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Regulation of GluR6-PSD95-MLK3 Signaling in KA-Induced Epilepsy

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1. Introduction

Glutamate receptors are classified into two groups: metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). The ionotropic glutamate receptors are superfamily of ligand-gated cation channels that encompass three receptor families identified by the agonists that selectively activate them: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainic acid (KA) receptors (Dingledine *et al.* 1999, Mayer & Armstrong 2004, Kew & Kemp 2005). KA is a potent exogenous agonist of KA receptors and AMPA 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 KA (Sperk *et al.* 1983). KA-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 (Ben-Ari 1985).

KA receptors are comprised of five different subunits: KA1, KA2, GluR5, GluR6 and GluR7 (Lilliu *et al.* 2002, Porter *et al.* 1997). It is reported that GluR6 subunit-deficient and *Jnk3* gene knock-out mice resistance to KA-induced seizures and neuronal toxicity (Yang *et al.* 1997, Mulle *et al.* 1998). And the Glur6 midiated JNK3 (c-Jun N-terminal kinase 3) signaling pathway has been pay more attention in the study of neuron damage during epilepsia. C terminus of GluR6 can bind to the PDZ1 domain of the postsynaptic density protein PSD95/SAP90 through specific interaction (Garcia *et al.* 1998, Mehta *et al.* 2001). Previous studies have also shown that MLK3 (mixed lineage kinase-3), an upstream kinase of JNK (Tibbles *et al.* 1996), can interact with the SH3 (Src homology) domain of PSD95 (Savinainen *et al.* 2001). The triple complex GluR6-PSD95-MLK3 may exist and facilitate JNK activation.

In our previous studies on brain ischemia, it has demonstrated that KA enhanced the assembly of GluR6-PSD95-MLK3 module, increased the autophosphorylation of MLK3 and the phosphorylation of MKK7 (mitogen-activated protein kinase kinase 7), JNK3, c-Jun and Bcl-2 (B-cell lymphoma 2), raised the expression of Fas-Ligand (FasL) and caused the release of Bax (Bcl-2 associated x protein) from Bcl-2/Bax dimmers and the release of cytochrome c from mitochondria (Pei *et al.* 2006). Consequently, the activation of Caspase 3 led to delayed neuronal death in the hippocampal CA1/CA3 subfield (Tian *et al.* 2005, Pei *et al.* 2005, Pan *et al.* 2005). The activation of mitochondrion-linked apoptotic signaling pathways after seizures, including activation of caspase-9, -3, and -8, has also been reported (Henshall *et al.* 2000). And we further found that KA-induced neuronal death is mediated by the GluR6-

PSD95-MLK3 signaling module via FasL/Fas and cytochrome c pathways in KA-induced seizures and interference of the interaction between GluR6 and PSD95 with a peptide can protect neurons from KA-induced death (Liu *et al.* 2006).

Regulation of GluR6 mediated apoptotic pathway has emerged as a possible approach to protect neuron damage against seizure. One idea is down-regulating excitatory GluR6-containing KA receptors by activation of inhibitory GABA receptors. GABA plays a key role in modulating neuronal activity via distinct receptor systems, the ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. It has been proposed that coactivation of $GABA_A$ and $GABA_B$ receptors induced by muscimol and baclofen respectively can result in neuroprotection during *in vitro* ischemia (Costa *et al.* 2004), and coapplication of the two agonists is more effective than when solely used (Zhang *et al.* 2007). Data acquired from our lab demonstrated that coapplication of muscimol with baclofen has neuroprotective effects in rat hippocampal CA1 and CA3 regions and inhibits the assembly of the GluR6-PSD95-MLK3 signaling module and subsequently activates JNK downstream signaling pathways (Li et al.).

2. Neuroprotective effects of peptide Tat-GluR6-9c and GABA receptors activation against neuronal death induced by KA in rat hippocampus

Based on our previous study, activation of GluR6-PSD95-MLK3 signaling is an important reason for neuron death in rat KA-induced epilepsy model. Interference of this signaling might have protective effects against neuron death, so we designed two different strategies to carry out the goal. First, we constructed a peptide comprising the conserved nine COOH-terminal residues of GluR6 (Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala, named GluR6-9c), which was fused to Tat protein (cell-membrane transduction domain of the human immunodeficiency virus-type 1, Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg). The Tat-GluR6-9c peptide can be delivered into hippocampal neurons and destroy the interaction between GluR6 and PSD95, and further suppress the GluR6-PSD95-MLK3 signaling. Second, elevating the inhibition of GABA receptors can significantly decrease KAR-mediated excitation in KA-induced epilepsy. The major finding is that Muscimol and/or Baclofen can suppress the assembly of the GluR6-PSD95-MLK3 signaling. One possible explanation is that the activation of $GABA_A$ receptor by muscimol can induce the hyperpolarization of postsynaptic nrurons via activating ligand-gated Cl- channels, which decrease the depolarization of the neurons (Attwell *et al.* 1993), and activation of G protein-coupled $GABA_B$ receptor by baclofen can attenuate glutamate release from presynaptic neurons (Moldavan *et al.* 2006). The mechanism and roles of these two strategies are described below.

2.1 Assembly of the GluR6-PSD95-MLK3 signaling module during seizure induced by KA in hippocampal CA1 and CA3/DG regions

Immunoprecipitation and immunoblotting were performed to examine the interactions of GluR6 and MLK3 with PSD95 at various times of KA injection. 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.

2.2 Tat-GluR6-9c peptide suppresses the increased assembly of the GluR6-PSD95-MLK3 signaling module induced by KA in hippocampal CA1 and CA3/DG regions

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*. Reciprocal immunoprecipitation experiments demonstrated that the peptide perturbed the GluR6-PSD95-MLK3 signaling module. 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.

2.3 Tat-GluR6-9c inhibits the activation of MLK3, MKK7, and JNK induced by KA in hippocampal CA1 and CA3/DG regions

KA treatment resulted in a remarkable increase in the phosphorylation of MLK3 in CA1and CA3/DG regions. Pretreatment with Tat-GluR6-9c significantly diminished the increase in the phosphorylation of MLK3. And, the activation of MKK7 and JNK at 6 h after KA injection was significantly suppressed by application of the Tat-GluR6-9c peptide in CA1 and CA3/DG regions.

2.4 Tat-GluR6-9c inhibits the phosphorylation of c-Jun and the expression of FasL induced by KA in hippocampal CA1 and CA3/DG regions

The phosphorylation and expression of transcription factor c-Jun was significantly increased at 6 and 12 h in both CA1 and CA3/DG regions after KA injection. Prior administration of Tat-GluR6-9c significantly diminished the increase in phospho-c-Jun at 6 h after KA treatment. The protein levels of c-Jun were not affected. The increased phosphorylation of c-Jun leads to increased expression of FasL. The expression of FasL increased rapidly at 6 h and returned to the basal level at 3 days in CA1 and CA3/DG regions. Prior application of Tat-GluR6-9c diminished the increased expression of FasL.

2.5 Tat-GluR6-9c decreases Bax expression and increases Bcl-2 expression induced by KA in hippocampal CA1 and CA3/DG regions

It is known that Bcl-2 is an anti-apoptotic protein, whereas Bax is an pro-apoptotic protein. 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. 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.

2.6 Tat-GluR6-9c attenuates Bax translocation and the release of cytochrome c induced by KA in Hippocampal CA1 and CA3/DG regions

A previous study reported mitochondrial Bax accumulation after seizure (Henshall *et al.* 2002). Tat-GluR6-9c can inhibit Bax translocation in the mitochondrial fraction in both CA1 and CA3/DG regions. Moreover, Tat-GluR6-9c inhibited the release of cytochrome c from mitochondria to the cytosol in CA1 and CA3/DG fields.

2.7 Tat-GluR6-9c inhibits the activation of caspase-3 and neuronal apoptosis induced by KA in hippocampal CA1 and CA3/DG regions

Tat-GluR6-9c pretreatment diminished the activation of caspase-3 at 6 h after KA injection. TUNEL (Terminal Transferase dUTP Nick End Labeling) staining was used to examine the apoptosis of CA1 and CA3 neuronal cells in the hippocampus. Administration of Tat-GluR6-

9c 40 min before KA injection significantly decreased TUNEL-positive cells. Tat-GluR6-9c significantly decreased neuronal degeneration (Fig. 1).

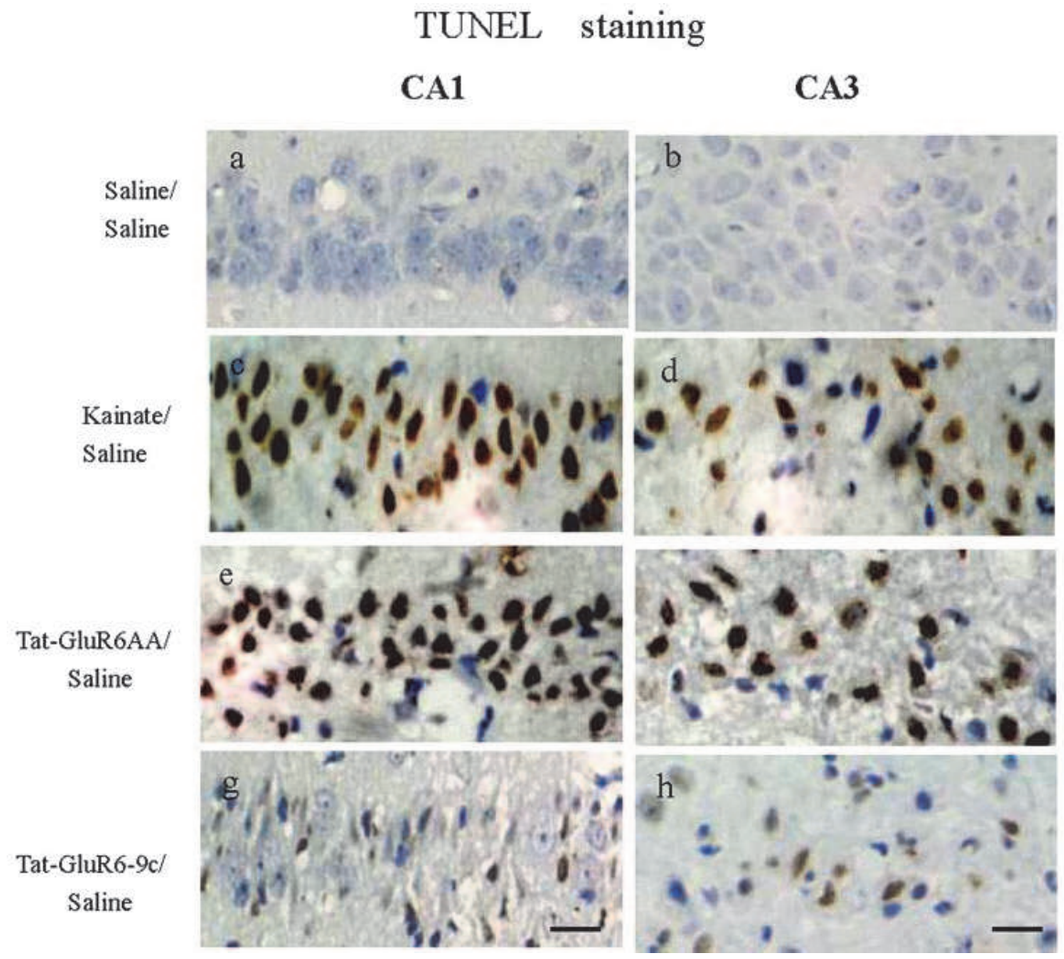

Fig. 1. Neuroprotection of peptide Tat-GluR6-9c in hippocampal CA1 and CA3 subfields.

2.8 Muscimol and baclofen suppress the increased assembly of the GluR6-PSD95-MLK3 signaling module induced by KA in hippocampal CA1 and CA3/DG regions

The GABA receptors can be activated by two GABA agonists: muscimol, a $GABA_A$ agonist, and baclofen, a $GABA_B$ agonist. Reciprocal immunoprecipitation experiments suggested that the administration of two agonists resulted in the disassembly of GluR6-PSD95-MLK3 signaling module. Co-administration of muscimol and baclofen 40 min prior to KA injection diminished the increased interactions of GluR6 and MLK3 with PSD95 at 6 h after KA treatment in CA1 and CA3/DG subregions, whereas the protein levels of GluR6, PSD95 and MLK3 were not altered.

2.9 Muscimol and baclofen inhibit the activation of MLK3, MKK7 and JNK3 induced by KA in hippocampal CA1 and CA3/DG regions

Pretreatment with muscimol and baclofen significantly diminished the increase of the MLK3 phosphorylation. Meanwhile, the activation of MKK7 at 6 h after KA injection was

significantly suppressed by co-application of muscimol and baclofen in CA1 and CA3/DG regions. Furthermore, similar results were obtained with JNK3.

2.10 Muscimol and baclofen inhibit the phosphorylation of c-Jun and the expression of FasL induced by KA in hippocampal CA1 and CA3/DG regions

Prior administration of muscimol and baclofen significantly diminished the increase of phospho-c-Jun at 6 h after KA treatment. Prior application of musimol and baclofen attenuated the increased expression of FasL at 6 h induced by KA in hippocampal CA1 and CA3/DG regions.

2.11 Muscimol and baclofen decrease Bax expression and increase Bcl-2 expression induced by KA in hippocampal CA1 and CA3/DG regions

Prior application of muscimol and baclofen 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.

2.12 Muscimol and baclofen attenuate Bax translocation and the release of cytochrome c induced by KA in hippocampal CA1 and CA3/DG regions

Muscimol and baclofen inhibited Bax translocation in the mitochondrial fraction at 6 h after KA administration compared with the saline control in both CA1 and CA3/DG regions. In the cytosolic fraction, cytochrome c immunoreactivity was evident as a single band at 6 h of KA injection. However, it was weakly detected in the saline group. 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, muscimol and baclofen inhibited the release of cytochrome c from mitochondria to the cytosol in CA1 and CA3/DG fields.

2.13 Muscimol and baclofen inhibit the activation of caspase-3 and neuronal apoptosis induced by KA in hippocampal CA1 and CA3/DG regions

Muscimol and baclofen pretreatment diminished the activation of caspase-3 at 6 h after KA injection. Furthermore, significant numbers of TUNEL-positive cells were observed in the KA-treated group after 7 days, but administration of muscimol and baclofen 40 min before KA injection significantly decreased TUNEL-positive cells.

3. Conclusion

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 assembly of the GluR6-PSD95-MLK3 signaling module. As a result, the peptide attenuated 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.

Co-application of muscimol ($GABA_A$ receptor agonist) and baclofen ($GABA_B$ receptor agonist) inhibited the assembly of the GluR6-PSD95-MLK3 signaling module. The two agonists attenuated the activation of MLK3 and JNK. Furthermore, muscimol and baclofen inhibited the activation of the nuclear and non-nuclear pathways of JNK induced by KA. Notably, the coapplication the two agonists had neuroprotective effects against rat epileptic brain damage. This highlighted that the balance between neuronal excitation and inhibition is critical for maintaining normal function.

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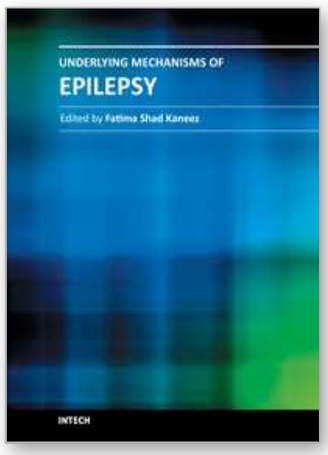

Underlying Mechanisms of Epilepsy

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This book is a very provocative and interesting addition to the literature on Epilepsy. It offers a lot of appealing and stimulating work to offer food of thought to the readers from different disciplines. Around 5% of the total world population have seizures but only 0.9% is diagnosed with epilepsy, so it is very important to understand the differences between seizures and epilepsy, and also to identify the factors responsible for its etiology so as to have more effective therapeutic regime. In this book we have twenty chapters ranging from causes and underlying mechanisms to the treatment and side effects of epilepsy. This book contains a variety of chapters which will stimulate the readers to think about the complex interplay of epigenetics and epilepsy.

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