Crosstalk between PSD-95 and JIP1-Mediated Signaling Modules: The Mechanism of MLK3 Activation in Cerebral Ischemia†

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ABSTRACT: Our previous study indicates that global ischemia facilitates the assembly of the GluR6•PSD-95•MLK3 signaling module, which in turn activated MLK3, leading to exacerbated ischemic neuron death. In addition, JIP1, functioning as a scaffold protein, could couple MLK3-MKK7-JNK to form a specific signaling module and facilitate the activation of the JNK signal pathway. However, the organization, regulation, and function between the two signaling modules and the effects they have on MLK3 activation remain incompletely understood. Here, we show that JIP1 maintains MLK3 in an inactive and monomeric state; once activated, MLK3 binds to PSD-95 and then dimerizes and autophosphorylates. In addition, a GluR6 C-terminus-containing peptide (Tat-GluR6-9c) and antisense oligonucleotides (AS-ODNs) against PSD-95 inhibit the integration of PSD-95 and MLK3 and the dimerization of MLK3, facilitate the interaction of JIP1 and MLK3, and, consequently, perform neuroprotection on neuron death. However, AS-ODNs against JIP1 play a negative role compared to that mentioned above. The findings show that the crosstalk occurs between PSD-95 and the JIP1-mediated signaling module, which may be involved in brain ischemic injury and contribute to the regulation of MLK3 activation. Thus, specific blockade of PSD-95−MLK3 coupling may reduce the extent of ischemia-reperfusion-induced neuronal cell death.

Among various hypotheses about the mechanism of ischemic injury, excitotoxicity mediated by kainate (KA) receptors has attracted our attention (*1*). KA receptor subunit glutamate receptor 6 (GluR6)[1] may mediate the activation of JNK3 in response to the excitotoxicity of kainate (*2*, *3*). Moreover, our previous paper reports they might form a GluR6•PSD-95•MLK3 signaling module and facilitate the JNK signal pathway (*1*, *4*). The peptide Tat-GluR6-9c we construct could perturb the assembly of the GluR6•PSD-95•MLK3 signaling module and, therefore, play a protective role against ischemic injury (*1*).

Previous work (*5*−*10*) has shown that the components of the JNK protein kinase cascade can be organized into a defined signaling module with a scaffold in vivo, which determines the JNK signaling specificity. Among the components of JNK-signaling modules, the MLK3-MKK7-JNK signaling module is the most important in the mechanism of neuronal apoptosis (*9*−*11*). Moreover, JNK-interacting protein 1 (JIP1), which is highly concentrated in the adult brain, and particularly enriched in the cerebral cortex and hippocampus (*12*), is identified as a scaffold and selectively mediates signaling by the MLK3-MKK7-JNK pathway by facilitating sequential interaction of this cascade (*9*−*11*); however, the mechanism of coupling between JIP1 and the signaling module during reperfusion after transient (15 min) brain ischemia was poorly understood. The information mentioned above forces us to consider whether Tat-GluR6-9c could inhibit the binding of JIP1 and the MLK3-MKK7-JNK signaling module. In the global ischemic model, we reveal the interactive mechanism of JIP1 with the MLK3-JNK signaling module following brain ischemia-reperfusion.

A recent study (*13*) proves that a JNK-induced pro-apoptosis program can be prevented by overproducing JIP1, and on the other hand, reducing JIP1 content could induce a robust increase in the level of basal apoptosis. These findings raise new questions regarding the function of JIP1 in cerebral ischemia-reperfusion and whether AS-ODNs against JIP1 could have the opposite effect on activation of JNK compared to overexpression of JIP1. In addition, JIP1 maintains MLK in a monomerization, unphosphorylation, and inactive state, and the activation of MLK requires dissociation from JIP1 (*14*, *15*). Moreover, MLK3 dimerization via its leucine/isoleucine zipper (LZs) motif is a prerequisite for MLK3 autophosphorylation, which indicates that dimerization plays a pivotal role in MLK3 activation (*15*). However,

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[1] Abbreviations: MLK3, mixed-lineage kinase 3; JIP1, JNK-interacting protein 1; PSD-95, postsynaptic density protein-95; SAP90, synapse-associated protein 90; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DAB, diaminobenzidine; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GluR6, glutamate receptor 6; JNK, c-Jun NH_2-terminal kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NBT, nitroblue tetrazolium; NP-40, Nonidet P40; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PNPP, *p*-nitrophenyl phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with 0.1% Tween 20.

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MLK3 could not only bind with PSD-95 but also interact with JIP1 (*10*, *16*). The above-mentioned facts imply that MLK3 exhibits an indispensable effect on the regulation of the JNK signaling module, and PSD-95 and JIP1 which function as scaffold proteins may have an important influence on the MLK3-JNK signaling transduction pathway; however, the mechanisms remain poorly elucidated.

To further clarify the mechanism of JIP1 and PSD-95 in the JNK signaling pathway induced by cerebral ischemia-reperfusion, our research shed light on the interaction of JIP1 and MLK3, PSD-95, and MLK3; the specific AS-ODNs against JIP1 and the specific AS-ODNs against PSD-95 were used. Fully stated, in this study, we clarify the crosstalk between the two signaling modules and the molecule mechanism underlying MLK3 activation in rat hippocampus after global ischemia.

MATERIALS AND METHODS

Antibodies and Reagents. The following primary antibodies were used. Rabbit polyclonal anti-MLK3 (sc-13072), anti-MEK7 (sc-13071), anti-p-JNKs (sc-6254), and anti-JIP1 (sc-15353) were from Santa Cruz Biotechnology. Mouse monoclonal anti-PSD-95 (CP35-100UL) was from Oncogene. Rabbit polyclonal anti-p-MLK3 and anti-p-MKK7 were acquired from Cell Signaling Biotechnology (Beverly, MA). Anti-JNK1/2 antibody, anti-PSD-95 antibody, and the secondary goat anti-rabbit IgG antibody used in our experiment were from Sigma (St. Louis, MO). The nitrocellulose filter was acquired from Amersham. BCIP and NBT were acquired from Promega. All other chemicals were from Sigma unless indicated otherwise.

Induction of Ischemia. Adult male Sprague-Dawley rats weighing 200−250 g were used. Transient cerebral ischemia was induced by a four-vessel occluded (4-VO) method as described previously (*26*). Briefly, rats were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally), and both vertebral arteries were occluded permanently by electrocautery. Then rats were allowed to recover for 24 h, and both carotid arteries were occluded with aneurysm clips to induce cerebral ischemia. After occlusion for 15 min, the aneurysm clips were removed for reperfusion. Rats who lost their right reflex within 30 s and those whose pupils were dilated and unresponsive to light were selected for the experiments. Rectal temperature was maintained at ∼37 °C during ischemia (15 min) and the 2 h reperfusion. Sham control rats were treated using the same surgical procedures except that the carotid arteries were not occluded.

Sample Preparation. Rats were decapitated immediately at various times after reperfusion, and then the hippocampi were isolated and quickly frozen in liquid nitrogen. Tissues were homogenized in ice-cold homogenization buffer containing 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS, pH 7.4) (Sigma), 100 mM KCl, 100 mM $MgCl_2$, 0.2 mM DTT, 1 mM EGTA, 1 mM Na_3VO_4 (Sigma), 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM *p*-nitrophenyl phosphate (PNPP), 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride (PMSF), and leupeptin, aprotinin, and pepstatin A (5 μg/mL each). The homogenates were centrifuged at 800*g* for 10 min at 4 °C.

Drug Administration. Peptides, which comprise the nine COOH-terminal residues of GluR6 (Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala; GluR6-9c), were conserved in sapiens and rodents. Tat protein (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) was fused to GluR6-9c and resulted in a 20-amino acid fusion peptide (Tat-GluR6-9c). Peptides Tat-GluR6-9c (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala) (100 μg) or control peptides, a Tat-GluR6-9c peptide in which the COOH-terminal ETMA motif contained four points mutation (Arg-Leu-Pro-Gly-Lys-Ala-Ala-Asp-Asp; Tat-GluR6AA), in 10 μL of saline were administered to rats 40 min before ischemia using a microinjector through both cerebral ventricles (anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma) (*3*). Ten nanomoles of specific antisence oligodeoxynucleotides against PSD-95 (Invitrogen Japan K.K., Tokyo, Japan) in 10 μL of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] was administered to the rats every 24 h for 3 days through cerebral ventriclar injection (anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma). The same dose of missense oligodeoxynucleotides and vehicle were used as the control. The antisense and missense sequences are as follows: AS, 5′-TGT-GATCTCCTCATACTC-3′; and MS, 5′-AAGCCCTTGT-TCCCATTT-3′. Ten nanomoles of specific antisence oligodeoxynucleotides against JIP1 also accepted the same treatment. The same dose of missense oligodeoxynucleotides and vehicle were used as a control. The antisense and missense sequences are as follows: AS, 5′-TCTCTCGCTC-CGCCATTC-3′; and MS, 5′-CTCTCTCACTGCGTCCCT-3′.

Immunoprecipitation and Immunoblotting. Tissue homogenates (400 μg of protein) were diluted 4-fold with 50 mM HEPES buffer (pH 7.4) containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and EDTA, EGTA, PMSF, and Na_3VO_4 (1 mM each). Samples were preincubated for 1 h with 20 μL of protein A Sepharose CL-4B (Amersham, Uppsala, Sweden) at 4 °C and then centrifuged to remove protein 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−agarose beads (20 μL, Sigma) were added to the tube, and incubation was continued for an additional 2 h. Samples were centrifuged at 10000*g* for 2 min at 4 °C, and pellets were washed with immunoprecipitation buffer three times. Bound protein were eluted by boiling at 100 °C for 5 min in SDS−PAGE loading buffer and then isolated with a centrifuge. The supernatants were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, U.K.). After being blocked for 3 h in Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 3% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugate secondary antibodies in TBST for 2 h and developed using NBT/BCIP color substrate (Promega, Madison, WI). The densities of the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP, Upland, CA).

When necessary, to examine monomer and dimeric forms of MLK3, 2× SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, and 20% glycerol] both with and without the reducing agent dithiothreitol (DTT) (final concentration, 200 mM) was added to the protein

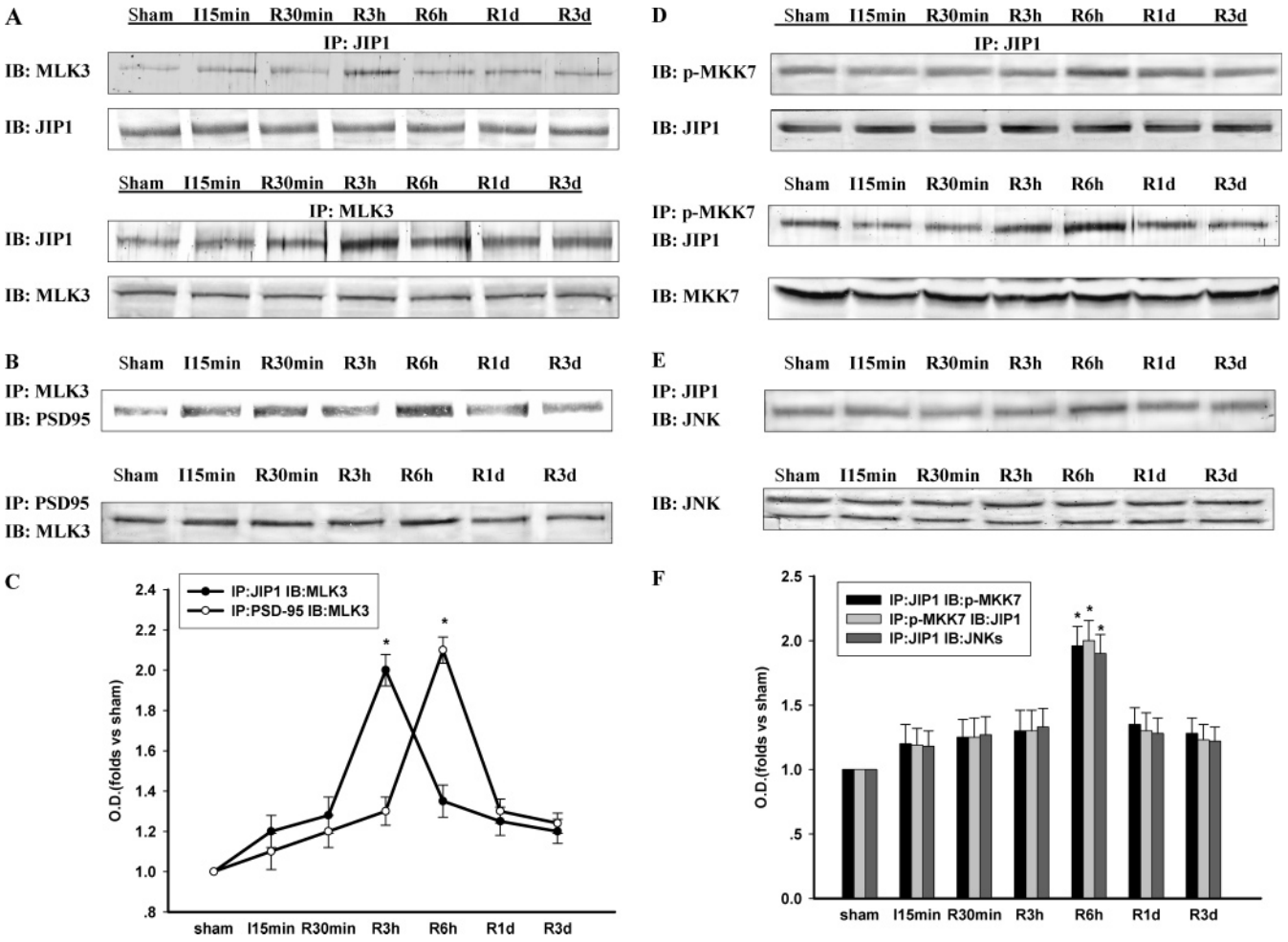

FIGURE 1: Time courses of reperfusion-induced alteration of interaction between JIP1 and the MLK3-JNK signaling module as well as MLK3 and PSD-95. Extracts were obtained from rat hippocampus derived from sham-operated (sham) or 15 min ischemic rats at the indicated times, and immunoprecipitation followed by immunoblotting analysis was used to detect the alteration of JIP1 with the MLK3-MKK7-JNK signal module and MLK3 with PSD-95. Bands were scanned, and the intensities are represented as *x*-fold vs sham control. Data are means ± the standard deviation from four independent animals ($n = 4$) and are expressed as *x*-fold vs control. The asterisk indicates a P of <0.05 vs control.

samples. After boiling for 3 min, the samples were separated by SDS−PAGE and transferred to nitrocellulose membranes. The remaining samples were treated with the process mentioned above.

Histology. Rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) under anesthesia after ischemia-reperfusion for 5 days. Brains were removed rapidly and further fixed with the same fixation solution at 4 °C overnight. Postfixed brains were embedded in paraffin, followed by preparation of coronal sections 5 μm thick using a microtome (RM2155, Leica, Nussloch, Germany). 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 were examined with light microscopy, and the number of surviving hippocampal CA1 pyramidal cells per 1 mm length was counted as the neuronal density.

Statistical Evaluation. Values from more than three independent animals were expressed as means ± the standard deviation. Statistical analysis of the results was carried out by one-way analysis of variance (ANOVA) followed by the Duncan's new multiple-range method or Newman-Keuls test. P values of <0.05 were considered significant.

RESULTS

Association of JIP1 with MLK3, MKK7, and JNK and Interaction of PSD-95 with MLK3 during Reperfusion after Ischemia for 15 min in Rat Hippocampus. JIP proteins facilitate MLK-dependent signal transduction to JNK possibly by aggregating the three components of a JNK module (*17*). MLK3 or DLK, MKK7, and JNK individually associate with JIP1 via a direct protein−protein interaction (*9*, *18*). To examine the underlying mechanism and effect of JIP1 on the JNK signaling pathway, we first investigated the interaction of JIP1 with the MLK3-MKK7-JNK signaling module during reperfusion after transient (15 min) global cerebral ischemia using immunoprecipitation and immunoblotting. As shown in Figure 1D, the level of association of JIP1 with p-MKK7 increased obviously after reperfusion for 6 h and then decreased gradually to the basal level, but the level of protein expression remained unchanged. The interaction of JIP1 with JNK produced similar results (Figure 1E). However, the time course of binding of MLK3 with JIP1 was interesting because the interaction peak was different from others. Figure 1A indicates that the level of binding of MLK3 to JIP1 gradually increased and reached the maximum after reperfusion for 3 h. These figures suggest MKK7 and

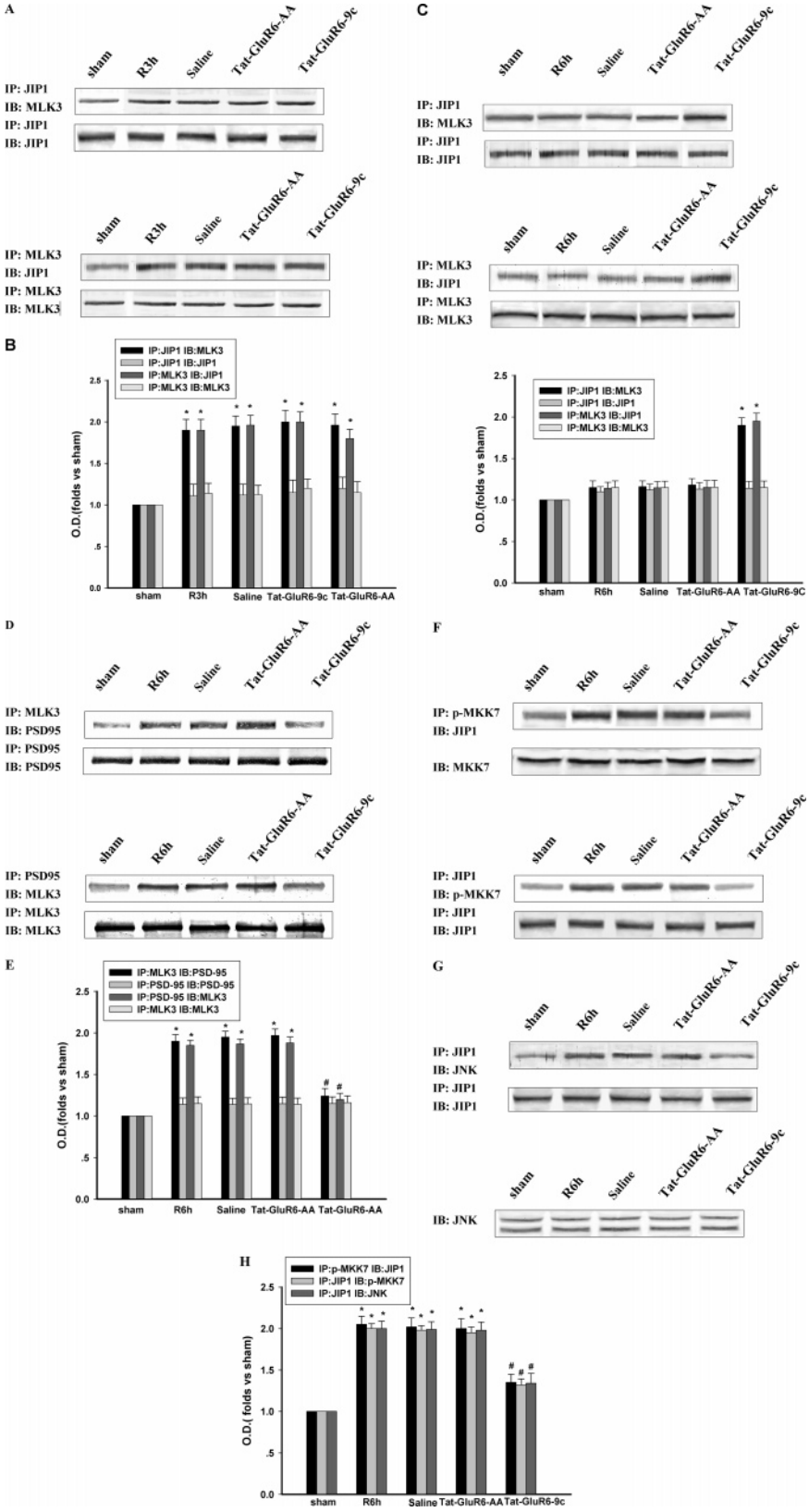

FIGURE 2: Effects of the pretreatment with Tat-GluR6-9c on the binding of JIP1 and the MLK3-JNK signaling module as well as MLK3 and PSD-95. Homogenates of rat hippocampus from animals at indicated reperfusion time points after ischemia for 15 min were used to examine the effects of the peptides on the interaction of them. Immunoprecipitation and immunoblotting analysis indicated that the peptides perturb the interaction peak of PSD-95 with MLK3 and JIP1 with p-MKK7 and JNK significantly after reperfusion for 6 h but have little influence on the association between JIP1 with MLK3 after reperfusion for 3 h; however, the peptides facilitate the interaction of JIP1 with MLK3 after reperfusion for 6 h. Results are expressed as means ± the standard deviation derived from four independent animals ($n = 4$) and are expressed as the power of control animals. The asterisk indicates a P of <0.05 vs control, and the number sign indicates a P of <0.05 vs a vehicle-treatment group.

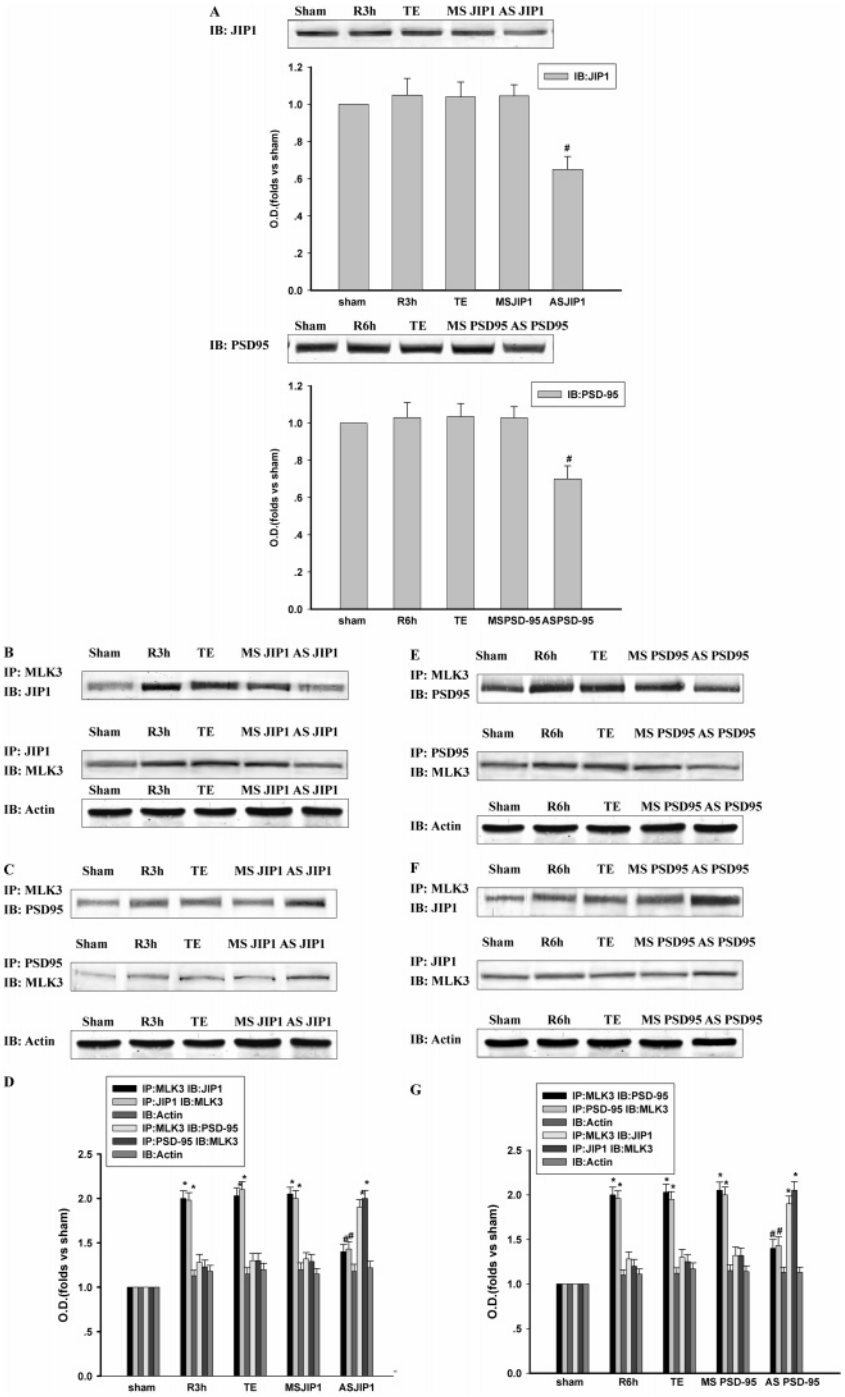

FIGURE 3: Effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on the interaction of MLK3 and JIP1 as well as MLK3 and PSD-95. (A) The expression protein of JIP1 or PSD-95 was weakened by treatment with AS-ODNs against JIP1 or specific AS-ODNs against PSD-95. (B and C) Pretreatment with AS-ODNs against JIP1 can attenuate the interaction of JIP1 and MLK3 after reperfusion for 3 h but enhances the interaction of PSD-95 and MLK3 at the same time point. (E and F) Pretreatment with AS-ODNs against PSD-95 can perturb the interaction of PSD-95 and MLK3 and facilitate the interaction of JIP1 and MLK3 after reperfusion for 6 h. Bands were scanned, and the intensities were represented as *x*-fold vs sham control. Data are means ± the standard deviation from four independent animals ($n = 4$) and are expressed as *x*-fold vs control. The asterisk indicates a P of <0.05 vs control, and the number sign indicates a P of <0.05 vs the vehicle-treatment group.

JNK congregate with JIP1 simultaneously following MLK3 dissociation.

Similarly, PSD-95 also functions as a scaffold protein and mediates the formation of the GluR6·PSD-95·MLK3 signaling module and thereby facilitates the activation of JNK protein kinase. We further detected the alteration of the interaction between MLK3 and PSD-95 because the association of MLK3 and PSD-95 is also important for the JNK signaling pathway. Figure 1B indicates that the interaction peak was at reperfusion for 6 h, which was different from the binding of MLK3 to JIP1. Our recent study also reports that MLK3 activation reached the maximum at the same time point (*19*, *20*). Together, these results suggest that JIP1 could not simultaneously bind the components of the JNK signaling module and, furthermore, that its interaction peak with MLK3 preceded the binding peak between PSD-95 and MLK3.

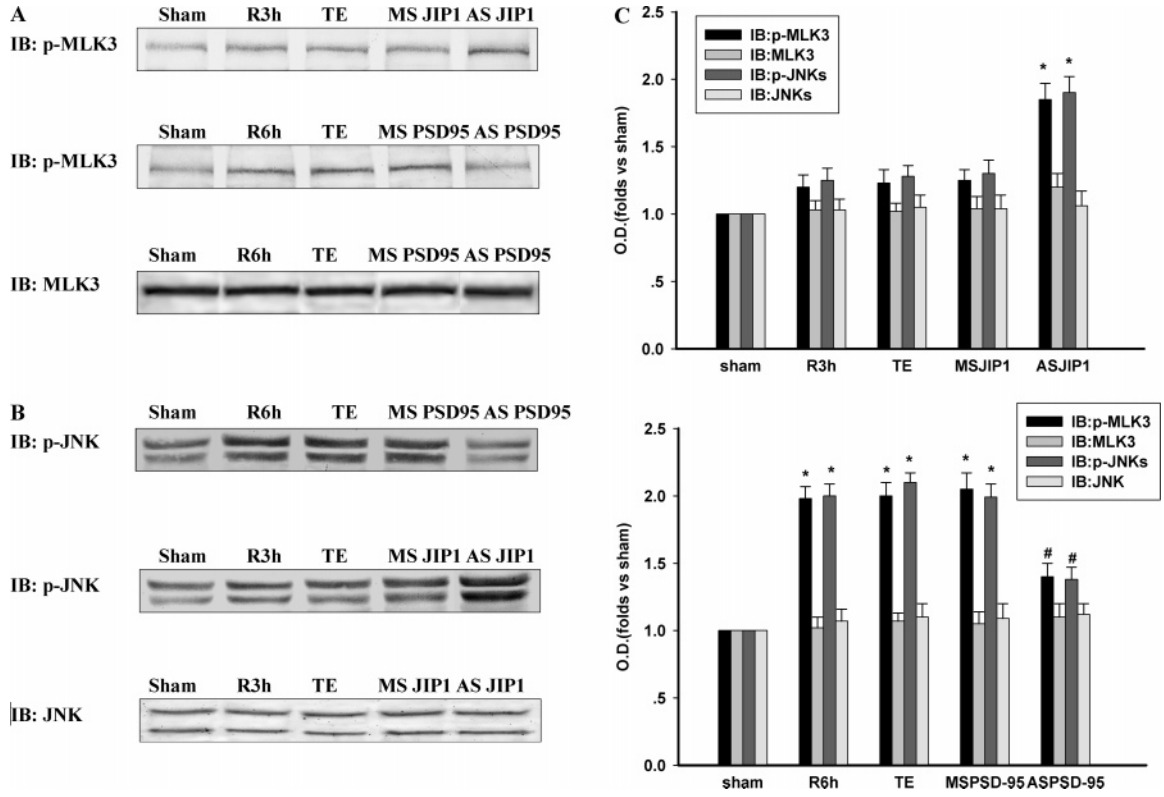

FIGURE 4: Effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on the activation of MLK3 and JNK. Pretreatment with AS-ODNs against PSD-95 can inhibit the phosphorylation of MLK3 after reperfusion for 6 h. However, administration with AS-ODNs against JIP1 can facilitate the activation of MLK3 after reperfusion for 3 h (A). At the same time, the activation of JNK was inhibited by treatment with AS-ODNs against PSD-95 after reperfusion for 6 h and was strengthened by administration with AS-ODNs against JIP1 after reperfusion for 3 h (B). Results are expressed as means ± the standard deviation derived from four independent animals ($n = 4$) and are expressed as the power of control animals. The asterisk indicates a P of <0.05 vs control, and the number sign indicates a P of <0.05 vs the vehicle-treatment group.

Effects of the Peptide on the Association of JIP1 with MLK3, MKK7, and JNK and Interaction of PSD-95 and MLK3. As mentioned above, the peptide Tat-GluR6-9c could inhibit the activity of MLK3 via suppression of the assembly of GluR6·PSD-95·MLK3 and performed neuroprotection against ischemia-reperfusion-induced neuron injury (*1*). However, whether the JIP1-mediated MLK3 signaling module was also involved in the injury or whether the peptide disturbed the signaling module remains unclear. To investigate the possibility, we examined the effects of the peptides on the interaction of JIP1 with MLK3, MKK7, and JNK and the interaction of PSD-95 with MLK3. Samples at each maximum interaction time point were selected, and both immunoprecipitation and immunoblotting were used to detect the alteration. The results show that the peptide could inhibit the strengthened interaction of JIP1 with p-MKK7 and JNK after reperfusion for 6 h after transient ischemia (Figure 2G−I) but had no significant effect on the interaction peak of JIP1 with MLK3 after reperfusion for 3 h (Figure 2A,B). Interestingly, the peptide diminished dramatically the increased level of interaction of PSD-95 with MLK3 but facilitated the association of JIP1 and MLK3 after reperfusion for 6 h (Figure 2C−F). The possible explanation is that the peptide Tat-GluR6-9c could inhibit the interaction of GluR6 and PSD-95 and further suppress the assembly of the GluR6·PSD-95·MLK3 signal module. Thus, MLK3 was maintained in a monomeric state and bound to JIP1 instead of PSD-95, which inhibits the following interaction between JIP1 and p-MKK7 and JNK. Taken together, our results suggest that the binding of JIP1 and MLK3 may negatively regulate MLK3 activity, while the interaction of PSD-95 with MLK3 could enhance the MLK3-JNK signal pathway. The incremental binding affinity of JIP1 and P-MKK7/JNK facilitates the activation of the JNK cascade.

Effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on the Interaction of MLK3 with JIP1 and PSD-95. To further investigate the mechanism of the interaction between MLK3 and JIP1 and PSD-95, we used 10 nmol of specific AS-ODNs against JIP1 and PSD-95 (Invitrogen Japan K.K.) to detect the alteration of the interaction between JIP1 and MLK3 as well as PSD-95 and MLK3. The same dose of missense oligodeoxynucleotides and vehicle were used as the control. Immunoblotting analyses indicate the protein expression of JIP1 is inhibited significantly by specific AS-ODNs against JIP1. However, the same dose of control has no influence on protein expression (Figure 3A), and protein expression of PSD-95 produced similar results (Figure 3A). As shown in Figure 3B−D, AS-ODNs against JIP1 inhibit the strengthened interaction between MLK3 and JIP1 after reperfusion for 3 h but facilitates the interaction of PSD-95 and MLK3 at the same time point. Furthermore, AS-ODNs against PSD-95 attenuated the enhancement of the association between PSD-95 and MLK3 after ischemia for 6 h but, however, strengthened the association of JIP1 and MLK3 at the same time point (Figure 3E−G). All the results suggest JIP1 and PSD-95 interact with MLK3

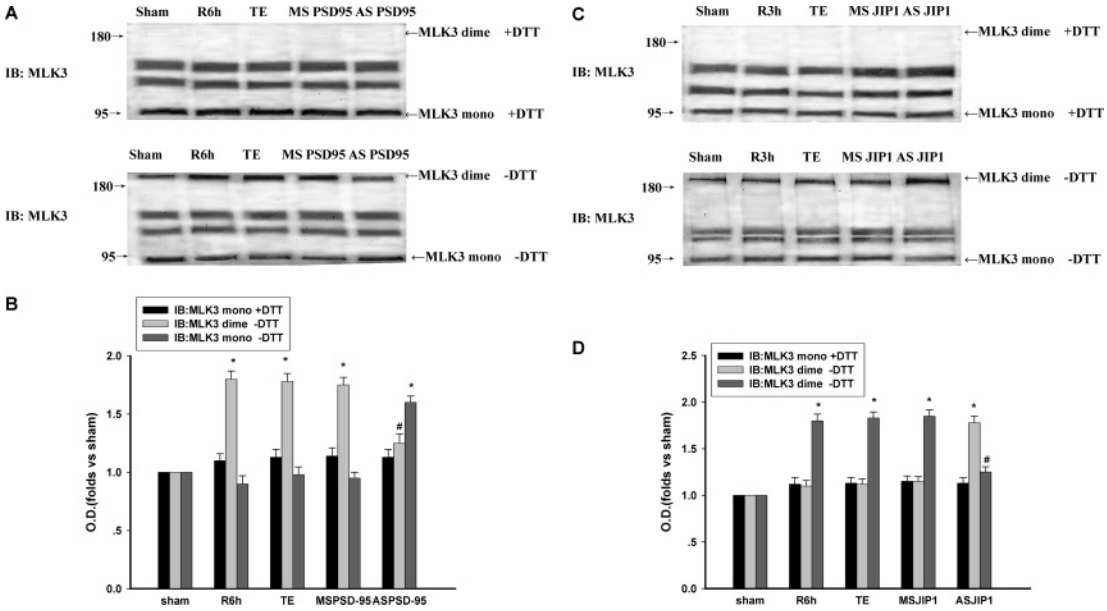

FIGURE 5: Effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on MLK3 dimerization. In the presence of DTT, the dimerization of MLK3 could not be detected and the two AS-ODNs did not have any influence on the MLK3 monomer. However, in the absence of DTT, pretreatment with AS-ODNs against PSD-95 diminished the MLK3 monomer and facilitated the MLK3 dimerization synchronously (A). Under the same conditions, conversely, AS-ODNs against JIP1 enhanced the monomer of MLK3 and suppressed the dimerization of MLK3. Results are expressed as means ± the standard deviation derived from four independent animals ($n = 4$) and are expressed as the power of control animals. The asterisk indicates a P of <0.05 vs control, and the number sign indicates a P of <0.05 vs the vehicle-treatment group.

competitively, and the dynamic interaction may be involved in the regulation of the JNK signal transduction pathway.

Effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on the Activation of MLK3 and JNK. On the basis of results given above, we further examined the effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on the activation of MLK3 and JNK to investigate the mechanism of MLK3 activation and the effect of the interaction between MLK3 and JIP1 and PSD-95 on the ischemia-induced JNK signal transduction pathway. As shown in Figure 4, immunoblotting analysis shows that after reperfusion for 3 h when the interaction of MLK3 with JIP1 reached the maximum strength, AS-ODNs against JIP1 facilitated the activation of MLK3 and JNK. However, after reperfusion for 6 h when the binding of MLK3 and PSD-95 reached the maximum level, AS-ODNs against PSD-95 had negative effects, which attenuated the phosphorylation of MLK3 and JNK. These data show that JIP1 and PSD-95 might play different roles in the activation of the JNK signal pathway, and reducing JIP1 may play a detrimental role in brain ischemia injury.

Effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on MLK3 Dimerization. A previous study confirms that dimerization is a prerequisite for MLK3 autophosphorylation (*15*). As mentioned above, JIP1 and PSD-95 perform different roles in the phosphorylation of MLK3 and JNK. However, the underlying mechanism of the activation of MLK3 was poorly understood, so we examined the effects of AS-ODNs against JIP1 and AS-ODNs against PSD-95 on MLK3 dimerization to clarify the mechanism. As shown in Figure 5, in the absence of dithiothreitol (DTT), AS-ODNs against JIP1 strengthen the expression of MLK3 dimerization and reduce the level of expression of the MLK3 monomer after reperfusion for 3 h. Under the same condition, AS-ODNs against PSD-95 attenuate the expression of MLK3 dimerization and facilitate the expression of the MLK3 monomer after reperfusion for 6 h. However, after the addition of dithiothreitol, MLK3 dimerization could not be detected, and the expression of the MLK3 monomer nearly has no alteration. From these results, we conclude that JIP1 keeps MLK3 in a monomeric, inactive state, and the activation of MLK3 requires the dissociation from JIP1 and then dimerization and phosphorylation.

Effects of Tat-GluR6-9c, AS-ODNs against JIP1, and PSD-95 on the Ischemia-Reperfusion-Induced Delayed Neuronal Cell Death in Rat Hippocampus. As mentioned above, we detect effects of the peptide Tat-GluR6-9c, specific AS-ODNs against JIP1, and specific AS-ODNs against PSD-95 on the assembly of the JNK signaling module as well as the activation of MLK3 and JNK. To further explore whether they play a neuroprotective role against ischemia-reperfusion-induced delayed neuronal cell death, cresyl violet staining was used. The surviving cells of CA1 pyramidal neurons in rat hippocampus, which were subjected to ischemia for 15 min followed by reperfusion for 5 days, were examined. Normal cells showed round and pale stained nuclei, while shrunken cells with pyknotic nuclei after ischemia were counted as dead cells. As shown in Figure 6, transient cerebral ischemia followed by reperfusion for 5 days induced severe cell death. However, administration of the peptides 40 min before cerebral ischemia or pretreatment of specific AS-ODNs against PSD-95 to the rats every 24 h for 3 days limited neuronal degeneration. However, pretreatment of specific AS-ODNs against JIP1 did not have a protective effect. Taken together, these data suggest JIP1 not only functions as a scaffold protein but also may be involved in the regulation of the JNK signal transduction pathway.

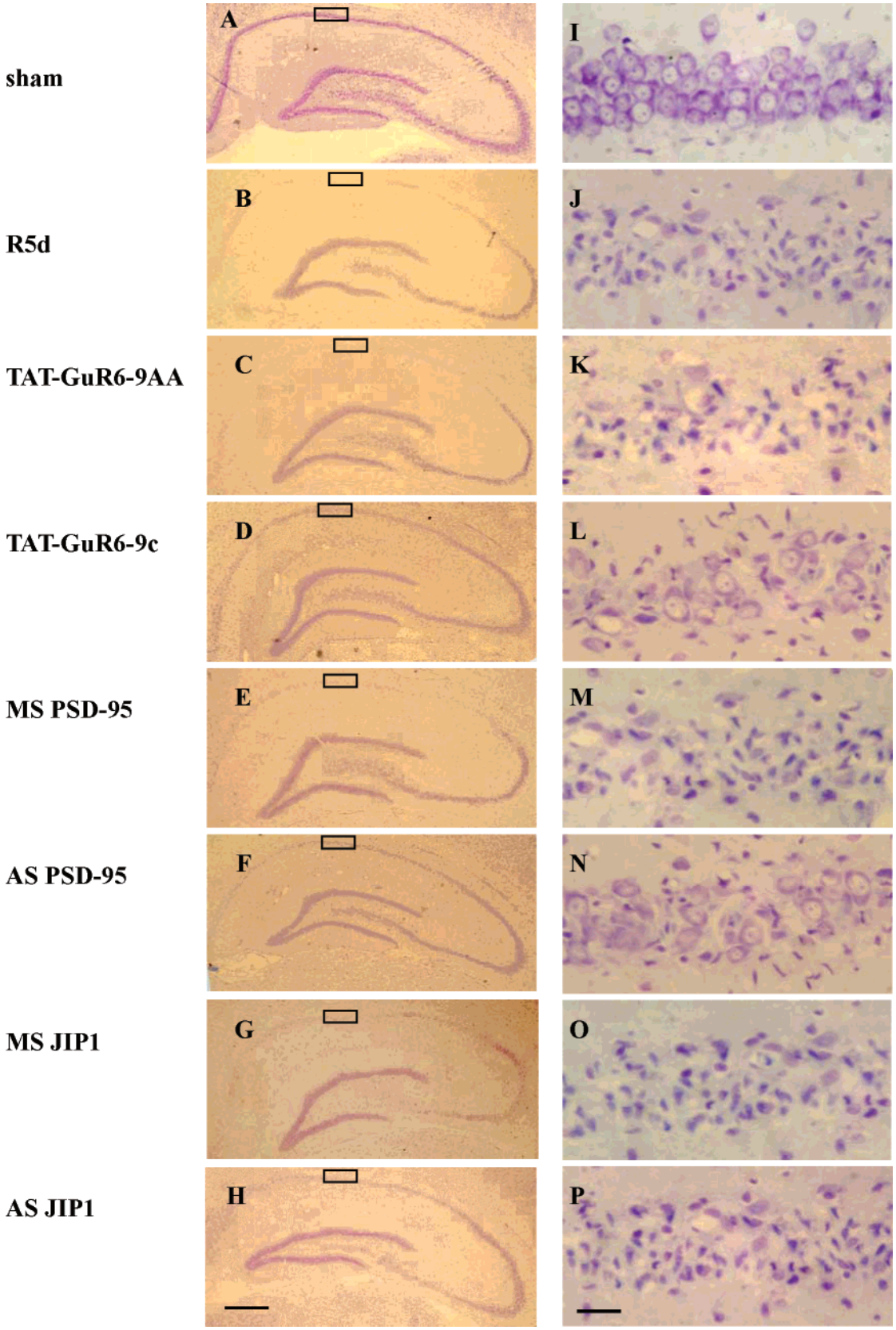

FIGURE 6: Effects of pretreatment with Tat-GluR6-9c, AS-ODNs against JIP1, and PSD-95 on delayed neuronal cell death induced by ischemia-reperfusion injury. Cresyl violet-stanied sections of the hippocampus in sham operation and rats subjected to reperfusion for 5 days after ischemia for 15 min and rats subjected to ischemia for 15 min followed by reperfusion for 5 days with peptide Tat-GluR6-9c (100 μg) 40 min before ischemia, and AS-ODNs against PSD-95 or JIP1 in 10 μL of TE buffer were administered to the rats every 24 h for 3 days through cerebral ventriclar injection. Data were obtained from six independent animals, and a typical experiment is presented. Boxed areas in the left column are shown at higher magnifications in the right column: (A−D) 40× and (E−H) 400×. The scale bar in the left column is 200 μm; the scale bar in the right column is 10 μm.

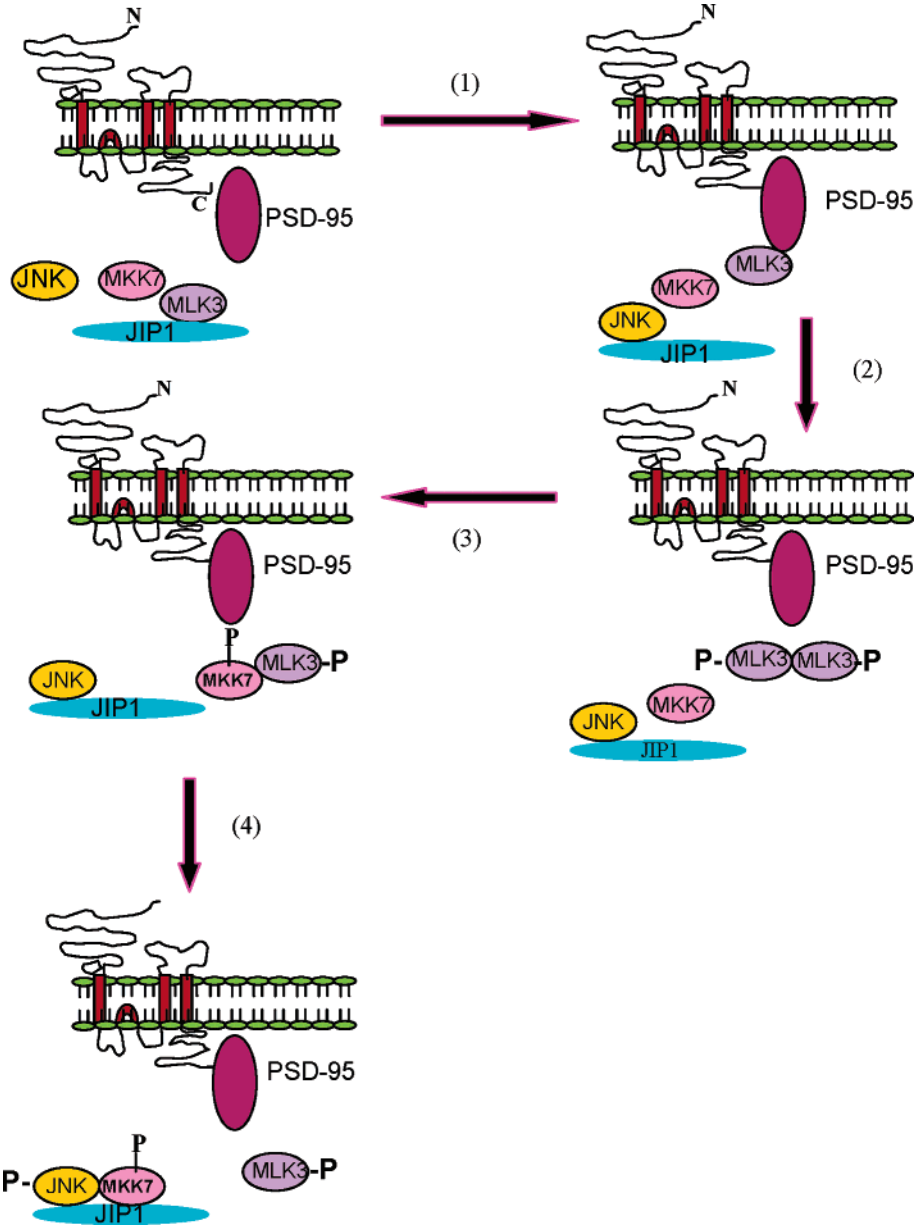

FIGURE 7: Model for the role of JIP1- and PSD-95-mediated signaling modules in the MLK3 activation during brain ischemia-reperfusion. This figure shows a schematic diagram of a theoretical model based on the literature and data presented here. This model illustrates the sequential events which occur on scaffolding proteins during brain ischemia-reperfusion: brain ischemia-reperfusion → JIP1−MLK3 binding → JNK−JIP1 binding → dissociation of MLK3 from JIP1 → binding of MLK3 to PSD-95 → MLK3 dimerization → MLK3 autophosphorylation → activation of the JNK cascade.

DISCUSSION

In this study, we demonstrate that JIP1 kept MLK3 in an inactive and unphosphorylated state; once it is activated, JNK recruitment to JIP1-induced MLK3 dissociates from JIP1 and congregates to PSD-95 and then dimerized and autophosphorylated. Subsequently, the activated MLK3 phosphorylates MKK7 to increase the binding affinity of MKK7-JIP1, which in turn activates JNK and ultimately induces the activation of JNK cascades. These findings support the hypothesis that during brain ischemia, the crosstalk between PSD-95 and the JIP1-mediated signaling module contributes significantly to neuron death in rat hippocampus via regulation of MLK3 activation.

As mentioned previously, PSD-95 can link GluR6 to facilitate JNK activation by anchoring MLK3, upstream activators of JNKs, to form the receptor complex (*15*, *21*, *22*). Our results show the interaction of JIP1 and MLK3 reached the maximum level after reperfusion for 3 h, but JIP1−MKK7 and JIP1−JNK interaction peaked after reperfusion for 6 h, which is consistent with the binding peak of MLK3 and PSD-95 during brain ischemia-reperfusion (Figure 1). These findings suggest that MKK7 and JNK remain simultaneously associated with JIP1 following dissociation of MLK3 from JIP1. Furthermore, the binding of MLK3 with JIP1 and PSD-95 is dynamic and proceeds orderly. The binding of JIP1 and MLK3 precedes the maximum level of interaction between PSD-95 and MLK3, and the phosphorylation peak of MLK3 and interaction peak of PSD-95 and MLK3 occurred concurrently during brain ischemia-reperfusion. This suggests JIP1 might keep MLK3

in an unphosphorylated state, and once MLK3 interacts with PSD-95, it will be activated and further facilitate the JNK cascade.

JIP proteins are assigned the role of either inhibitor or facilitator of JNK activation since they were identified initially (*6*, *17*). Whitmarsh and co-workers suggested that overexpression of JIP1 inhibits JNK activation, which may reflect the sequestration of limiting JNK pathway components into separate complexes (*9*). Our results presented herein show that the peptide Tat-GluR6-9c significantly inhibits the interaction of MLK3 and PSD-95 and also enhances the binding of MLK3 and JIP1, which may suggest that during brain ischemia-reperfusion, the interaction of JIP1 with MLK3 may be involved in the negative regulation of the JNK signal pathway.

On the basis of the mathematical modeling of a two-component scaffold, Levchenko et al. argue that JIP may inhibit or facilitate JNK module signal transduction depending on the relative concentration of the scaffold and component bind to scaffold in a manner independent of one another, exhibiting no cooperation in binding (*23*). Although those observations suggested that JIP1 specifically interacts with the MLK3-JNK signaling module and facilitates the JNK signal pathway, experimental evidence regarding the function of JIP1 during brain ischemia is lacking and experimental evidence for signal transduction requires investigation. As noted above, during ischemia-reperfusion, the alterations of binding affinity between MLK3 and PSD-95 as well as MLK3 and JIP1 were different. In the inactive state, the binding affinity of MLK3 with JIP1 was increased. Once stimulated appropriately, MLK3 integrates with PSD-95 distinctly and reached the maximum height, but the interaction of JIP1 and MLK3 was decreased to the basal level (Figure 1). Moreover, Tat-GluR6-9c inhibited the strengthened interaction between PSD-95 and MLK3 but enhanced the weakened interaction between JIP1 and MLK3 after reperfusion for 6 h (Figure 2).

What is mentioned above suggests that the association of PSD-95 with MLK3 may facilitate the activation of MLK3 and the binding affinity of JIP1 with MLK3 may play a negative role in MLK3 phosphorylation. To further validate the intracellular mechanism of interaction of MLK3 with JIP1 and PSD-95 during reperfusion induced by transient cerebral ischemia, specific AS-ODNs against JIP1 and specific AS-ODNs against PSD-95 were used to detect the interaction of MLK3 with JIP1 and PSD-95, respectively, as well as the phosphorylation of MLK3 and JNK (Figures 3 and 4). These results suggest that during ischemia-reperfusion, the couplings between MLK3 and JIP1/PSD-95 were a dynamic process and the binding affinity between them directly affected MLK3 activation.

A recent model postulates that JIP1, serving as a scaffold protein, binds an MLK, specifically DLK in a monomeric, unphosphorylated, inactive state under basal conditions (*14*). Once stimulated, JNK−JIP1 binding affinity increased and released MLK from JIP1, allowing MLK dimerization, autophosphorylation, activation, and subsequently reinforcement of JNK activity (*18*). Furthermore, MLK3 contains several domains that are predicted to mediate protein−protein interactions, including an SH3 domain, a leucine zipper (LZs), and a Cdc42/Rac interactive binding (CRIB) motif (*24*). In addition, other in vitro studies indicate that dimerization via LZs is crucial for MLK3 autophosphorylation and subsequent phosphorylation of the downstream signaling pathway (*15*). With other MLK family members, the extent of MLK3's catalytic activity is associated with its degree of autophosphorylation (*18*, *25*).

To further investigate the mechanism of MLK3 activation in response to brain ischemia, AS-ODNs against JIP1 and PSD-95 were selected. Our data show AS-ODNs against PSD-95 attenuate the dimerization, subsequently inhibit the phosphorylation of MLK3 after reperfusion for 6 h, and exhibit neuroprotection against programmed neuronal cell death (Figures 4−6). However, AS-ODNs against JIP1 enhance the MLK3 dimerization and activation and have little effect on the delayed neuron death (Figures 4−6). The findings mentioned above suggest that the dissociation from JIP1 and the interaction with PSD-95 facilitate the activation of MLK3, and the dimerization is a prerequisite for MLK3 activation in ischemic rat hippocampus.

In summary, we report the molecular mechanism of MLK3 activation for the first time in rat hippocampus during brain ischemia-reperfusion. The data presented here support the proposal of a modified model of JIP1 and PSD-95 scaffold functions. In these models, MLK3 binds JIP1 in a monomeric, catalytically inactive state under basal conditions. Upon appropriate stimulation, MLK3 dissociated from JIP1 and interacts with PSD-95, and then dimerizes, autophosphorylates, and becomes catalytically active. Subsequently, phosphorylated MLK3 further activates MKK7, which in turn phosphorylates JNK and induces the activity of the JNK cascade. This study sheds new light on the crosstalk between JIP1 and PSD-95-mediated signaling modules on MLK3 activation during brain ischemia-reperfusion and provides new therapeutic targets against ischemic neuron death.

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