

CHINESE MEDICAL SCIENCES JOURNAL

ORIGINAL ARTICLE

PSD95 Gene Specific siRNAs Attenuate Neuropathic Pain through Modulating Neuron Sensibility and Postsynaptic CaMKII α Phosphorylation $\triangle$

Le Shen[†], Xu Li[†], Wen Chen, Li Xu, Wei Liu, Xue-rong Yu, and Yu-guang Huang*

Department of Anesthesiology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China

Key words: PSD95; siRNAs; neuropathic pain; glutamate excitotoxicity; calcium/calmodulin-dependent protein kinase II α

Objective To observe the effects of PSD95 gene specific siRNAs on neuropathic pain relief, neuron viability, and postsynaptic calcium/calmodulin-dependent protein kinase II α (CaMKII α) phosphorylation *in vitro* and *in vivo*.

Methods Gene-specific siRNAs of rat PSD95 were synthesized chemically for transfection. Adult male Sprague-Dawley (SD) rats were randomly divided into 3 groups: naïve group ($n=6$), sham group ($n=6$), and sciatic nerve chronic constriction injury (CCI) group ($n=24$). The CCI group was further divided into 4 groups ($n=6$ in each group), which were pretreated with normal saline, transfection vehicle, negative control siRNAs, and PSD95 gene specific siRNAs respectively. All the subgroups received corresponding agents intrathecally for 3 days, started one day before the CCI of sciatic nerve. Both mechanical allodynia and thermal hyperalgesia were measured on post-operative day 3 and 7. PSD95 gene silenced NG108-15 cells were further stimulated by glutamate, with the cell viability and the expression/phosphorylation of CaMKII α measured by MTT cell proliferation assay and Western blot, respectively.

Results The siRNAs decreased PSD95 mRNA level significantly both *in vivo* and *in vitro*. Neuropathic pain rats pretreated with PSD95 gene specific siRNAs exhibited significant elevation in the mechanical withdrawal threshold and paw withdrawal thermal latency, without affecting the baseline nociception. PSD95 gene silencing enhanced neuronal tolerance against the glutamate excitotoxicity, meanwhile the phosphorylation of CaMKII α Thr286 was attenuated.

Conclusion Pre-emptive administration of PSD95 gene specific siRNAs may attenuate the central sensitization CaMKII α -related signaling cascades, leading to the relief of neuropathic pain.

Chin Med Sci J 2011; 26(4):201-207

Received for publication May 5, 2011.

[†]Equal contribution to this paper.

*Corresponding author Tel/Fax: 86-10-65295580, E-mail: pumchhyg@yahoo.com.cn

$\triangle$ Supported by National Natural Science Foundation of China (30672029, 30872436) and Central Committee Foundation of Health Care Program (B2009B076).

POSTSYNAPTIC density (PSD) is an electron-dense specialization of excitatory postsynaptic membranes that contains a high concentration of glutamate receptors and associated signaling and cytoskeletal proteins, which is identified as a band of electron-dense material 20-30 nm thick and 300 nm long.¹ In the nervous system, excitatory synapses, and in particular their PSDs, contain many postsynaptic density-95/discs large/zona occludens-1 (PDZ) domain proteins.² The best characterized synaptic PDZ domain protein is postsynaptic density protein 95 (PSD95), an abundant component of the PSD.³ PSD95, also known as discs, large homolog 4 (DLG4) or synapse-associated protein 90, is a member of the family of membrane-associated guanylate kinase proteins, which has many recognized binding partners and large number of copies in PSDs. PSD95 interacts with postsynaptic glutamate receptors, both N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, in postsynaptic signal transduction.

Postsynaptic signal transduction plays an important role in the central sensitization of neuropathic pain. Postsynaptic depolarization leads to an opening of the NMDA receptor-gated channel, which in turn leads to calcium entry and postsynaptic sensitization. Another possible mechanism of postsynaptic sensitization involves an increased trafficking of AMPA receptors to the cell surface.⁴ Because of the close relationship between PSD95 and these glutamate receptors, several PSD95 gene silencing therapeutics on neuropathic pain have been attempted. Garry et al⁵ revealed that PSD95 plays a key role in neuropathic reflex sensitization, and the NMDA receptor-dependent hyperalgesia and allodynia of neuropathic pain were eliminated in mutant mice expressing a truncated form of PSD95. Tao et al⁶ demonstrated that PSD95 is important in the development of peripheral nerve injury-induced neuropathic pain, and that rats injected intrathecally with PSD95 antisense oligodeoxynucleotide exhibited a significant reduction in mechanical and thermal hyperalgesia. However, the underlying cellular and molecular mechanism of PSD95 gene silencing in neuropathic pain still remains unknown. In this study, the PSD95 gene expression was silenced with siRNAs both *in vivo* and *in vitro*. The possible cellular or molecular implications of PSD95 gene silencing in neuropathic pain relief was discussed.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague-Dawley (SD) rats (Beijing Vitalriver

Co., Ltd) of eight-week old and weighing 220-250 g were fed standard rat chow and acclimated for one week before experiments. Animals were treated in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals issued by the International Association for the Study of Pain.

siRNAs preparation

The siRNAs sequence for the rat PSD95 (accession no. NM_019621.1) was from nucleotides 1237 to 1255 relative to the start codon (siR-P). The sequences were as follows: sense, 5'-CCAGAAGAGUAUAGUCGAUtt-3'; antisense, 5'-AUCGACUAUACUCUUCUGGtt-3'. Basic local alignment search tool search of the nucleotide sequences in the GenBank database showed no substantial homology with other genes. A non-silencing sequence was designed as the negative control (siR-N): sense, 5'-UUCUCCGAACGUGUCACGUtt-3'; antisense, 5'-ACGUGACACGUUCGGAGAAtt-3' (Shanghai GeneChem Co., Ltd). siRNAs were formed in a concentration of 20 μ mol/L stocks in universal buffer as manufacturer described, annealed for 2 minutes at 90°C, cooled down to room temperature, and stored overnight at 4°C. The siRNAs stocks were aliquoted and stored at -80°C.

Cell culture and *in vitro* transfection

The NG108-15 neuronal cells (Cell center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) were routinely propagated in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Cell center) and 100 U/mL penicillin G sodium and 100 μ g/mL streptomycin sulfate (Cell center). The cells were transfected with siRNAs/Lipofectamine 2000™ (Invitrogen) complexes, with the final siRNA concentration at 25, 50, 100, and 200 nmol/L. Twenty-four hours after transfection, the cells were harvested and total RNA was isolated. PSD95 transcripts were detected by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Lumbar catheterization of the spinal subarachnoid space

Lumbar catheterization of the spinal subarachnoid space was carried out as reported.⁷ A polyethylene catheter (PE-10; nominal i.d. 0.28 mm, length 15 cm) was inserted to the 5th to 6th lumbar intrathecal space cephalad to reach the lumbar spinal cord. To confirm correct catheter positioning, 10 μ L of 2% lidocaine was administered through the catheter after complete recovery from the surgery in post-operative days 2-3. A correct intrathecal catheter placement was proved by paralysis of the hind limbs without affecting normal forelimb motor function.

The cannulated animals were allowed 4-5 days to recover. The rats were given saline, transfection vehicle, the siR-P, or siR-N through the catheter once per day for three consecutive days, and on the 2nd day during injection, rats underwent the chronic constriction injury (CCI) operation of the sciatic nerve.

Chronic constriction injury rats and *in vivo* delivery of siRNAs

A CCI of the sciatic nerve was induced according to the method of Bennett and Xie,⁸ when the rats completely recovered from the intrathecal catheter placement surgery on post-operative day 4. Briefly, in the CCI group rats ($n=24$), the common sciatic nerve of the left thigh was exposed at mid-thigh level, a 7 mm portion of nerve was freed of adhering tissue and 4 loose ligatures (4.0 chromic gut suture) were tied around the sciatic nerve (1-1.5 mm apart). A sham group ($n=6$) received the procedure of the common sciatic nerve exposure without ligatures. Rats in the naïve group ($n=6$) were not treated at all before sacrifice.

The CCI group was further divided into 4 groups ($n=6$ in each group), which were pretreated with normal saline (CCI-NS), transfection vehicle (CCI-TV), negative control siRNAs (CCI-siR-N), and PSD95 gene specific siRNAs (CCI-siR-P) respectively. On the day of the injection, one aliquot of the siRNAs (siR-N or siR-P) was thawed on ice and then mixed with the *in vivo* transfection vehicle, iFect (Neuromics Antibodies, Northfield, MN, USA), to reach a final concentration of 0.2 $\mu\text{g}/\mu\text{L}$. A total of 100 μL of the siRNAs solution or vehicle (20 μL of suspension buffer in 80 μL of iFect, 1 : 4 v/v) was locally delivered to lumbar spinal cord through implanted intrathecal catheter. The catheter was flushed with 10 μL sterile saline.

Mechanical allodynia and heat allodynia test

The mechanical and thermal nociceptive threshold were measured one day (i.e. post-operative day 3, POD3) and five days (i.e. post-operative day 7, POD7) after the final injection, followed by the execution of the rats and the isolation of L3-L4 lumbar spinal cord.

Mechanical allodynia was tested using an electronic von Frey filament (IITC Life Sciences, Woodland Hills, CA, USA). The rats were acclimated to mesh bottom cages for 5-15 minutes. Testing consisted of applying punctate mechanical pressure to the plantar surface of each hind paw from below with the electronic von Frey filament through the mesh floor, a total of 3 trials per hind paw per day. The intensity of mechanical stimulation applied at the time of paw withdrawal was recorded as the mechanical withdrawal threshold (MWT). Rats were pretested before

CCI surgery to acclimate them to the testing protocol. Pretesting was carried out for at least 3 consecutive days before CCI surgery.

For heat allodynia, rats were introduced into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode. The plate was heated to $55\pm0.3^{\circ}\text{C}$ to produce paw licking or jumping that can be measured in terms of reaction time, which were recorded as paw withdrawal thermal latency (PWT_L).

Quantitative RT-PCR

Total RNA was isolated from NG108-15 neuronal cells or lumbar spinal cord using TRIZOL™ (Invitrogen). First-strand cDNA was prepared from equal amounts of total RNA per sample (2 μg) using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Real-time PCR was subsequently performed to quantitatively determine the relative amounts of gene-specific cDNAs using the iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany). All samples were run in triplicate using an annealing temperature of 60°C . Primers for the amplification of PSD95 were: PSD95-forward, 5'-TCTATGTCATGCGCCGAAACCC-3'; PSD95-reverse, 5'-CAATCTGCAACCTGCCATCCTTG-3'. Those for β -actin were: actin-forward, 5'-TGTTGTCCTGTATGCCTCTGGT-CG-3'; actin-reverse, 5'-CAGCTGTGGTGGTGAAGCTGTAG-CC-3'. All the primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Expression of PSD95 was normalized to the expression of β -actin.

Neuronal viability assay

The MTT (Biomol, Enzo Life Sciences, Farmingdale, NY, USA) cell proliferation assay was applied to measure the glutamate-induced neuronal toxicity of NG108-15 cells, with NMDA receptor antagonist, D(-)-2-amino-5-phosphonopentanoic acid (APV), as the positive control. 3000 NG108-15 cells were dispensed into each well of a 96-well plate 72 hours before the assay. After 24 hours in culture, cells were either treated with normal culture media or transfected with siRNAs for another 24 hours. Then the cells were incubated in culture media containing different concentrations of glutamate (50, 100, 200, 500, 1000, and 2000 $\mu\text{mol/L}$) for 24 hours, with 50 $\mu\text{mol/L}$ APV added as the positive control. Data were normalized to the relative neuronal viability expressed as the ratios between the absorbance of different glutamate concentration and the absorbance of the cells treated with normal culture media without glutamate.

Western blot

For total and phosphorylated CaMKII α (CaMKII α , pThr286) (Calbiochem, Merck KGaA, Darmstadt, Germany; NB12 and NB13, respectively) Western blot analysis, NG108-15 cells transfected with different siRNAs were treated with 500 μ mol/L glutamate for 3, 6, or 24 hours. The harvested cell lysates at each time point were then quantitated and boiled for 5 minutes before separating with sodium dodecyl sulfate polyacrylamide gel electrophoresis. An equal volume of prepared lysate was run per condition and transferred to Hybond-P PVDF membrane (Amersham, GE Healthcare UK Ltd., England, UK). After blocking, the membranes were incubated in primary antibody overnight at 4°C. HRP-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used at a dilution of 1:5000 for 1 hour at room temperature, and visualization by Ultra enhanced chemi-luminescence (MultiSciences Biotech, Hangzhou, China) was captured by FluorChem® FC2 (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

All the experiments were performed in triplicate. Data were presented as means $\pm$ SD. SPSS 11.5 software was applied for statistical analysis. The *P* values were calculated using Student's *t*-test and one way analysis of variance (ANOVA). In the pain behavioral studies, the mechanical thresholds evaluated by von Frey filaments were expressed in grams. Pain behavioral data were compared between pre- and post-injection conditions using repeated measures ANOVA. *P*<0.05 was considered statistically significant.

RESULTS

PSD95 gene silencing by siRNAs *in vitro* and *in vivo*

Quantitative RT-PCR analysis of total RNA extracts from NG108-15 cells showed a dose-dependent knockdown of the PSD95 expression by siR-P transfection compared with negative control (i.e. siR-N-transfected cells). With siRNAs

at different final concentrations, PSD95 transcripts were decreased by different percentages (25 nmol/L, 76.4% $\pm$ 26.9%; 50 nmol/L, 91.5% $\pm$ 51.6%; 100 nmol/L, 94.4% $\pm$ 28.1%; 200 nmol/L, 97.9% $\pm$ 0.8%; all *P*<0.05, Fig. 1). Based on the above results, 50 nmol/L was selected as the optimal final concentration to be used in the following *in vitro* experiments.

As shown by the mRNA levels of PSD95 in the lumbar spinal cord determined by quantitative RT-PCR, siR-P treated group had a decreased PSD95 transcript level of 62.0% $\pm$ 17.0% (*P*<0.05). Saline, siR-N, or transfection vehicle pretreatment did not alter the PSD95 mRNA level in the lumbar spinal cord.

Intrathecal administration of PSD95 gene specific siRNAs reversed mechanical allodynia and thermal hyperalgesia

PSD95 gene specific siRNAs at 2 μ g per day significantly attenuated the mechanical and thermal nociception, when compared with other groups or controls. Neither vehicle nor siRNAs treatment precipitated overt signs of behavioral toxicity or motor impairment in the animals. The inhibitory effect of siRNAs treatment on mechanical allodynia and thermal hyperalgesia were obvious (*P*<0.05) and lasted for at least 5 days after the final injection (Table 1, Fig. 2).

PSD95 gene silencing antagonized glutamate-induced neuronal excitotoxicity

Glutamate caused a significant decrease in neuronal viability (*P*<0.05 for glutamate at 50 μ mol/L and above compared with control). Culture media that contained different concentrations of glutamate decreased relative neuronal viability of normal or siR-N-transfected NG108-15 cell in a dose-dependent manner (Fig. 3). APV completely reversed the decrease of neuronal viability caused by glutamate. Transfection of NG108-15 cells with siR-P also significantly antagonized the glutamate-induced neuronal excitotoxicity (*P*<0.05, Fig.3).

Table 1. PSD95 gene specific siRNAs reverses mechanical allodynia and thermal hyperalgesia (*n*=6)[§]

Groups	MWT (g)			PWTL (sec)		
	Baseline	POD3	POD7	Baseline	POD3	POD7
Naïve	52.6 $\pm$ 8.7	50.3 $\pm$ 8.1	52.8 $\pm$ 7.0	13.47 $\pm$ 4.08	13.81 $\pm$ 4.29	13.74 $\pm$ 4.35
Sham	51.4 $\pm$ 5.3	54.0 $\pm$ 7.5	48.2 $\pm$ 7.7	12.67 $\pm$ 2.17	13.99 $\pm$ 2.92	13.63 $\pm$ 3.67
CCI-NS	52.1 $\pm$ 9.5	22.5 $\pm$ 7.4	11.8 $\pm$ 4.4	15.32 $\pm$ 3.96	11.33 $\pm$ 4.15	12.50 $\pm$ 6.48
CCI-TV	50.4 $\pm$ 14.3	16.6 $\pm$ 5.6	12.8 $\pm$ 6.7	10.78 $\pm$ 3.53	7.84 $\pm$ 2.14	5.93 $\pm$ 1.51
CCI-siR-N	53.0 $\pm$ 7.7	16.7 $\pm$ 6.6	12.4 $\pm$ 4.4	11.72 $\pm$ 2.72	7.33 $\pm$ 2.21	5.80 $\pm$ 1.93
CCI-siR-P	51.4 $\pm$ 9.9	31.4 $\pm$ 11.0*	40.0 $\pm$ 31.0*	13.95 $\pm$ 3.78	14.79 $\pm$ 3.92*	16.08 $\pm$ 5.45*

§: Plus-minus values are means $\pm$ SD.

CCI: chronic constriction injury; NS: normal saline; TV: transfection vehicle; siR-N: negative control siRNA; siR-P: PSD95 gene specific siRNA; MWT: mechanical withdrawal threshold; PWTL: paw withdrawal thermal latency; POD3: post-operative day 3; POD7: post-operative day 7

**P*<0.05 compared with CCI-siR-N.

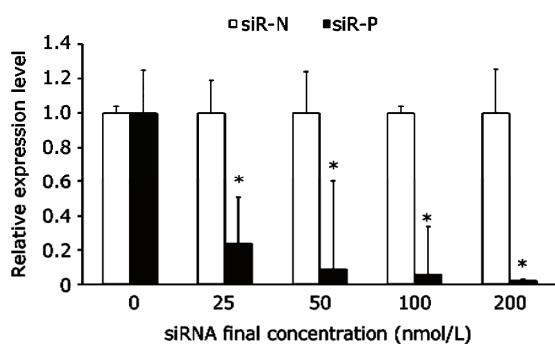


Figure 1. PSD95 gene silencing by siRNAs in dose-dependent manner *in vitro*.

* $P < 0.05$ compared with siR-N.

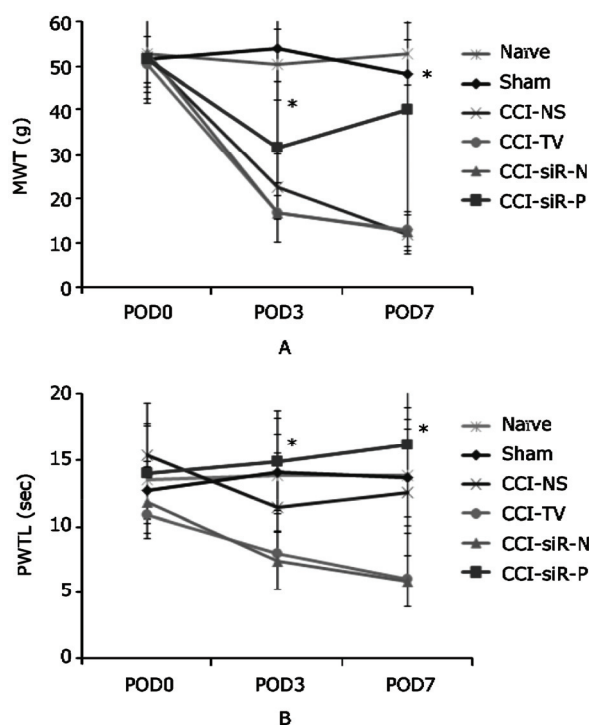


Figure 2. PSD95 gene specific siRNAs reverses mechanical allodynia and thermal hyperalgesia ($n=6$).

Chronic constriction injury rats were given siR-P, siR-N, transfection vehicle, or normal saline 24 hours before the operation. Both mechanical withdrawal threshold (MWT) (A) and paw withdrawal thermal latency (PWTL) (B) were evaluated on different post-operative days (POD), with naïve and sham as the controls.

* $P < 0.05$ compared with siR-N.

PSD95 gene silencing attenuated CaMKII α expression and phosphorylation

Significant increase of both total and phosphorylated CaMKII α (CaMKII α , pThr286) were observed in normal or siR-N-transfected NG108-15 cells ($P < 0.05$, Fig. 4). This effect was attenuated when cells were transfected with siR-P ($P < 0.05$).

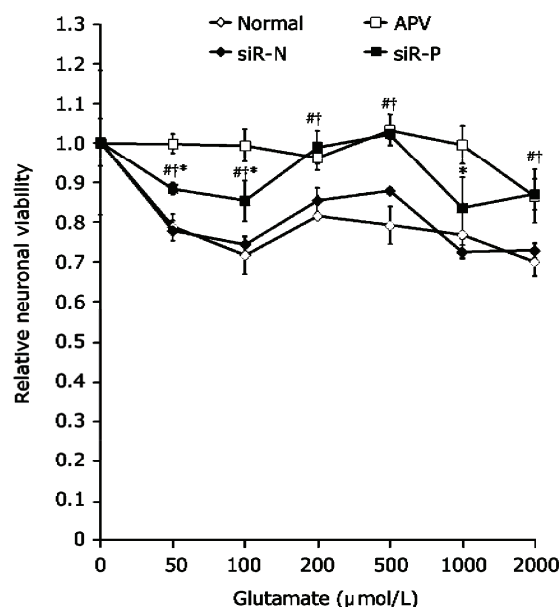


Figure 3. Glutamate-induced neuronal excitotoxicity.

NG108-15 neuronal cells in normal culture media or transfected with non-silencing siRNAs exhibited apparent susceptibility to glutamate even in some lower concentrations, which was significantly blocked when cells were treated with NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (APV) or with PSD95 gene specific siRNAs.

* $P < 0.05$ compared with normal; # $P < 0.05$ compared with siR-N; † $P < 0.05$ compared with APV.

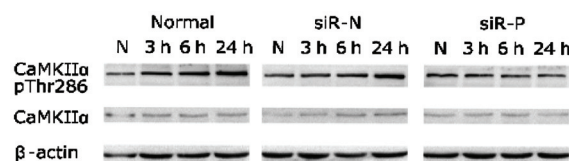


Figure 4. Western blot of total and phosphorylated CaMKII α .

CaMKII α expression and Thr286 phosphorylation in both normally cultured NG108-15 cells and the cells transfected with non-silencing siRNAs increased in a time-dependent manner when stimulated with 500 $\mu\text{mol/L}$ glutamate for 3, 6, and 24 hours. Those changes were attenuated in NG108-15 cells transfected with PSD95 gene specific siRNAs under the same glutamate stimulation.

DISCUSSION

Chronic pain, especially neuropathic pain, due to nerve injury is resistant to current analgesics. Multiple sites along the neural axis are altered after nerve injury. Both peripheral sensitization and central sensitization contribute to the origination and development of neuropathic pain.⁴ Central sensitization means the increased responsiveness of nociceptive neurons in the central nervous system to

their normal or subthreshold afferent input.

Glutamate, the fast transmitter of primary afferent neurons, binds to several receptors on postsynaptic neurons in the dorsal horn of spinal cord.⁹ Sustained release of glutamate by nociceptors leads to glutamate binding to the NMDA receptor and allows entry of calcium into the neuron, which then increases the excitability of nociceptive neurons and activates numerous intracellular pathways that then contribute to the maintenance of central sensitization. In the present study, glutamate was used to stimulate NG108-15 cell to achieve the cellular model of glutamate excitotoxicity.

High level glutamate causes neuronal excitotoxicity, and can be blocked by NMDA receptor antagonist.¹⁰ Profound research revealed that glutamate-receptor-based calcium inflow and signal are of great importance in neuronal excitotoxic damage.¹¹ Calcium influx through NMDA receptors plays key roles in synaptic transmission, neuronal development, and plasticity. Excessive calcium influx triggers excitotoxicity, damaging neurons in diverse neurological disorders. NMDA receptor-mediated postsynaptic nitric oxide synthesis and changes in NMDA receptor gene expression are both associated with neurotoxicity.¹²

In cultured cortical neurons, suppression of PSD95 expression selectively attenuates excitotoxicity triggered via NMDA receptors, but not other glutamate or calcium ion channels.¹³ Moreover, the activation of NMDA receptors initiates the neuronal apoptosis, which also contributes to the excitotoxic neuronal death.¹⁴ Hou et al¹⁵ clarified that antisense oligonucleotides-induced PSD95 gene silencing delayed cell death in postischemic rat hippocampus. PSD95's interaction with glutamate receptors (especially NMDA receptor) was disrupted by PSD95 gene silencing, which then enhanced neuronal viability and restrained the development of neuropathic pain. The results of the present study with NG108-15 neuronal cells are in accord with these previous findings.

CaMKII is abundant in the central nervous system, which is mainly composed of two major isoforms: CaMKII α and CaMKII β ,¹⁶ showing very broad substrate specificity and phosphorylating many proteins in neurons.¹⁷ CaMKII α was identified as a major PSD protein. Following strong synaptic activation, CaMKII α autophosphorylates at its Thr286 and translocates from the cytosol to the PSD or presumed postsynaptic sites where it phosphorylates target proteins in long-term potentiation.¹⁸

CaMKII α and the NMDA receptor are key regulators of synaptic plasticity underlying learning and memory. Direct binding of CaMKII α to the NMDA receptor subunit NR2B is induced by Ca²⁺/calmodulin, which mediates CaMKII α

translocation to synapses and regulates synaptic strength.^{19, 20} CaMKII α activity and Thr286 autophosphorylation are essential for normal NMDA receptor-dependent forms of long-term potentiation in the hippocampal CA1 region and hippocampus-dependent behaviors, such as spatial learning and memory,^{18, 21} and also for plasticity in other central nervous system regions.²² Central sensitization of neuropathic pain can elevate Thr286 autophosphorylation of CaMKII α and the interaction between CaMKII α and NMDA receptor subunits, which were disrupted in mice expressing a truncated PSD95 protein.¹¹ CaMKII α in spinal dorsal horn acts on the downstream of NR2B subunit phosphorylation at its Tyr1472 and is involved in the maintenance, rather than generation, of mechanical allodynia in neuropathic pain.^{23, 24} In the present study, PSD95 gene silencing also attenuated the CaMKII α Thr286 autophosphorylation without affecting NMDA receptor, implying the key role of PSD95 in NMDA receptor-PSD95-CaMKII α postsynaptic signaling cascade. However, other cascades and/or cellular factors downstream to the NMDA receptor may also participate in the maintenance of neuropathic pain.

In conclusion, central sensitization is of great importance for neuropathic pain. Both glutamate-induced NMDA receptor activation and subsequent postsynaptic calcium-dependent signaling contribute to the origination and maintenance of the central sensitization. The present study illustrated the key role of PSD95 in NMDA receptor signaling, especially in NMDA receptor-PSD95-CaMKII postsynaptic signaling cascade, which was blocked by PSD95 gene specific siRNAs. Therefore, PSD95 gene specific siRNAs may relieve neuropathic pain mainly through attenuating the central sensitization signaling cascades.

Acknowledgments

We would like to thank Prof. Bao-sheng Chen, Ms. Hong Xue, Dr. Xin-bo Zhang, and Dr. Yu-hui Gao for their generous assistance in molecular biology. We owe great thanks to Dr. Chao Ma (Assistant Professor of Department of Anesthesiology, Yale University School of Medicine) for his exquisite and detailed revisions of this paper.

REFERENCES

1. Boeckers TM. The postsynaptic density. *Cell Tissue Res* 2006; 326:409-22.
2. Swilius MT, Kubota Y, Forest A, et al. Structure and composition of the postsynaptic density during development. *J Comp Neurol* 2010; 518:4243-60.
3. Kim E, Sheng M. PDZ domain proteins of synapses. *Nat*

- Rev Neurosci 2004; 5:771-81.
4. Campbell JN, Meyer RA. Mechanisms of neuropathic pain. *Neuron* 2006; 52:77-92.
 5. Garry EM, Moss A, Delaney A, et al. Neuropathic sensitization of behavioral reflexes and spinal NMDA receptor/CaM kinase II interactions are disrupted in PSD95 mutant mice. *Curr Biol* 2003; 13:321-8.
 6. Tao F, Tao YX, Mao P, et al. Role of postsynaptic density protein-95 in the maintenance of peripheral nerve injury-induced neuropathic pain in rats. *Neuroscience* 2003; 117:731-9.
 7. Størkson RV, Kjørsvik A, Tjølsen A, et al. Lumbar catheterization of the spinal subarachnoid space in the rat. *J Neurosci Methods* 1996; 65:167-72.
 8. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33:87-107.
 9. Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* 2009; 10:895-926.
 10. Choi DW, Maulucci-Gedde M, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 1987; 7:357-68.
 11. Arundine M, Tymianski M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* 2003; 34:325-37.
 12. Beas-Zarate C, Rivera-Huizar SV, Martinez-Contreras A, et al. Changes in NMDA-receptor gene expression are associated with neurotoxicity induced neonatally by glutamate in the rat brain. *Neurochem Int* 2001; 39:1-10.
 13. Sattler R, Xiong Z, Lu WY, et al. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 1999; 284:1845-8.
 14. Wang H, Yu SW, Koh DW, et al. Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. *J Neurosci* 2004; 24:10963-73.
 15. Hou XY, Zhang GY, Wang DG, et al. Suppression of postsynaptic density protein 95 by antisense oligonucleotides diminishes postischemic pyramidal cell death in rat hippocampal CA1 subfield. *Neurosci Lett* 2005; 385:230-3.
 16. Petersen JD, Chen X, Vinade L, et al. Distribution of postsynaptic density (PSD)-95 and Ca²⁺/calmodulin-dependent protein kinase II at the PSD. *J Neurosci* 2003; 23:11270-8.
 17. Soderling TR, Chang B, Brickey D. Cellular signaling through multifunctional Ca²⁺/calmodulin-dependent protein kinase II. *J Biol Chem* 2001; 276:3719-22.
 18. Shen K, Meyer T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* 1999; 284:162-6.
 19. O'Leary H, Liu WH, Rorabaugh JM, et al. Nucleotides and phosphorylation bi-directionally modulate Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) binding to the N-methyl-D-aspartate (NMDA) receptor subunit GluN2B. *J Biol Chem* 2011; 286:31272-81.
 20. Sanhueza M, Fernandez-Villalobos G, Stein IS, et al. Role of the CaMKII/NMDA receptor complex in the maintenance of synaptic strength. *J Neurosci* 2011; 31:9170-8.
 21. Fink CC, Meyer T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr Opin Neurobiol* 2002; 12:293-9.
 22. Fang L, Wu J, Lin Q, et al. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *J Neurosci* 2002; 22:4196-204.
 23. Katano T, Nakazawa T, Nakatsuka T, et al. Involvement of spinal phosphorylation cascade of Tyr1472-NR2B, Thr286-CaMKII, and Ser831-GluR1 in neuropathic pain. *Neuropharmacology* 2011; 60:609-16.
 24. Matsumura S, Kunori S, Mabuchi T, et al. Impairment of CaMKII activation and attenuation of neuropathic pain in mice lacking NR2B phosphorylated at Tyr1472. *Eur J Neurosci* 2010; 32:798-810.