PSD-93 mediate the dialogue between neuron and microglia and facilitate acute ischemic stroke by binding 357-395 amino acid sequence of CX3CL1

CURRENT STATUS: UNDER REVIEW

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DOI: 10.21203/rs.2.20106/v1

SUBJECT AREAS Neurobiology of Disease

KEYWORDS yeast two-hybrid, protein interaction, PSD-93, CX3CL1, cerebral ischemia-reperfusion
Abstract

Background: Our previous experiments demonstrated that PSD-93 mediates glutamate excitotoxicity induced by ischemic brain injury, which promotes the release of inflammatory cytokines in early ischemic brain injury by activating the NMDA receptor. Glutamate activity is the key to neuronal excitatory toxicity and microglial cell inflammatory response in the joints. However, the underlying mechanisms of how does PSD-93 mediate the dialogue between neurons and microglia in the postsynaptic dense region remain elusive. And CX3 chemokine ligand 1 (CX3CL1) is a chemokine that is specifically expressed in neurons. Its only receptor CX3CR1 is highly expressed in microglia and its main forms are membrane binding and soluble. In this study, we aim to clarify the specific amino acid sequence of the binding of psd-93 and CX3CL1 and investigate role of PSD-93 on regulating the crosstalk between neuron and microglia in acute ischemic stroke.

Methods: In this study, male C57BL/6 mice aged 8-12 weeks and weighted 22-26g were applied with Middle Cerebral Artery Occlusion (MCAO) model and randomly divided into different groups. Firstly, co-immunoprecipitation and immunoblotting were used to detect the binding of PSD-93 and CX3CL1 at different time points 3h, 6h, 12h 24h, 48h and 72h following cerebral ischemic/reperfusion. Meanwhile, ELISA was used to investigate the expression of soluble CX3CL1 at the same time points to confirm the relationship between of the expression of soluble CX3CL1 and the combination of PSD-93 and CX3CL1. Secondly, two bait plasmids pSos-PSD-93-full length, pSos-CX3CL1-full length and five mutant plasmids: pMyr-PSD-93-mut1, pMyr-PSD-93-mut2, pMyr-PSD-93-mut3, pMyr-PSD-93-mut4, and pMyr-CX3CL1-mut, were constructed and used a yeast two-hybrid system to screen and identify positive
clones and to determine the sequence in which the two proteins bind to each other. Thirdly, the proteins corresponding to the three positive clones obtained in the yeast two-hybrid experiment were used to construct plasmids for transfection of eukaryotic cells and the protein expression binding was verified again by in vitro co-immunoprecipitation. Finally, a specific fusion small peptide Tat-CX3CL1 were designed according to above experiment to inhibit the integration of PSD-93 and CX3CL1 and to explore their role on neuron death following reperfusion.

Results: We found that the binding capacity of PSD-93 and CX3CL1 proteins peaked at 6h after ischemia/reperfusion and then decreased gradually. The specific amino acid sequence of PSD-93 and CX3CL1 binding was obtained by yeast double hybridization and in vitro immunoprecipitation. We identified that their binding sites are located in the 420-535 amino acid sequence of PSD-93 and 357-395 amino acid sequence of CX3CL1. And a specific fusion small peptide Tat-CX3CL1 (357-395aa) were designed to inhibit the integration of PSD-93 and CX3CL1 and perform neuroprotection on neuron death following reperfusion.

Conclusions: Our results suggest that PSD-93 promotes the formation of its soluble form by binding to CX3CL1, which is recruited to the surface of microglia to bind to CX3CR1, thereby activating microglia to initiate inflammation. Thus, specific blockade of PSD-93-CX3CL1 coupling can reduce ischemia-reperfusion induced neuronal cell death, which provide a new target to treat ischemic stroke.

Introduction

Stroke is the second leading cause of death and the first cause of disability in worldwide \(^{[1]}\). Neuronal glutamate excitatory toxin and microglia mediated neuroinflammatory response are important pathological mechanisms leading to
brain injury after ischemia\textsuperscript{[2-4]}. Therefore, to clarify the relationship and mechanism between excitotoxicity and neuroinflammatory will provide a theoretical basis for clinical development of new target drugs.

Accumulating studies in recent years have indicated that ionic glutamic acid receptor agonists can promote the proliferation and activation of microglia cells, and promote the release of iNOS, IL-1, TNF-\(\alpha\) and NO\textsuperscript{[5-6]}. The release of these pro-inflammatory factors can aggravate the damage of neurons in turn and form a vicious cycle in the process of ischemic brain injury. On the other hand, neurons bind to microglia cells through specific chemokines and their receptors, such as CD22/CD45, CD47/CD172a, CD200/CD200R, CX3CL1/CX3CR1, etc. and to communicate and activate a series of downstream signaling pathways, ultimately regulating neuron damage\textsuperscript{[7-11]}.

Post-synaptic densitries-93 (PSD-93), one of the PSDs scaffold proteins, is composed of the PDZ domain, the src homology domain SH3 and the GUK domain\textsuperscript{[12, 13]}. Previous study have shown that PSD-93 bind directly to the carboxy terminus of NMDA receptor subunits NR2A and NR2B via the PDZ domain\textsuperscript{[14]} and transports it to the postsynaptic membrane as a major regulator of synaptic maturation. Our recent studies found that in the early reperfusion of mouse MCAO model, the loss of PSD-93 gene can inhibit pro-inflammatory factors and promote the expression of anti-inflammatory factors\textsuperscript{[15]}, while the application of NMDA receptor inhibitors can provide brain protection. The above results suggest that PSD-93 promotes a series of responses to cerebral ischemia-reperfusion injury through the activation of NMDA receptors through the postsynaptic dense fraction complex, including the regulation of inflammatory factor release in early ischemic cells.
As mentioned above excitatory amino acid toxicity of neurons interacts with microglial-induced inflammatory responses in cerebral ischemia-reperfusion injury [16, 17]. After ischemic stroke, neurons activate microglia through a variety of regulatory factors and activated microglia promote neuronal damage by releasing pro-inflammatory factors and aggravated brain damage. So it can be seen that the activation of NMDAR is closely related to the activation of microglia. And CX3 chemokine ligand 1 (CX3CL1) is a chemokine that is specifically expressed in neurons. Its only receptor CX3CR1 is highly expressed in microglia and its main forms are membrane binding and soluble. When neurons are damaged or exposed to glutamate, soluble CX3CL1 disintegrates from its structural domain and binds to CX3CR1 on microglia to activate microglia [18-19], directly inducing multiple functions of microglia. Therefore, neurons and microglia can communicate through CX3CL1/CX3CR1 signals.

Based on the above research, we hypothesized that PSD-93 might form a PSD-93•NR2B•CX3CL1 complex to promote the production of soluble CX3CL1, allowing it to be recruited to the surface of microglia and binds to CX3CR1, which activates microglia and initiates the occurrence of inflammatory response. Therefore, we used yeast double hybridization and co-immunoprecipitation to clarify the binding amino acid sequence and construct a small peptide to disturb the combination between PSD-93 and CX3CL1 to validate its impact on ischemic brain injury. The study helps us to understand the role of PSD-93 in inflammatory response and PSD-93 mediate dialogue between neurons and microglia to induce inflammation through regulating CX3CL1/CX3CR1 signaling.

Material and Method
Antibodies and Reagents

The peptide was synthesized by QiangYao Company. The peptide sequence (N→C) is as follows: 5-FITC-(Acp)-MFAYQSLQGCPRKMAGEMVEGLRYVPRSCGSNSYVLVPV, its purity is 95%. The following primary antibodies were used: Rabbit polyclonal anti-CX3CL1 (ab25091), rabbit monoclonal anti-PSD-93 (ab151721) and mouse monoclonal anti-NMDAR2B (ab93610) were from Abcom. The secondary goat anti-rabbit IgG antibody used in our experiment were from Sigma (St. Louis, MO). ELISA kits were purchased from S&D.

Experiment Animals

Eighty-four male C57BL/6 mice, weighing 22-26 g, were purchased from Jinan pengyue experimental animal breeding co. LTD and were maintained in our animal facility in a temperature-controlled room (22-25 °C) with a 12-h dark-light cycle. All animal procedures were reviewed and approved by the international guidelines for the ethical use of laboratory animals.

Middle Cerebral Artery Occlusion Model (MCAO)

Adult male mice weighing 22g to 26g were exposed to transient middle cerebral artery occlusion (tMCAO) by suture-occlusion method. Mice were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally). In anesthetized mice, the right side of the common carotid artery was exposed and isolated. The right internal carotid artery was clipped by microartery clip. The middle cerebral artery (MCA) was occluded by inserting a suture into the internal carotid artery, which was advanced further until it closed the origin of the MCA and fixed line bolt. After occlusion for 60 min, the aneurysm clips were removed for reperfusion. Rectal temperature was
maintained at 37 ± 0.5 °C by means of a heating blanket and heating lamp throughout the surgery. Sham-operated control mice received the same surgical procedures except that the carotid arteries were not occluded.

**Immunoprecipitation and Western Blot**

Tissue homogenates (1000μg of protein) were diluted with 50 mM HEPES buffer (pH 7.4) containing 10% glycerol, 150 mM NaCl, EDTA, EGTA, PMSF, Na3VO4 (1 mM each) and 1% Triton X-100, 0.5% NP-40. The protein samples were incubated with 1-2μg of primary antibodies for 4 h or overnight at 4 °C. Protein A/G PLUS-Agarose (20 μL, SANTA CRUZ) were added to the tube, and incubation was continued for an additional 2 h. Samples were centrifuged at 10000g 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. Equal amounts of proteins were isolated on 10 % SDS polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon Transfer Membrane, Millipore, USA). 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 1% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugate secondary antibodies in TBST for 2 h and developed using ECL luminescent solution kit (BioRad). The densities of the bands on the membrane were scanned with Bio-rad gel imager and analyzed.

**ELISA**

Soluble C-X3-C motif ligand 1 levels were analyzed by enzyme immunoassay (R&D
Systems, Stillwater, MN, USA) using ischemic tissues according to manufacturer’s instructions. Prepare related reagents and samples. Standard holes, blank holes and blank holes were set respectively and 2 secondary holes were made for each sample. Adding 50μl Assay Diluent RD1W solution to each well, then add another 50μl reference material for comparison or sample. Gently beat for 1min to mix and cover with tape and incubate for 2h at room temperature. Drain and rinse each hole 5 times in total. Rinse each well with 300μl Washing buffer. Add 100μl Mouse Fractalkin Conjugate to each well, cover with adhesive strips, and incubate at room temperature for 2h. Repeat the suction/wash steps above. 100μl Substrate Solution was added to each well, incubated at room temperature for 30min to avoid light, and 100μl Stop Solution was added and flapped to make it even. The absorbance test was performed within 30min using a 450nm laser, or 540nm or 570nm if the wavelength was optional.

**Lateral ventricular injection**

The mice were treated with peptide administered by injection in the right cerebral ventricle (from the bregma: anteroposterior-1 mm; lateral 1 mm; depth 2 mm) 30 min before ischemia on the first day. The sham group and I/R group rats were injected with same volume of DMSO. Selected mice were randomly divided into five groups: which were I/R (ischemia/reperfusion and normal saline treatment), 1μg/μl (I/R 7day and 1μg/μl peptide treatment), 5μg/μl (I/R 7day and 5μg/μl peptide treatment), 10μg/μl (I/R 7day and 10μg/μl peptide treatment) and DMSO (I/R 7day and DMSO treatment).

**Triphenyl tetrazolium chloride (TTC) staining**
Mice were sacrificed and the brains were rapidly stored at -80°C for 5 min. Brain tissues were sectioned into 2-mm thick slices along the coronal plane and slices were obtained and then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, USA) at 37°C for 15 min in the dark. Then he coronal sections were fixed in 4% paraformaldehyde and filter paper absorb the liquid for photography. The infarct area ratio (%) was then measured by utilizing Image J (version 1.8.0), computer based program. The infarcted regions and the areas of both hemispheres were calculated for each brain slice.

**Construction of expression vectors**

To construct plasmids for recombinant protein expression, including pSos-PSD-93-full length, pSos-CX3CL1-full length, pMyr-PSD-93-mut1, pMyr-PSD-93-mut2, pMyr-PSD-93-mut3, pMyr-PSD-93-mut4, pMyr-CX3CL1-full length and pMyr-CX3CL1-mut were amplified by PCR and cloned into the respective vectors. The primers used for amplifying PSD-93 and CX3CL1 are listed in table 2.

**Yeast two-hybrid (Y2H) screening**

After we confirmed that PSD-93 does not have self-activating activity and is not toxic to yeast strain cdc25Hα, the bait PSD-93 was transformed into cdc25Hα and hybridized with the CX3CL1 using the Yeast Two-Hybrid System. Gently mix the contents of the tube, add 2μl of β-mercaptoethanol to each tube, and incubate for 30 min at room temperature. The mixture table 3 was shocked for 20 min in an environment of 42 °C, and then placed on ice for 3 min, and the cells were collected. They were centrifuged at 14000 rpm for 30 s at room temperature. The supernatant was removed, and the cells were resuspended in sorbitol. Each mixture
was plated on a 100 mm SD/glucose plate and incubated at room temperature until
the clone appeared about 4-6 days, at least 3 clones were selected and transferred
to SD/glucose (-UL) and SD/galactose (-UL) plates at 37 °C for the detection of
protein-protein interactions. The clones to be screened were transferred to wells,
resuspended in high pressure water and 2.5 μl of the yeast/H2O suspension was
dropped onto two SD/galactose (-UL) and two SD/glucose (-UL) plates respectively.
One placed at 37 C and the other at 22-25 C, counting growth clones after 5 days of
culture, SD/galactose (-UL) plates may take 7-10 days.

**Plasmid transfection**

293T cells were transfected with plasmids using Lipofectamine™ 3000 Transfection
Reagent (L3000015; Thermo Fisher, USA), according to the manufacturer's
instructions. The mixtures were mixed well and placed in the cell culture box for
incubation with 5% CO₂, 37°C. After 6h, they were replaced with 2ml DMEM medium
containing 10% fetal bovine serum. Cell culture supernatants were harvested at 24 h post infection. The transfected cells were analyzed by immunoprecipitation and
western blotting.

**Statistical analysis**

Data were analyzed using SPSS software, version 18.0 (SPSS Inc., Chicago). One-
way ANOVA and Bonferroni correction were used to analyze these data. Values were
presented as mean± standard deviation (SD). The results were considered
statistically significant if \( P<0.05 \). Each experiment consisted of at least three
replicates per condition.
Results

Interaction of PSD-93 with CX3CL1 and NR2B during Reperfusion after Ischemia

As a scaffold protein in the postsynaptic membrane, PSD-93 mediates the release of inflammatory factors in early cerebral ischemia through NMDA receptor, and CX3CL1 is a specific transmembrane protein expressed on neurons. To examine the underlying mechanism and effect of PSD-93 on the crosstalk with CX3CL1 signaling pathway, we first investigated the interaction of PSD-93 with the NR2B and CX3CL1 signaling module during reperfusion after transient (60 min) cerebral ischemia using immunoprecipitation and immunoblotting. As shown in Fig 1A, B, C, F, the level of association of CX3CL1 with NR2B increased obviously after reperfusion at 6 h ($P<0.01$, $n=5$) and then decreased gradually. And the similar results of binding between CX3CL1 with PSD-93 were obtained ($P<0.001$, $n=5$) (Fig 1A, D).

Furthermore, we also detected the interaction of NR2B with PSD-93 and found that the peak binding between the two proteins happen at 6 h ($P<0.01$, $n=5$) Fig 1B, E).

Together, these results suggest that CX3CL1, PSD-93 and NR2B can form macromolecular complexes after cerebral ischemia reperfusion and the combination of the three proteins peaks at reperfusion 6h.

The expression of soluble CX3CL1 after ischemia/reperfusion

When neurons are damaged or exposed to glutamate, membrane-bound CX3CL1 is cleaved into soluble CX3CL1 by integrin and metalloproteinase. So we further detected the expression of soluble CX3CL1 after ischemia/reperfusion different time points using Enzyme Linked Immunosorbent Assay. As shown in Fig 2, ELISA analysis
shows that the expression of sCX3CL1 reached the maximum strength after reperfusion for 6h ($P<0.001$, n=5, I/R 6h vs Sham), which coincided with the formation of PSD-93NR2B-CX3CL1 complexes. These data showed that interaction of PSD-93 with CX3CL1 might play significant roles in promoting the formation of sCX3CX1 in brain ischemia reperfusion injury.

**PSD-93 interacts with CX3CL1 in Yeast two-hybrid system**

To confirm the interaction between PSD-93 and CX3CL1, bait plasmids and mutant plasmids were successfully constructed by yeast two-hybrid experiment, and the recombinant plasmids were identified by enzyme digestion and sequencing. pSos-PSD-93-full length with pMyr-CX3CL1-full length and pMyr-CX3CL1-mut, pSos-CX3CL1-full length with pMyr-PSD-93-mut1, mut2, mut3, and mut4 were co-transformed into yeast strain cdc25Hα (seen in table1) and grown on synthetically defined media (see supplement 1 and 2). In the yeast transformation test of psd-93 and CX3CL1, colony growth was observed in SD/glucose(-ul) and SD/galactose(-ul) solid medium at 22-25°C (supplement 3). Colonies in 37°C SD/glucose(-ul) solid medium did not grow (supplement 4). However, in 37°C SD/galactose(-ul) solid medium, yeast cells co-transformed with pSos-PSD-93-full length and pMyr-CX3CL1-full length, pSos-CX3CL1-full length with pMyr-PSD-93-mut3 and mut4, and the positive control pSos MAFB+pMyr MAFB can grow (As shown in Fig 3A, C, G, H and Table 4), which indicated that PSD-93 and CX3CL1 can bind to each other. Moreover, the sequence (420-535aa) on PSD-93 and the sequence (357-395aa) on CX3CL1 are important segments for the mutual binding of the two proteins.

**PSD-93 interacts with CX3CL1 in 293T cells**
To further verify the interaction between PSD-93 and CX3CL1 in vitro, the labeled positive recombinant plasmids obtained from yeast transformation experiments were transfected with all kinds of HA-CX3CL1 and Flag-PSD-93 into 293T cells in pairs. After that, co-immunoprecipitation and Western blotting were used for verification. The target protein complex in the protein mixture was precipitated with Flag antibody, and then the subsequent Western was performed with HA antibody and Flag antibody, respectively. As shown in Fig4C, D, PSD-93-mut3 and PSD-93-mut4 were co precipitated with the full-length CX3CL1 protein, respectively. However, mut1 and mut2 of PSD-93 mutates could not co-precipitate with CX3CL1 (Fig 4A, B), which indicated that the mutated somatome mut1 and mut2 sequence of PSD-93 could not bind CX3CL1. As mentioned above, PSD-93-mut4 (1-661aa) contains sequences of PSD-93-mut3 (1-535aa), co-immunoprecipitation assay demonstrated that PSD-93-mut3 (1-535aa) and PSD-93-mut4 (1-661aa) can bind CX3CL1-full length in transfected 293T cells (as shown in Fig 4). While the sequence on PSD-93-mut2 (1-420aa) could not bind CX3CL1, which mean that the effective amino acid binding sequence of the two proteins is (420-535aa) of PSD-93 and (357-395aa) of CX3CL1. These results are consistent with the conclusions obtained from yeast two-hybrid experiments.

Effects of peptide against PSD-93 and CX3CL1 on cerebral infarction volume.

To investigate whether inhibiting PSD-93 and CX3CL1 can reduce neuron cells death, we designed a small peptide that inhibit the combination of both. Three concentration gradients were designed to observe the volume of cerebral infarction after 7 days of reperfusion. As shown in Fig 5A and B, comparing with the MCAO group, 5μg/μl and 10μg/μl groups both improved the neurological impairment
significantly caused by ischemia-reperfusion ($P<0.05$). However, the reduction in infarct volume in 10μg/μl group was more pronounced, which suggesting that 10μg/μl concentration drug provided better neuroprotective effects.

Discussion

Our previous research indicated that PSD-93, as a scaffold protein in the postsynaptic membrane, mediated the release of inflammatory factors in the early stage of cerebral ischemia through NMDA receptors [15]. In addition, PSD-93 can combine with specific protein with its function structure domain and change the structure and function of the protein [20-21]. Furthermore, as a transmembrane protein specifically expressed in neurons, CX3CL1 can be cut into soluble CX3CL1 and reach the surface of the microglia to bind to CX3CR1 and initiating microglia activity when neurons are damaged or exposed to glutamate [18-19]. In this study, we clarify that PSD-93 could bind to NR2B, CX3CL1 to form a complex and promote CX3CL1 shear into a soluble form. Moreover, we obtained the amino acid binding sequence of PSD-93 and CX3CL1 through yeast double hybridization. On this basis, we designed small peptide to disturb the combination of PSD-93 and CX3CL1 and found that the peptide perform neuroprotective effect following ischemic brain injury.

In recent years, the relationship between PSDs protein and early post-ischemic inflammatory response has received increasing attention. Christopherson et al [22] showed that PSD-95, NMDAR and nNOS can combine with each other and assemble into a macromolecular signal complex. And disrupting ischemia-induced interaction of nNOS with PSD-95 can improve cerebral ischemia and regenerative repair after
stroke [23-25]. And recent research focusing on PSD-93 have found that PSD-93 can also bind to NR2A and nNOS and facilitate ischemic brain injury [15, 26]. On other hand, neuronal excitatory toxicity and microglia-induced inflammatory response interact in ischemic brain injury [27-28]. After ischemic stroke, neurons activate microglia through a variety of neurotransmitters or regulators such as glutamate, fractalkine (FKN, CX3CL1), and NO. In contrast, activated microglia promote the development of neuronal injury by releasing pro-inflammatory factors such as NO, oxygen free radicals, prostaglandin E2, and cytokines such as TNF-, il-1, and il-6 [3, 29].

The CX3CL1/CX3CR1 signaling pathway has been shown to play an important role in regulating communication between neurons and microglia. CX3CL1 is a unique chemokine that is thought to be a membrane-bound protein that regulates cell-to-cell communication and adhesion and cell recruitment and survival by binding to CX3C chemokine receptor 1 (CX3CR1) [30-32]. In addition, neurotoxic pathways are also affected by CX3CL1/CX3CR1 signaling through the synergistic cooperation of adenosine systems [33]. CX3CL1 exists in two different forms, the full-length membrane-bound and soluble forms. When neuron is damaged or exposed to glutamate, its extracellular domain is lysosomal cysteine protease, Cathepsin S and ADAM family members are isolated from membrane-bound cleavage form to a soluble form [34-37], which has chemotactic effects and can be used as a signaling molecule after cleavage to bind to CX3CR1 receptor expressed on microglia [38]. In turn, it affects the activation of microglia and regulates the recruitment of circulating white blood cells at the site of injury [39, 40]. Studies have shown that
soluble CX3CL1 directly enhances microglial phagocytosis of neuronal fragments via phosphatidylserine (PS) [41]. From the above studies, the mechanism of promoting the release of soluble CX3CL1 is the key to illuminate dialogue between neuronal and microglia.

Our findings provide a scientific basis for PSD-93 regulating the mechanism of CX3CL1/CX3CR1 signaling mediating acute ischemic stroke. In this study, we first examined the interaction of PSD-93 with CX3CL1 after I/R by using Co-IP assay. The results showed the level of association of CX3CL1 with PSD-93 increased obviously after reperfusion 6 h and then decreased gradually. Secondly, we further study binding amino acid sequences between mentioned above using yeast two-hybrid system screening and finally identify two candidate sequences. In addition, we also validated the combination of them by using co-immunoprecipitation in 293T cells. PSD-93 has five functional domains, which contain the binding site both of them and this provides a basis for us to explore binding site of proteins.

To further investigate whether inhibiting the binds of PSD-93 and CX3CL1 has a neuroprotective effect against ischemia-reperfusion, we constructed small peptides to antagonize the combination of proteins. In vivo experiments, our data showed that inhibiting the combination of the proteins can reduce neurons death. Therefore, the small peptides has a neuroprotective effect, which will lay a foundation for further verify the mechanism between proteins.

Conclusion

In summary, our study revealed the interaction between PSD-93 and CX3CL1 and we further explored the mechanism of PSD-93 regulating CX3CL1/CX3CR1 signaling mediated acute ischemic stroke. The complex PSD-93–NR2B–CX3CL1 promotes the
production of soluble CX3CL1 and mediates the crosstalk between neurons and microglia during acute cerebral ischemia. This study sheds new light on the role of the crosstalk between CX3CL1 and PSD-93 after brain ischemia-reperfusion and provides new therapeutic targets against ischemic neuron death.

Declarations

Ethics approval and consent to participate
All animal experiments were performed at Xuzhou Medical University according to Animal Care and Use Committee of Xuzhou Medical University. Mice were euthanized by cervical dislocation.

Consent for publication
Not applicable.

Availability of data and materials
All data are available upon reasonable request to correspondence author.

Competing interests
The authors declare that they have no competing interests.

Funding
This study was supported by the National Natural Science Foundation of China (No. 81671149, 81301120 and 81971179), the Natural Science Foundation of Jiangsu Province (No.BK20161167, BK20151168 and BK20191463), the Natural Science Foundation of the Colleges and Universities in Jiangsu Province (No. 13KJB320027) and the Xuzhou Medical Young Talents Project.

Author contributions
QZ, LH, HY, MC, XL, XC, and XL performed the experiments, QH, ZC, and TL validated the experiments, XW analyzed data, QZ and LH wrote the paper, QZ and
LR designed and supervised the study.

Acknowledgements

Not applicable

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Tables

Table 1  PCR primers

| Name                      | Primer                                      |
|---------------------------|---------------------------------------------|
| pSos-CX3CL1-full-length   | CX3CL1-S-BamHI: 5' CGCGGATCCGCGATGGGCTCCTCGCCGCTC 3' |
|                           | CX3CL1-AS-SalI: 5' ACGCGTCGACTCAGTGGACACCAGGAC 3' |
| pMyr-PSD-93-mut1          | PSD-93-mut1-S-EcoRI: 5' CGGAATTCATGGTCTTTGATGTAT 3' |
|                           | PSD-93-mut1-AS-SalI: 5' CGCGTCGACCAACCAGTCTTGAATAT 3' |
| pMyr-PSD-93-mut2          | PSD-93-mut2-S-EcoRI: 5' CGGAATTCATGTTCTTTGATGTAT 3' |
|                           | PSD-93-mut2-AS-SalI: 5' GTCGACGCGGCTCCCCCTTCCAG 3' |
| pMyr-PSD-93-mut3          | PSD-93-mut3-S-EcoRI: 5' CGGAATTCATGGTCTTTGATGTAT 3' |
|                           | PSD-93-mut3-AS-SalI: 5' GCGTCGACCTGATTGGCTCAAGG 3' |
| pMyr-PSD-93-mut4          | PSD-93-mut4-S-EcoRI: 5' CGGAATTCATGTTCTTTGATGTAT 3' |
|                           | PSD-93-mut4-AS-SalI: 5' GCGTCGACGTTATTATATTCCTGCT 3' |
| CX3CL1-mut                | CX3CL1-mut-S-EcoRI: 5' CGGAATTCAGCACCTCGGATGACG 3' |
|                           | CX3CL1-mut-AS-SalI: 5' GCGTCGACGATGCGCACCAGGCA 3' |

Table 2 Mutant Plasmid
| Plasmid                  | Number                    | Length |
|-------------------------|---------------------------|--------|
| pMyr-PSD-93-mut1        | Q91XM9, NM_011807.3       | 1-192aa|
| pMyr-PSD-93-mut2        |                           | 1-420aa|
| pMyr-PSD-93-mut3        |                           | 1-535aa|
| pMyr-PSD-93-mut4        |                           | 1-661aa|
| pMyr-CX3CL1-full length | O35188NM_009142           | 1-395aa|
| pMyr-CX3CL1-mut         |                           | 25-357aa|

**Table 3**  Yeast Conversion Mixture
| Number | Plasmid                                           | Weight | Volume | Medium          |
|--------|--------------------------------------------------|--------|--------|-----------------|
| 1      | pSos MAFB+pMyr MAFB                              | 300ng  | 100μl  | SD/glucose (UL) |
| 2      | pSos MAFB+pMyr Lamin C                        | 300ng  | 100μl  | SD/glucose (UL) |
| 3      | pSos-PSD-93-full length+pMyr-CX3CL1-mut       | 300ng  | 100μl  | SD/glucose (UL) |
| 4      | pSos-PSD-93-full length+pMyr-CX3CL1-full length | 300ng  | 100μl  | SD/glucose (UL) |
| 5      | pSos-CX3CL1-full length+ pMyr-PSD-93-mut1     | 300ng  | 100μl  | SD/glucose (UL) |
| 6      | pSos-CX3CL1-full length+ pMyr-PSD-93-mut2     | 300ng  | 100μl  | SD/glucose (UL) |
| 7      | pSos-CX3CL1-full length+ pMyr-PSD-93-mut3     | 300ng  | 100μl  | SD/glucose (UL) |
| 8      | pSos-CX3CL1-full length+ pMyr-PSD-93-mut4     | 300ng  | 100μl  | SD/glucose (UL) |
| 9      | pSos MAFB+ pMyr-CX3CL1-mut                    | 300ng  | 100μl  | SD/glucose (UL) |
| 10     | pSos MAFB+ pMyr-CX3CL1-full length             | 300ng  | 100μl  | SD/glucose (UL) |
| 11     | pSos MAFB+ pMyr - PSD-93-mut1                 | 300ng  | 100μl  | SD/glucose (UL) |
| 12     | pSos MAFB+ pMyr - PSD-93-mut2                 | 300ng  | 100μl  | SD/glucose (UL) |
| 13     | pSos MAFB+ pMyr - PSD-93-mut3                 | 300ng  | 100μl  | SD/glucose (UL) |
| 14     | pSos MAFB+ pMyr - PSD-93-mut4                 | 300ng  | 100μl  | SD/glucose (UL) |

Table 4  Yeast Transformation results
| Yeast transformation | (-UL)/37°C | (after patching) |
|----------------------|-----------|------------------|
|                      | Glucose   | Galactose        |
| pSos MAFB + pMyr MAFB |           |                  |
| pSos MAFB + pMyr Lamin C |       |                  |
| pSos-PSD-93-full length + pMyr-CX3CL1-full length |   |                  |
| pSos-PSD-93-full length + pMyr-CX3CL1-mut |   |                  |
| pSos-CX3CL1-full length + pMyr-PSD-93-mut1 |   |                  |
| pSos-CX3CL1-full length + pMyr-PSD-93-mut2 |   |                  |
| pSos-CX3CL1-full length + pMyr-PSD-93-mut3 |   |                  |
| pSos-CX3CL1-full length + pMyr-PSD-93-mut4 |   |                  |
| pSos MAFB + pMyr-CX3CL1-mut |   |                  |
| pSos MAFB + pMyr-CX3CL1-full length |   |                  |
| pSos MAFB + pMyr - PSD-93-mut1 |   |                  |
| pSos MAFB + pMyr - PSD-93-mut2 |   |                  |
| pSos MAFB + pMyr - PSD-93-mut3 |   |                  |
| pSos MAFB + pMyr - PSD-93-mut4 |   |                  |
Figures

Figure 1

Interaction of PSD-93 with CX3CL1 and NR2B during reperfusion after ischemia.
The expression of soluble CX3CL1. ELISA analysis shows that the expression of sCX3CL1 increase remarkably at 3, 6, 12, 24, 48, and 72 hours. One asterisk indicates a P of <0.05 vs control and three asterisks indicate a P of <0.001 vs control.

Figure 3

PSD-93 interacts with CX3CL1 in Yeast two-hybrid system. A: the positive control
PSD-93 interacts with CX3CL1 in 293T cells. According to the positive clones in the yeast two-hybrid experiment and immunoblotting analysis, the interaction of PSD-93 with CX3CL1 in different sequences is demonstrated.

Infarct sizes were assessed 7 days after ischemia-reperfusion. Pale unstained sections were considered to be infarcted. The concentration of peptides was investigated to determine the cerebral protective effects. Significant differences were observed in the 5 µg/µl and 10 µg/µl groups compared to the control and DMSO groups.

Supplementary Files
This is a list of supplementary files associated with the primary manuscript. Click to download.

- supplemental 2.pdf
- supplemental 1.pdf
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