Neuroprotection of Tat-GluR6-9c against Neuronal Death Induced by Kainate in Rat Hippocampus via Nuclear and Non-nuclear Pathways*

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Previous studies have suggested that glutamate receptor 6 (GluR6) subunit- and JNK-deficient mice can resist kainate-induced epileptic seizure and neuronal toxicity (Yang, D. D., Kuan, C.-Y., Whitmarsh, A. J., Rinoč, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) Nature 389, 865–870; Mulle, C., Seiler, A., Perez-Otano, I., Dickinson-Anson, H., Castillo, P. E., Bureau, I., Maron, C., Gage, F. H., Mann, J. R., Bettle, B., and Heinemann, S. F. (1998) Nature 392, 601–605). In this study, we show that kainate can enhance the assembly of the GluR6-PSD95-MLK3 module and facilitate the phosphorylation of JNK in rat hippocampal CA1 and CA3/dentate gyrus (DG) subfields. More important, a peptide containing the Tat protein transduction sequence (Tat-GluR6-9c) perturbed the assembly of the GluR6-PSD95-MLK3 signaling module and suppressed the activation of MLK3, MKK7, and JNK. As a result, the inhibition of JNK activation by Tat-GluR6-9c diminished the phosphorylation of the transcription factor c-Jun and downregulated Fas ligand expression in hippocampal CA1 and CA3/DG regions. The inhibition of JNK activation by Tat-GluR6-9c attenuated the translation of the release of cytochrome c, and the activation of caspase-3 in CA1 and CA3/DG subfields. Furthermore, kainate-induced neuronal loss in hippocampal CA1 and CA3 subregions was prevented by intracerebroventricular injection of Tat-GluR6-9c. Taken together, our findings strongly suggest that the GluR6-PSD95-MLK3 signaling module mediates activation of the nuclear and non-nuclear pathways of JNK, which is involved in brain injury induced by kainate. Tat-GluR6-9c, the peptide we constructed, gives new insight into seizure therapy.

As the major excitatory neurotransmitter in the central nervous system, glutamate gates three types of ionotropic receptors: N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate, and kainate receptors. Kainate receptors are composed of five subunits, glutamate receptor (GluR)α5, GluR6, GluR7, KA1, and KA2 (1). Kainic acid (KA) is a potent exogenous agonist of kainate receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors, and systemic administration of KA produces epilepsy in rats and mice accompanied by neuronal damage mainly in limbic structures. In particular, hippocampal pyramidal neurons are highly vulnerable to the excitotoxicity of kainate (2). Kainate-induced seizures in rodents have been widely used as a model of human temporal lobe epilepsy on the basis of both behavioral and pathological similarities (3).

Although the expression of a number of cell death regulatory genes and receptors has been investigated in the seizure model, the exact molecular regulation mechanisms of this process remain poorly understood. Recent studies have indicated that GluR6 subunit-deficient and Jnk3 gene knock-out mice share similar phenotypes, including resistance to KA-induced seizures and neuronal toxicity (4, 5). Additional studies have indicated that the RLPGETMA motif of the C terminus of GluR6 can bind to the PDZ1 domain of the postsynaptic density protein PSD95/SAP90 through specific interaction (6, 7). Previous studies have also shown that MLK3, an upstream kinase of JNK (8), can interact with the SH3 (Src homology) domain of PSD95 (9). Thus, the triple complex GluR6-PSD95-MLK3 may exist, which can facilitate JNK activation. However, whether signaling module-mediated JNK activation exists in epileptic rat hippocampal CA1 and CA3 regions is still unknown.

Our previous study on neuronal cell death induced by global ischemia focused on two different possible pathways (10). We showed that activated JNK translocates into the nucleus and phosphorylates transcription factor c-Jun, leading to increased AP-1 transcription activity and cell apoptosis. The activation of JNK may enhance the expression of the Fas ligand (FasL) via c-Jun/AP-1-mediated transcriptional regulation, which could ultimately contribute to Fas receptor-mediated apoptosis. On the other hand, partially activated JNK remains in the cytosol and regulates the activation of non-nuclear substrates, including Bcl-2 family members such as the pro-apoptotic proteins Bax and Bid, and promotes ischemic cell death by inducing the release of cytochrome c (11, 12), which could ultimately contribute to mitochondrial-mediated apoptosis. Studies have shown that seizures induce a mixed pattern of death that includes features consistent with both apoptosis and necrosis (13–15). Furthermore, the activation of mitochondrial-linked apoptotic signaling pathways after seizures, including activation of caspase-9, -3, and -8, has been reported (16–18). However, it is still unclear whether KA-induced neuronal death is mediated by the GluR6-PSD95-MLK3 signaling module via nuclear and non-nuclear pathways, which occurs during ischemic injury.

In a previous study (19), we demonstrated that Tat-GluR6-9c, a GluR6 C-terminus-containing peptide conjugated to the cell membrane transduction sequence of the human immunodeficiency virus Tat protein, can be delivered into hippocampal neurons in vitro and in vivo. In the present study, we demonstrate that Tat-GluR6-9c can perturb the interaction of GluR6 with PSD95 and suppress the assembly of the
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GluR6-PSD95-MLK3 signaling module. Furthermore, Tat-GluR6-9c decreases neuronal death induced by kainate in hippocampal CA1 and CA3 subregions via the nuclear and non-nuclear pathways of JNK.

EXPERIMENTAL PROCEDURES

Antibody and Reagents—The following primary antibodies from Santa Cruz Biotechnology, Inc., were used: goat polyclonal anti-GluR6 (sc-7618), mouse monoclonal anti-phospho-JNKs (sc-6254), rabbit polyclonal anti-MLK3 (sc-13072), rabbit polyclonal anti-Bcl-2 (sc-492), rabbit polyclonal anti-FAsl (sc-6237), rabbit polyclonal anti-c-Jun (sc-1694), and rabbit polyclonal anti-actin (sc-10731). Rabbit polyclonal anti-GluR6 (sc-10731) antibody was obtained from Upstate Biotechnology, Inc. Rabbit polyclonal anti-c-Jun (sc-7618), mouse monoclonal anti-phospho-JNKs (sc-6254), rabbit polyclonal anti-MLK3 antibodies were from Cell Signaling Biotechnology, Inc. Rabbit polyclonal anti-GluR6 antibody (catalog no. 06-309) was obtained from Upstate Biotechnology, Inc. Mouse monoclonal anti-PSD95 (P246) antibody was bought from Sigma. Monoclonal antibody against cytochrome c oxidase subunit IV was obtained from Molecular Probes. The secondary antibodies used in our experiment were goat anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-goat IgG (Sigma). Non-specific mouse or rabbit IgG was also purchased from Sigma.

The ApopTag® peroxidase in situ apoptosis detection kit (catalog no. S7100) was purchased from Chemicon International, Inc. KA was from BIOMOL. Tat-GluR6-9c (Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala, GluR6-9c), a 20-amino acid fusion peptide with Tat protein (Tyr-Gly-Arg-Lys-Arg-Arg-Gln-Arg-Arg-Arg), and Tat-GluR6AA (Tat-Arg-Leu-Pro-Gly-Lys-Ala-Ala-Asp-Asp) were constructed in our laboratory (19).

Seizure Model—Adult male Sprague-Dawley rats weighing 200–250 g were used. Seizures were induced by intraperitoneal injection of KA (12 mg/kg) dissolved in sterile saline. Animals were monitored behaviorally for seizures for at least 6 h after injection. The seizures were scored using a modified scale devised by Racine (20): 1) behavioral arrest and staring spells, 2) head bobbing and gnawing, 3) unilateral forelimb clonus, 4) bilateral forelimb clonus, 5) severe seizures with loss of postural control, and 6) seizure-induced death. Only animals with Stage 4 or 5 seizure were used for this study. Controls were injected with sterile saline.

Administration of Drugs—Tat-GluR6-9c (100 μg) or Tat-GluR6AA (control) peptide in 10 μl of saline was administrated to the rats 40 min before KA injection through intracerebroventricular infusion (antero-posterior, 0.8 mm; lateral, 1.5 mm; and depth, 3.5 mm from the bregma).

Sample Preparation—Rats were decapitated immediately after different times of KA injection, and the hippocampi were then separated into CA1 and CA3/dentate gyrus (DG) regions and quickly frozen in liquid nitrogen. The hippocampi were homogenized in ice-cold homogenization buffer containing 50 mM MOPS (pH 7.4; Sigma), 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM MgCl2, 0.2 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM Na2VO4 (Sigma), 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM p-nitrophenyl phosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin A. The homogenates were centrifuged at 800 × g for 10 min at 4 °C. Supernatants were collected, and protein concentration was determined by the method of Lowry et al. (21). Samples were stored at −80 °C and were thawed only once until used.

When necessary, the hippocampal CA1 ad CA3/DG regions were immediately isolated to prepare mitochondrial fractions. All procedures were conducted in a cold room. Generally, unfrozen brain tissue was used to prepare mitochondrial fractions because freezing tissue causes release of cytochrome c from mitochondria. The hippocampal CA1 and CA3/DG tissues were homogenized in 1:10 (w/v) ice-cold homogenization buffer. The homogenates were centrifuged at 800 × g for 10 min at 4 °C. The pellets were discarded, and supernatants were centrifuged at 17,000 × g for 20 min at 4 °C to obtain the cytosolic fraction in the supernatants and the crude mitochondrial fraction in the pellets. The protein concentrations were determined by the method of Lowry et al. (21).

Nuclear Extraction—The homogenates were centrifuged at 800 × g for 10 min at 4 °C. Supernatants (cytosolic fraction) were collected, and protein concentrations were determined. The nuclear pellets were extracted with 20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 0.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and enzyme inhibitors for 30 min at 4 °C with constant agitation. After centrifuged at 12,000 × g for 15 min at 4 °C, supernatants (nuclear fraction) were collected, and protein concentrations were determined. Samples were stored at −80 °C and were thawed only once until used.

Immunoprecipitation—Tissue homogenates (400 μg of protein) were diluted 4-fold with immunoprecipitation buffer (50 mM HEPES buffer (pH 7.4) containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na2VO4). Samples were preincubated for 1 h with 20 μl of protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) at 4 °C and centrifuged to remove proteins that had adhered nonspecifically to protein A. The supernatants were incubated with 1–2 μg of primary antibodies for 4 h or overnight at 4 °C. Protein A was added to the tube for another 2-h incubation. Samples were centrifuged at 10,000 × g for 2 min at 4 °C, and the pellets were washed three times with immunoprecipitation buffer. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS-PAGE loading buffer and then isolated by centrifugation. The supernatants were used for immunoblot analysis.

Immunoblotting—Proteins were separated on polyacrylamide gels and then electrotransferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). After blocking for 3 h in Tris-buff ered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin, membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 3% bovine serum albumin. Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibodies in TBST for 2 h and developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color substrate (Promega Corp.). The density of the bands on the membrane was scanned and analyzed with LabWorks image analysis software (UVP, Inc.).

Histological Analysis and Immunohistochemistry—Rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) under anesthesia after 7 days of KA injection. Brains were removed quickly and further fixed with the same fixation solution overnight at 4 °C. Post-fixed brains were embedded in paraffin, followed by preparation of coronal sections (5 μm thick) using a microtome. The paraffin-embedded brain sections were deparaffinized with xylene and rehydrated with ethanol at graded concentrations of 100–70% (v/v), followed by washing with water. The sections were stained with 0.1% (w/v) cresyl violet and examined by light microscopy, and the number of surviving hippocampal CA1 and CA3 pyramidal cells/1 mm of length was counted as the neuronal density.

Immunoreactivity was determined by the avidin/biotin/peroxidase method. Briefly, sections were deparaffinized with xylene and rehydrated with ethanol at graded concentrations and distilled water. High temperature antigen retrieval was performed in 1 mM citrate buffer. To block endogenous peroxidase activity, sections were incubated for 30 min in 1% H2O2. After being blocked with 5% (v/v) normal goat serum in phosphate-buffered saline for 1 h at 37 °C, sections were incubated with
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FIGURE 1. Time courses of the interactions of GluR6 and MLK3 with PSD95 derived from saline-treated rats or rats at various times of KA injection. A, B, D, and E, shown are the results from co-immunoprecipitation analysis of interactions of GluR6 and PSD95 with MLK3 in CA1 and CA3/DG regions. Sample proteins from the hippocampus were immunoprecipitated (IP) with anti-GluR6, anti-MLK3, or anti-PSD95 antibody and then immunoblotted (IB) with anti-PSD95, anti-GluR6, or anti-MLK3 antibody. C and F, shown is the quantitative representation of interactions of GluR6 and PSD95 with MLK3. Data are the means ± S.D. and are expressed as -fold versus the saline control. *p < 0.05 versus the saline group (n = 6). G, homogenates (400 μg of total protein) from saline controls and 6-h KA-injected groups were immunoprecipitated with anti-GluR6, anti-MLK3, or anti-PSD95 antibody or nonspecific IgG (n.s.IgG), and the precipitates were analyzed by immunoblotting with anti-PSD95, anti-GluR6, or anti-MLK3 antibody. In the input lane, 100 μg of protein without immunoprecipitation were loaded. d, days.
FIGURE 2. Tat-GluR6-9c suppresses the assembly of GluR6 and PSD95 with MLK3. Shown are the effects of pretreatment with Tat-GluR6-9c on the increased interactions of GluR6 and MLK3 with PSD95 in CA1 (A and B) and CA3/DG (C and D) sub-regions in rats at 6 h after KA treatment. Sample proteins were immunoprecipitated (IP) with anti-GluR6, anti-PSD95, or anti-MLK3 antibody and then immunoblotted (IB) with anti-GluR6, anti-PSD95, or anti-MLK3 antibody. Data are the means ± S.D. and are expressed as fold versus the saline control. *, p < 0.05 versus the saline group; †, p < 0.05 versus the respective KA-administered groups (n = 6).
rabbit polyclonal anti-phospho-c-Jun antibody (1:50 dilution) at 4 °C for 2 days. These sections were then incubated overnight with biotinylated goat anti-rabbit secondary antibody and subsequently with avidin-conjugated horseradish peroxidase for 1 h at 37 °C. Finally, sections were incubated with the peroxidase substrate diaminobenzidine until the desired stain intensity developed.

TUNEL staining was performed using the ApopTag® peroxidase in situ apoptosis detection kit according to the manufacturer’s protocol with minor modifications. The paraﬁn-embedded coronal sections were deparafﬁnized and rehydrated and then treated with protease K (20 μg/ml) for 15 min at room temperature. Sections were incubated with reaction buffer containing terminal deoxynucleotidyltransferase at 37 °C for 1 h. After washing with stop/wash buffer, sections were treated with anti-di–digoxigenin conjugate for 30 min at room temperature and subsequently developed color in peroxidase substrate. The nuclei were lightly counterstained with 0.5% methyl green.

**RESULTS**

Assembly of the GluR6-PSD95-MLK3 Signaling Module during Seizure Induced by Kainate in Hippocampal CA1 and CA3/DG Regions—To investigate the alternation of the assembly of the GluR6-PSD95-MLK3 signaling module during seizure, rats were injected with KA at different times and decapitated immediately at several time points, and then the hippocampi were removed for homogenization. Immunoprecipitation and immunoblotting were performed to examine the interactions of GluR6 and MLK3 with PSD95 at various times of KA injection. As shown in Fig. 1, our results demonstrated that the interactions of GluR6 and MLK3 with PSD95 increased rapidly after KA injection, reached peak levels at 6 h, and then gradually decreased to control levels at 3 days in both CA1 and CA3/DG regions. However, the interactions of GluR6 and MLK3 with PSD95 were not affected by saline. When immunoprecipitated with nonspeciﬁc mouse or rabbit IgG, no signiﬁcant bands corresponding to PSD95, GluR6, or MLK3 were detected (Fig. 1G).

The Tat-GluR6-9c Peptide Suppresses the Increased Assembly of the GluR6-PSD95-MLK3 Signaling Module Induced by Kainate in Hippocampal CA1 and CA3/DG Regions—Our results showed that the association of GluR6-PSD95-MLK3 reached its peak level at 6 h after KA injection. To test the effects of administration of the peptide on the assembly of the GluR6-PSD95-MLK3 module, the association of GluR6 and MLK3 with PSD95 was examined by immunoprecipitation and immunoblotting. Reciprocal immunoprecipitation experiments demonstrated that the peptide perturbed the GluR6-PSD95-MLK3 signaling module. As shown in Fig. 2 (A–D), administration of Tat-GluR6-9c 40 min prior to KA injection diminished the increased interactions of GluR6 and MLK3 with PSD95 at 6 h after kainate treatment in CA1 and CA3/DG subregions, whereas the protein levels of GluR6, PSD95, and MLK3 were not altered. Conversely, the same dose of the Tat-GluR6AA control peptide did not affect the increased associations of GluR6 and MLK3 with PSD95.

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As shown in Fig. 3 (A and B), KA treatment resulted in a remarkable increase in the phosphorylation of MLK3 in CA1 and CA3/DG regions. Pretreatment with Tat-GluR6-9c signiﬁcantly diminished the increase in the phosphorylation of MLK3, whereas the protein level of MLK3 was unchanged. The same dose of the Tat-GluR6AA control peptide did not affect the increased phosphorylation of MLK3 in both CA1 and CA3/DG regions. We further examined the effects of pretreatment with Tat-GluR6-9c on the activation of the downstream pathway of MLK3. The results of Western blotting revealed that the activation of MKK7 at 6 h after kainate injection was signiﬁcantly suppressed by application of the Tat-GluR6-9c peptide in CA1 and CA3/DG regions (Fig. 3, C and D). Furthermore, similar results were obtained with JNK (Fig. 3, E and F).

**Tat-GluR6-9c Inhibits the Phosphorylation of c-Jun and the Expression of FasL Induced by Kainate in Hippocampal CA1 and CA3/DG Regions**—To elucidate the downstream mechanisms of JNK, the effects of Tat-GluR6-9c on the activation and expression of c-Jun after KA injection were investigated. As shown in Fig. 4 (A–D), the results of Western blotting showed that the phosphorylation and expression of c-Jun were signiﬁcantly increased at 6 and 12 h in both CA1 and CA3/DG regions after KA injection, whereas the saline control showed no signiﬁcant inﬂuence. As shown in Fig. 4 (E and F), prior administration of Tat-GluR6-9c signiﬁcantly diminished the increase in phospho-c-Jun at 6 h after kainate treatment, but the same dose of the control peptide did not have the same effect. The protein levels of c-Jun were not affected by Tat-GluR6-9c or Tat-GluR6AA.

Similar results of decreased phospho-c-Jun immunoreactivity were observed via immunohistochemistry (Fig. 4G). In the saline group (Fig. 4G, panels a and b), weak phospho-c-Jun immunoreactivity was detected in the nuclei of hippocampal CA1 and CA3 regions. At 6 h after kainate injection (Fig. 4G, panels c and d), phospho-c-Jun immunoreactivity signiﬁcantly increased compared with the saline group. There was no inhibitory effect of intracerebroventricular pre-infusion of Tat-GluR6AA on phospho-c-Jun immunoreactivity at 6 h after KA treatment (Fig. 4G, panels e and f). Phospho-c-Jun immunoreactivity was signiﬁcantly inhibited by pre-infusion of Tat-GluR6-9c (Fig. 4G, panels g and h).

Next, to investigate whether the Fas receptor-mediated pathway is involved in the apoptotic proﬁle induced by kainate, the effects of Tat-GluR6-9c on the expression of FasL and Fas were analyzed by Western blotting. As shown in Fig. 5 (A–D), the expression of FasL increased rapidly at 6 h and returned to the basal level at 3 days in CA1 and CA3/DG regions, whereas the expression of Fas showed no signiﬁcant changes. Prior application of Tat-GluR6-9c diminished the increased expression of FasL at 6 h induced by kainate in hippocampal CA1 and CA3/DG regions (Fig. 5, E and F). The same dose of the control peptide did not affect the increase in the expression of FasL. The protein level of Fas was not affected by Tat-GluR6-9c and the control peptide.

**Tat-GluR6-9c Decreases Bax Expression and Increases Bcl-2 Expression Induced by Kainate in Hippocampal CA1 and CA3/DG Regions**—It is known that Bcl-2 is an anti-apoptotic protein, whereas Bax is a pro-apoptotic protein. We ﬁrst investigated the Bax/Bcl-2 ratio after KA injection. As shown in Fig. 6 (A and B), the expression of Bax increased dramatically at 6 h after KA injection and lasted 3 days, whereas the level of Bcl-2 decreased sharply at 6 h after KA injection and reached the lowest at 3 days in the CA1 region. Fig. 6 (C and D) shows similar results in the CA3/DG regions, whereas the saline control showed no signiﬁcant effect on the expression of Bax and Bcl-2.

Next, we examined the effects of Tat-GluR6-9c on the levels of Bax and Bcl-2 in hippocampal CA1 and CA3/DG regions. As shown in Fig.
FIGURE 3. Tet-GluR6-9c inhibits the phosphorylation of MLK3, MKK7, and JNK in CA1 and CA3/DG regions in rats. A, phospho-(p)-MLK3 and MLK3 were immunoblotted (IB) with anti-phospho-MLK3 or anti-MLK3 antibody. C, phospho-MKK7 and MKK7 expression was examined by immunoblot analysis. E, phospho-JNKs and the protein expression of JNK were examined by immunoblot analysis using anti-phospho-JNKs or anti-JNK antibody. B, D, and F, bands were scanned, and the intensities were determined by optical density (O.D.) measurement. Data are the means ± S.D. and are expressed as -fold versus the respective saline control. a, p < 0.05 versus the saline group; b, p < 0.05 versus 6-h KA-injected groups (n = 6).
FIGURE 4. Tat-GluR6-9c inhibits the phosphorylation of c-Jun in rat hippocampal CA1 and CA3 regions. A and C, shown are the time courses of phospho-(p)-c-Jun and c-Jun proteins in CA1 and CA3/DG subregions. E, the effects of pretreatment with Tat-GluR6-9c on increased phospho-c-Jun at 6 h after KA injection are shown. B, D, and F, bands were scanned, and the intensities were determined by optical density (O.D.) measurement. Data are the means ± S.D. and are expressed as fold versus the respective saline control. a, p < 0.05 versus the saline group; b, p < 0.05 versus the 6-h KA-treated groups (n = 6). G, shown is the immunohistochemical staining of phospho-c-Jun in CA1/CA3 subfields. Examples are shown of the immunohistochemical staining of sections of hippocampi from rats killed at 6 h after saline injection (panels a and b), rats subjected to 6 h of KA injection (panels c and d), and rats subjected to 6 h of KA injection with administration of 100 µg of Tat-GluR6AA (panels e and f) or Tat-GluR6-9c (panels g and h) 40 min before KA administration. Data were obtained from six independent animals in each experimental group, and the results of a typical experiment are presented. Magnification ×400 in panels a–h. Scale bars = 10 µm. d, days; IB, immunoblot.
FIGURE 5. Tat-GluR6-9c diminishes the increased protein levels of FasL in rat hippocampal CA1 and CA3/DG regions. A–D, time courses of the expression of FasL and Fas in hippocampal CA1 and CA3/DG regions derived from saline-treated rats or KA-injected rats at various time points. E and F, effects of Tat-GluR6-9c on the expression of FasL and Fas at 6 h after KA injection in the hippocampus. Samples were probed with anti-FasL or anti-Fas antibody. Bands were scanned, and the intensities were determined by optical density (O.D.) measurement. Data are the means ± S.D. and are expressed as -fold versus the saline control. a, p < 0.05 versus the saline group; b, p < 0.05 versus the 6-h KA-injected groups (n = 6). d, days; IB, immunoblot.
FIGURE 6. Tat-GluR6-9c inhibits the normalization of the pro-apoptotic Bax/Bcl-2 ratio in rat hippocampal CA1 and CA3/DG regions. A–D, time courses of the expression of Bax and Bcl-2 in the cytosol including mitochondria in samples derived from saline-treated rats and KA-injected groups. E and F, effects of pretreatment with Tat-GluR6-9c on the expression of Bax and Bcl-2 at 6 h after KA injection in hippocampal CA1 and CA3/DG regions. Data are the means ± S.D. and are expressed as fold versus the saline control. *, p < 0.05 versus the saline group; †, p < 0.05 versus 6-h KA-treated groups (n = 6). d, days.
prior application of Tat-GluR6-9c resulted in the decreased expression of Bax at 6 h after KA treatment in both CA1 and CA3/DG regions, whereas the level of Bcl-2 was obviously increased at 6 h after KA injection.

**Tat-GluR6-9c Attenuates Bax Translocation and the Release of Cytochrome c Induced by Kainate in Hippocampal CA1 and CA3/DG Regions**—A previous study demonstrated mitochondrial Bax accumulation after seizure (22). To further elucidate the involvement of the mitochondrion-mediated apoptotic pathway induced by KA, the expression of Bax and cytochrome c in both mitochondria and the corresponding cytosol was examined by Western blotting. We first demonstrated that the inhibitory effect of Tat-GluR6-9c on Bax translocation in the mitochondrial fraction reached a statistical difference at 6 h after KA administration compared with that of the Tat-GluR6AA control peptide in both CA1 and CA3/DG regions (Fig. 7, A and B).

In the cytosolic fraction, cytochrome c immunoreactivity was evident as a single band of 15 kDa at 6 h after injection. However, it was barely detected in the saline group (Fig. 7, C and D). A significant amount of mitochondrial cytochrome c was detected in the saline group and it decreased at 6 h after KA injection corresponding to a marked increase in the cytosolic fraction. Moreover, Tat-GluR6-9c inhibited the release of cytochrome c from mitochondria to the cytosol compared with the 6-h KA groups and Tat-GluR6AA-treated groups (Fig. 7, C and D) in CA1 and CA3/DG fields. To further investigate whether other mitochondrial protein was released from mitochondria, we examined cytochrome c oxidase levels in the cytosolic and mitochondrial fractions using anti-cytochrome c oxidase subunit IV antibody. Cytochrome c oxidase subunit IV was detected only in the mitochondrial fraction, but not in the cytosolic fraction of the saline group, kainate-treated group, or peptide-treated groups.

**Tat-GluR6-9c Inhibits the Activation of Caspase-3 and Neuronal Apoptosis Induced by Kainate in Hippocampal CA1 and CA3/DG Regions**—In this study, we examined the inhibitory effects of Tat-GluR6-9c on the activation of caspase-3 (p20) at 6 h after KA injection in CA1 and CA3/DG regions. As shown in Fig. 8 (A–C), immunoblot analysis indicated that Tat-GluR6-9c pretreatment diminished the activation of caspase-3 at 6 h after KA injection. The same dose of the control peptide did not affect the increase in the activation of caspase-3.

Furthermore, we investigated the role of Tat-GluR6-9c in kainate-induced neuronal apoptosis by TUNEL staining in hippocampal CA1 and CA3 regions. Rats were pretreated with Tat-GluR6-9c or Tat-GluR6AA by cerebroventricular injection 40 min before KA injection. After 7 days, rats were perfused and fixed with paraformaldehyde. TUNEL staining was used to examine the apoptosis of CA1 and CA3 neuronal cells in the hippocampus. As shown in Fig. 8D, compared with the saline group (panels a and b), significant numbers of TUNEL-positive cells were observed in the KA-treated group (panels c and d) after 7 days, with some of them showing characteristic appearances such as shrunken condensed nuclei and apoptotic bodies. Administration of Tat-GluR6-9c 40 min before KA injection significantly decreased
A Neuroprotective Role of Tat-GluR6-9c against Neuronal Injury Induced by Kainate in Hippocampal CA1 and CA3 Pyramidal Neurons—To investigate whether pretreatment with Tat-GluR6-9c could have a neuroprotective effect against kainate-induced cell death, rats were pretreated with Tat-GluR6-9c or Tat-GluR6AA by cerebroventricular injection 40 min before KA administration. After 7 days, rats from the saline group, KA-administered group, and Tat-GluR6-9c- and control peptide-pretreated groups were perfusion-fixed with paraformaldehyde. Cresyl violet staining was used to examine the survival of CA1 and CA3 pyramidal cells in the hippocampus. The histology results showed that normal CA1 and CA3 neuronal cells showed round and palely stained nuclei (Fig. 9, A, panels a and b; and B, panels a and b), whereas KA-induced dead cells showed pyknotic nuclei (A, panels c and d; and B, panels c and d). Administration of Tat-

TUNEL-positive cells (panels g and h). However, as a control, Tat-GluR6AA did not show any protective effect (panels e and f). The number of TUNEL-positive cells/1 mm of length in the saline group, seizure-insulted group, Tat-GluR6AA-treated group, and Tat-GluR6-9c-treated group was 21.4 ± 6.8, 177.0 ± 24.7, 169.5 ± 11.2, and 67.2 ± 5.6 in the CA1 region and 25.6 ± 7.8, 153.0 ± 20.5, 145.3 ± 23.4, and 58.3 ± 4.9 in the CA3 region, respectively.

FIGURE 8. Tat-GluR6-9c decreases the activation of caspase-3 in the cytosol in rat hippocampal CA1 and CA3/DG regions. A and B, cleaved caspase-3 and the protein levels of caspase-3 were examined by immunoblot (IB) analysis. C, bands corresponding to caspase-3 were scanned, and the intensities are represented as -fold versus the saline control. Data are the means ± S.D. and are expressed as -fold versus the respective saline group. *p < 0.05 versus the saline group; ±p < 0.05 versus 6-h KA-injected groups (n = 6). D, the representative hippocampal CA1 and CA3 subfield photomicrographs show TUNEL staining and counterstaining with methyl green. Rats were subjected to saline (panels a and b) or to 7 days of KA injection (panels c and d) with prior administration of 100 µg of Tat-GluR6AA (panels e and f) or Tat-GluR6-9c (panels g and h) 40 min before KA administration. Data were obtained from seven independent animals, and the results of a typical experiment are presented. Magnification ×400 in panels a–h. Scale bars = 10 µm.
GluR6-9c 40 min before KA injection significantly decreased neuronal degeneration (Fig. 9, A, panels g and h; and B, panels g and h). At the same time, as the control, Tat-GluR6AA did not show any protection against the degeneration induced by KA (Fig. 9, A, panels e and f; and B, panels e and f). The neuronal density of the saline group, seizure-insulted group, Tat-GluR6AA-treated group, and Tat-GluR6-9c-treated group was 250.0 ± 19.8, 37.2 ± 8.5, 32.6 ± 7.3, and 121.3 ± 17.8 in the CA1 region and 238.0 ± 25.3, 48.7 ± 6.5, 52.4 ± 6.5, and 136.3 ± 12.7 in the CA3 region, respectively.

DISCUSSION

The data obtained in this study show that GluR6 plays an important role in kainate-induced JNK activation and neuronal cell death in vivo. We constructed a peptide comprising nine C-terminal amino acids of GluR6, and pretreatment with the peptide diminished the increased interactions of GluR6 with PSD95 and of MLK3 with PSD95 induced by kainate. These results suggest that the peptide can competitively bind to the PDZ1 domain of PSD95 and suppress the interaction of GluR6 with PSD95 and MLK3. Furthermore, Tat-GluR6-9c inhibited the activation of MLK3, MKK7, and JNK at 6 h after KA treatment, subsequently diminishing apoptosis via inhibition of the nuclear and non-nuclear pathways. We also found that pretreatment with Tat-GluR6-9c led to marked protection against neuronal loss induced by KA in rat hippocampal CA1 and CA3 regions.

Various mechanisms have been proposed to account for the pathological manifestation observed after systemic administration of kainate. Previous studies have suggested that kainate-induced neuronal damage in the hippocampus is sensitive to protection by activation of dopamine D2 receptors (23), adenosine A receptors (24), and N-methyl-d-aspartate receptors (25). The activation of the kainate receptor subunit GluR6 induced neuronal degeneration in the hippocampus, and GluR6-deficient mice exhibited resistance to neuronal degeneration and seizure induced by KA. However, the molecular mechanism of GluR6 involved in the apoptotic signaling pathway induced by KA is less well understood. Savinaiaen et al. (9) showed that GluR6, PSD95, and MLK3 form a signaling module and facilitate MLK3 and JNK phosphorylation and activation in vitro; however, it is still unclear whether inhibition of the assembly of the GluR6-PSD95-MLK3 signaling module could attenuate JNK activation and epileptic brain damage in vivo. This study has demonstrated our hypotheses that the GluR6-PSD95-MLK3 signaling module exists in seizure and that Tat-GluR6-9c attenuates JNK activation induced by kainate.

All MLK family members regulate the JNK signaling pathway by phosphorylation-dependent regulation of MKK4 and MKK7. MKK4 and MKK7 are dual-specificity kinases that phosphorylate tyrosine and threonine residues in the catalytic domains of JNKs (26). Many studies have demonstrated that activation of the JNK signaling pathways is critical for both development and pathological neuronal cell death. Previous studies suggested that the activation of JNK3 is mediated by the MLK-MKK7-JNK signaling module (27) and that JNK3 is related to neuronal death induced by ischemia/reperfusion (28). Does the same mechanism exist in KA-induced brain injury in vivo? We have shown that pretreatment with Tat-GluR6-9c attenuated the activation of MLK3 induced by KA and subsequently diminished the phosphorylation levels of MKK7 and JNK. Because pretreatment with the peptide inhibited the activation of MLK3 and JNK induced by KA in hippocampal CA1 and CA3/DG subfields, we inferred that application of Tat-GluR6-9c would also rescue hippocampal CA1 and CA3 neurons from degeneration. The results from this study show that, in fact, the peptide had the ability to prevent hippocampal CA1 and CA3 neurons from degeneration 7 days after KA treatment, but it is surprising that the peptide could provide neuroprotection when one considers that α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors are also activated by kainate. One possibility is that the peptide could bind to other PDZ domain-containing proteins, which are involved in the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor signaling or other signaling pathways. Collectively, our results indicate that the GluR6-containing kainate receptor is involved in KA-induced brain injury and that the peptide plays a neuroprotective role through inhibiting the assembly of the GluR6-PSD95-MLK3 signaling module.

JNK is a serine/threonine protein kinase. Activated JNK can phosphorylate nuclear substrates such as c-Jun and cytosolic substrates such as some members of the Bcl-2 family, leading to neuronal death. Therefore, activated JNK phosphorylates the transcription factor c-Jun and leads to increased AP-1 transcription activity to modulate transcription of a number of apoptosis genes such as FasL (29). Our results from Western blotting and immunohistochemistry showed that pretreatment with Tat-GluR6-9c diminished the increased phosphorylation of c-Jun in both hippocampal CA1 and CA3 regions. Furthermore, pretreatment with Tat-GluR6-9c also significantly attenuated the increased expression of FasL induced by KA. Taken together, these results show that the GluR6-mediated signal pathway is involved in neuronal apoptosis induced by seizure via the nuclear pathway.

In addition to the nuclear pathway, JNK can also promote neuronal cell apoptosis by regulating the activation of some non-nuclear substrates such as Bcl-2 family members. Many studies have shown that Bax plays an important role as a regulatory target for JNK. Bax is a potent regulator of mitochondrial-dependent apoptosis, and a substantial proportion of Bax is bound to 14-3-3 proteins in the cytosol of healthy cells. In response to stress stimuli, Bax dissociates from 14-3-3 and translocates to mitochondria (30). In the event of seizure induced by kainate stimulation, Bax plays the same role in dissociation from 14-3-3, resulting in mitochondrial Bax accumulation after seizure (22). After translocation to mitochondria, Bax induces cytochrome c release either by forming a pore by oligomerization in the outer mitochondrial membrane or by opening other channels (31–33). A recent study based on assays using isolated mitochondria has suggested that activated JNK may directly interact with mitochondria and result in the release of cytochrome c and Smac from mitochondria (34). Once cytochrome c is released into the cytoplasm from mitochondria, it can interact with Apaf-1 and dATP, forming the apoptosome and leading to activation of the cytochrome c-dependent caspase cascade, and eventually leads to apoptosis (35, 36). Our study demonstrated that Bax translocated to mitochondria from the cytoplasm following JNK activation induced by seizure and then promoted the release of cytochrome c into the cytosol, which occurred in hippocampal CA1 and CA3/DG subregions, whereas pretreatment with Tat-GluR6-9c prevented Bax translocation to mitochondria and diminished the release of cytochrome c and the activation of caspase-3, which ultimately attenuated mitochondrial-mediated apoptosis. Moreover, results from TUNEL assay provided strong evidence that the peptide could protect the hippocampal CA1 and CA3 neurons from apoptosis. These results suggest that the GluR6-mediated signal pathway is involved in neuronal apoptosis induced by seizure via the non-nuclear pathway, i.e., the mitochondrial-dependent apoptotic pathway.

It is well known that the vulnerable area in response to cerebral ischemia is the CA1 (but not CA3) subfield. The extracellular signal-regulated kinase ERK5, an important survival signal, is selectively activated in the hippocampal CA3/DG region, which is relevant to the N-methyl-D-aspartate receptor and L-type voltage-gated calcium channel in ischemic brain damage (37). Although cumulative studies have shown that
kainate receptors are more abundant in the CA3 subfield compared with the CA1 subfield of the hippocampus (38), it is interesting that kainate stimulation induces similar severe neuronal damages in both CA3 and CA1 subfields. Analysis of GluR6-deficient mice has demonstrated the involvement of the GluR6 subunit in the high sensitivity of CA3 neurons to kainate and in synaptic transmission in the mossy fiber.

Role of GluR6 in Kainate-induced Seizures

FIGURE 9. Neuroprotection of Tat-GluR6-9c against KA-induced brain injury in rat hippocampal CA1 and CA3 regions. A, in the CA1 subfield, rats were subjected to saline (panels a and b) or KA for 7 days (panels c and d) with prior administration of 100 μg/10 μl control peptide (panels e and f) or Tat-GluR6-9c (panels g and h) 40 min before KA injection. B, the same model is shown for the hippocampal CA3 subfield. Data were obtained from seven independent animals, and the results of a typical experiment are presented. Magnification ×40 in panels a, c, e, and g; magnification ×400 in panels b, d, f, and h. Scale bars = 200 μm (panels g) and 10 μm (panels h).
Role of GluR6 in Kainate-induced Seizures

In summary, our results show that KA induced the assembly of the GluR6-PSD95-MLK3 signaling module and subsequently activated JNK downstream signaling pathways, ultimately resulting in neuronal cell death. Application of Tat-GluR6-9c, a GluR6 C-terminus-containing peptide, suppressed the clustering of GluR6 in the postsynaptic regions by competitively binding to the PDZ1 domain of PSD95 and subsequently inhibited the activation of MLK3 and JNK. Furthermore, Tat-GluR6-9c inhibited the activation of the nuclear and non-nuclear pathways of JNK induced by KA. Notably, the peptide had neuroprotective effects against rat epileptic brain damage. In conclusion, the kainate receptor subunit GluR6 plays an important role in brain damage induced by KA, and Tat-GluR6-9c provides a new approach for epileptic seizure therapy.

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