methods may irritate inflamed skin, and repeated treatments may not be possible. Delivery by peripheral intramuscular or subcutaneous injection presents the simplest and least invasive option, because it does not require surgery or body cavity penetration. It also offers the unique advantage of being able to target specific neurons innervating a particular muscle or region of skin.

Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin. Polyplexes formed from polyethyleneimine or plasmid DNA have previously been used to achieve transfection of neurons innervating a particular muscle or region of skin.

Specificity is a major concern in the use of RNA interference. Nonspecific silencing may result from sequence-specific off-target effects or induction of interferon response. Throughout this study, there was no evidence that mismatched NR1 siRNA had an effect on target gene expression. Two NR1 siRNAs targeting different sequences of NR1 had gene-silencing effects, and the results from the formalin assays revealed that the therapy had antinociceptive effects. We also showed that the levels of the NR2 subtypes were not affected by NR1 siRNA, thus excluding any potential nonspecific effects produced by the NR1 siRNA. Furthermore, the results of the real-time PCR analysis demonstrated that the NR1 siRNA did not induce an interferon-α response.

In summary, this study demonstrates that local subcutaneous injection of siRNA targeting the NR1 subunit of the NMDA receptor effectively silences the expression of the NMDA receptor, resulting in significant attenuation of CFA- and formalin-induced nociceptive behaviors. The fact that the siRNA did not induce significant side effects and that it provided an antinociceptive effect for up to 7 days suggests that NR1 siRNA has potential therapeutic value in the treatment of pathologic pain induced or maintained by peripheral nociceptor activity. These results highlight the potential of this technology as a valuable tool for the study of nociceptive processes and the development of new analgesic drugs.

References
2. Dickenson AH, Sullivan AF: Evidence for a role of the NMDA receptor in frequency dependent potentiation of deep rat dorsal horn nociceptive neurons following C fibre stimulation. Neuropharmacology 1987; 26:1235–8
37. Bursztajn S, Rutkowski MD, Deleo JA: The role of the N-methyl-D-aspartate receptor NR1 subunit in peripheral nerve injury-induced mechanical allodynia, glial activation and chemokine expression in the mouse. Neuroscience 2004; 125:269–75