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Peptide OM-LV20 exerts neuroprotective effects against cerebral ischemia/reperfusion injury in rats

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ABSTRACT

Ischemia/reperfusion (I/R) is a common injury leading to ischemic stroke. At present, I/R treatment remains limited, highlighting the urgent need for the discovery and development of new protective drugs for brain injury. Here, we investigated the neuroprotective effects of short peptide OM-LV20 previously identified from amphibian against I/R rats. Results showed that intraperitoneal administration of OM-LV20 (20 ng/kg) significantly reduced infarct area formation, improved behavioral abnormalities, and protected cortical and hippocampal neurons against death caused by I/R. Moreover, the underlying molecular mechanism was involved with the regulation of the MAPK and BDNF/AKT signaling pathways, as well as the levels of cyclic adenosine monophosphate, pituitary adenylate cyclase-activating polypeptide receptor, and tryptophan hydroxylase 1. To the best of our knowledge, this research was the first report to describe the neuroprotective effects of an amphibian skin secretion-derived peptide in I/R rats and highlighted OM-LV20 as a promising drug candidate for the development of novel anti-stroke therapies.

1. Introduction

Stroke is the second leading cause of death worldwide and first in China due to its high incidence, mortality, disability, and recurrence rates [1]. Globally, nearly 6 million people die of stroke every year, and more than 30 million people are disabled which affects life's quality seriously [2]. Stroke can be divided into hemorrhagic and ischemic stroke (IS), and IS accounted for 84.4% of the total events [3]. Cerebral vascular occlusion is rarely permanent in human stroke, most people receive spontaneous or thrombolytic therapy reperfusion after IS, i.e., cerebral ischemia/reperfusion (I/R); moreover, the brain damage caused by I/R can be higher than that of simple cerebral IS [4,5]. Currently, the only treatment drug approved for IS worldwide is recombinant tissue plasminogen activator (rt-PA) [6], but based on the huge number of stroke patients diagnosed worldwide (>13.7 million new patients/year), rt-PA alone is often insufficient for successful treatment [7]. Therefore, preventing the occurrence of IS and improving its treatment, especially that of I/R, are important areas of research.

Due to their low molecular weight, high efficiency, and specific targeting ability, certain peptides are reported to have protective effects on brain injury [8]. For example, PcTx1 (from the tarantula Psalmopoeus cambridgei venom) is reported to reduce cortical infarct area by 70% in middle cerebral artery occlusion (MCAO) rats [9]. Hi1a derived from the venom of Hadronyche infensa is reported to significantly reduce cerebral infarct area at 8 h after IS [10]. And intraperitoneal administration of Exendin-4 can significantly reduce brain damage in neonatal hypoxic-ischemic brain injury

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[11]. Thus, peptides have aroused significant attention in regard to neuroprotective drug discovery and development [8]. At present, novel peptides from amphibian skin secretions are widely recognized as a treasure trove with antimicrobial, antioxidant, wound-healing, and hypoglycemic potency [12–15], however, our knowledge on the neuroprotective potency of peptides from amphibians remains in its infancy.

In previous reports, we identified a peptide OM-LV20 (amino acid sequence: LVGKLLKGAVGDVCGLLPIC) from amphibian skin secretions, and functional results indicated that OM-LV20 accelerated the regeneration of full-thickness wounds and regulated the levels of blood glucose in mice [12,13], which laid solid foundations for us to speculate that the biological effects of OM-LV20 was similar to Exendin-4, and might reduce nerve damage on brain injury as well. In this study, we investigated the neuroprotective effects of OM-LV20 by using rat I/R models and explored the underlying neuroprotective mechanism. To the best of our knowledge, this study was the first report to elucidate the neuroprotective effects of an amphibian skin secretion-derived peptide in I/R rats, thus highlighting OM-LV20 as a promising drug candidate for the development of novel anti-stroke therapies.

2. Materials and methods

2.1. Peptide synthesis and prediction of advanced structure

With a purity of >95%, the peptide OM-LV20 (LVGKLLKG AVGDVCGLLPIC) was commercially synthesized by Wuhan Bioyeargene Biotechnology Co. Ltd. (Wuhan, China). The online server PEP-FOLD3 (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms Pep-fold3) was used to construct the initial model of the polypeptide, and obtained the final OM-LV20 structure [16].

2.2. Analysis of OM-LV20 stability

The stability of OM-LV20 in 4 °C, 37 °C and in plasma was tested as per previous research [17]. After centrifugation at 12,000×*g* for 30 min, the supernatant was obtained to detect the content of OM-LV20 until the peptide was completely degraded.

2.3. Animals

Male Sprague-Dawley rats (n = 83, 280–320 g) were obtained from the Laboratory Animal Department of Kunming Medical University. All animal experiments, cares and handling procedures were conducted in accordance with the requirements of the Ethics Committee of Kunming Medical University (License Number: KMMU20180012). Rats were maintained under specific pathogen-free conditions in the laboratory animal room in accordance with institutional guidelines. Animals were kept in a conventional animal facility under a 12 h light/12 h dark cycle at a constant temperature of 22 ± 2 °C, and all rats had *ad libitum* access to standard water and food pellets.

2.4. MCAO/reperfusion protocol

Rats were randomly separated into five groups: Sham, I/R, I/R + OM-LV20 (3 ng/kg; 8 ng/kg; 20 ng/kg) groups. OM-LV20 groups had an intraperitoneal injection of peptide once daily for 7 days. The I/R model (ischemia for 2 h and reperfusion for 24 h) was manufactured 1 h after the injection on day 7. The MCAO/R model was established by a thread ligation (Beijing Cinontech Co. Ltd., Beijing, China) as per previous research [18].

2.5. Evaluation of neurological deficits

Neurological deficits were evaluated after I/R. Rats were assessed by using the Longa score with receiving scores of 0 or 4 excluded, then further assessed by using the modified neurological severity score (mNSS) [19]. Scores were evaluated by three researchers based on the single-blind method. All score results were summarized and statistically calculated.

2.6. Evaluation of cerebral infarction area

Rats in each group were sacrificed with overdose anesthesia, their brains were rapidly removed on ice and frozen in a −20 °C refrigerator, then cut into six equal parts along the coronal plane, finally placed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, MO, USA) solution for 30 min (37 °C). The infarcted tissue showed white and normal brain tissue showed red. Infarct areas were measured and calculated using ImageJ software (National Institutes of Health, Bethesda, USA).

2.7. Hematoxylin and eosin (H&E) and Nissl staining in rats

After rats were sacrificed, the brains were fixed with 4% paraformaldehyde perfusion and embedded in paraffin. Samples were sectioned into 6-μm thick slices by using an ultra-thin semi-automatic microtome (Leica, Heidelberg, Germany), and slices were stained with H&E (Biosharp Life Science, Anhui, China) and Nissl (Solarbio, Beijing, China) for histological analysis. The numbers of surviving neurons was analyzed by ImageJ.

2.8. Western blotting assay

Cerebrums were rapidly removed from decapitated animals and then the right hemispheres of the infarcted brains (without the olfactory bulb and cerebellum) were homogenized in a lysis buffer mixture of pre-cooled RIPA and phenylmethanesulfonyl fluoride (PMSF) (Meilun Biotechnology, Dalian, China) before being centrifuged at 12,000×*g* for 20 min at 4 °C. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were used to verify the influence of OM-LV20 on protein expression. 10% SDS-PAGE was used for the separation of protein samples (20 μg). After transfer onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA), proteins were sealed with 5% skimmed milk (Solarbio, Beijing, China) for 2 h at room temperature. Membranes were then incubated at 4 °C overnight with corresponding primary antibodies, including p-ERK 1/2, ERK1/2, p-JNK, JNK, p38 and p-AKT (1:2 000), p-p38 (1:1 000) (Cell Signaling Technology, MA, USA), BDNF (1:1 000), TPH1 (1:500) (Abcam, MA, USA), AKT (1:2 000), and GAPDH (1:3 000) (Proteintech, Hubei, China). On the second day, the membranes were incubated with secondary antibodies (Proteintech, Hubei, China) at room temperature for 1 h. The positive reaction bands were detected by using an enhanced chemiluminescence kit (Biosharp Life Science, Anhui, China), and quantified by ImageJ software.

2.9. qRT-PCR assay

Total RNA of the infarcted cerebral tissues was extracted by using a total RNA extraction kit (Tiangen Biotech, Beijing, China). The cDNA was reverse transcribed by using a Prime Script Reagent kit (GeneCopoeia, Guangzhou, China). Specific primers for PAC1R (5′-ACTACCTGTCGGTGAAGGCTCTC-3′ and 5′-CGGAAGCGGCA-CAGGATGACC-3′) and ACTIN (5′-CAGCCTTCCTTCCTGGGTATG-3′ and 5′-TAGAGCCACCAATCCACACAG-3′) were from Sangon Biotech (Shanghai, China). qRT-PCR was tested by the PCR instrument (Life

Technologies, Thermo, USA), and the relative expression of samples to the control was calculated by the $2^{-\Delta\Delta CT}$ method [20].

2.10. cAMP assay

Rat brain tissue samples were collected and homogenized according to the protocols of the Mouse/Rat cAMP Assay kit (Creative & Diagnostics, New York, USA). The optical density was detected at 450 nm by using a microplate plate reader (SpectraMax 190, California, USA). The sham group was considered as 100% and data were normalized accordingly.

2.11. Statistical analysis

Data were depicted as means ± standard error of the mean (SEM). Results were analyzed using GraphPad Prism 8.0 (La Jolla, CA, USA) and pairwise comparisons were conducted using one-way analysis of variance (ANOVA) with the Bonferroni post-hoc test. All statistical analyses used the Student's t-test with Welch correction. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Molecular characteristics of OM-LV20

The advanced structure prediction indicated that the main structure of OM-LV20 was the aperiodical coil (Fig. 1A and B), and the C-terminal formed a pair of intramolecular disulfide bonds located between C^{14}–C^{20} (Fig. 1C).

The stability of OM-LV20 at 4 °C, 37 °C, and in rat plasma was tested in Fig. 1D, E and F. At 4 °C, the content of OM-LV20 remained stable even in week 20; but at 37 °C, the content of OM-LV20 disappeared in week 16. In addition, the half-life of OM-LV20 in rat plasma was 2.843 h. These results indicated that OM-LV20 had high stability.

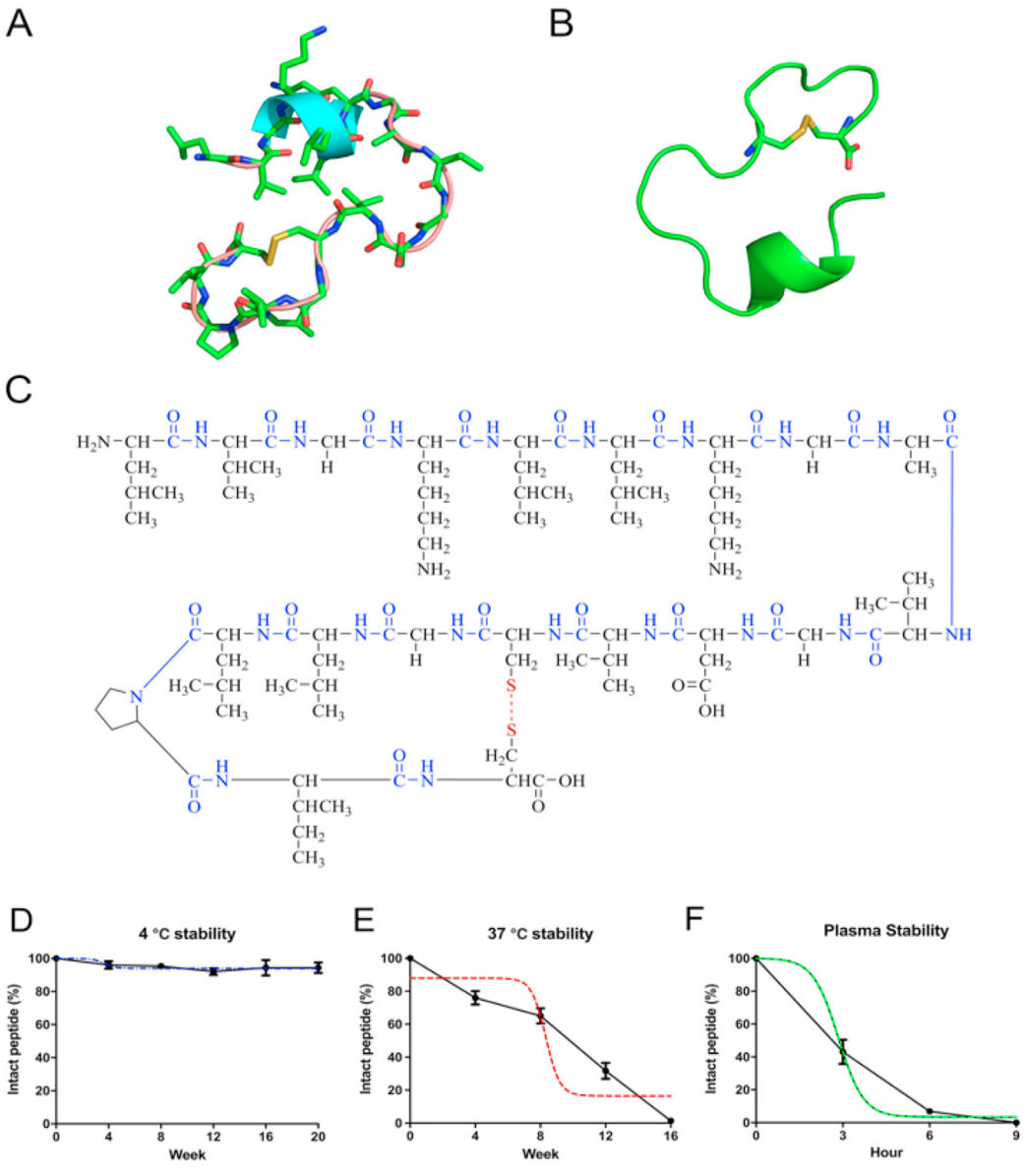

Fig. 1. Predicted advanced structure, chemical structure, and stability of OM-LV20. A, B. Front view of predicted advanced structure. C. Structure showing peptide bonds (in blue) and disulfide bonds (in red). Stability of OM-LV20 at 4 °C (D), 37 °C (E), and in plasma (F). Data are means ± SEM of three independent experiments.

3.2. OM-LV20 improved neurological impairment and reduced cerebral infarction volume caused by I/R injury

I/R injury can lead to cerebral infarction, resulting in neurobehavioral deficits. After I/R, a large area of cerebral infarction appeared, correspondingly, the application of 8 and 20 ng/kg OM-LV20 reduced the infarct size with a rescue of cerebral penumbra area infarction (Fig. 2B). The mNSS scores were used to evaluate the neurological deficits after I/R. As shown in Fig. 2C, compared with the sham group, obvious behavioral abnormalities were observed in the I/R group; however, a significant improvement was found following OM-LV20 pretreatment, especially in the 20 ng/kg group.

3.3. OM-LV20 reduced pathological changes and neuronal loss

Both H&E and Nissl staining were used to show pathological changes in the rat cortex and hippocampus (CA1 area). As shown in Fig. 3A and C, the number of living cells decreased, the damaged cells shrunken their bodies, pyknotic nuclei, the stain color deepened, and the CA1 structure became disordered after I/R (red arrows), however, this damage was visibly rescued by OM-LV20 (yellow arrows). Also, OM-LV20 (20 ng/kg) increased the neuron's survival rate by 31.31% ± 6.9% and 19.31% ± 4.5% in cortex and hippocampus, respectively (Fig. 3B, D). These results indicated that the peptide showed a protective effect of helping neuronal survival, especially in the penumbra.

3.4. OM-LV20 regulated MAPK and BDNF/AKT signaling pathways activation after I/R injury

Western blotting was used to detect the influences of OM-LV20 on the MAPK and BDNF/AKT signaling pathways. As shown in Fig. 4A, B and C, levels of p-MAPK were significantly increased in the I/R group, in comparison, pretreatment with OM-LV20 reduced the phosphorylation. In addition, the BDNF and pAKT levels were further increased in OM-LV20 groups (Fig. 4D and E), and in the 20 ng/kg group, the pAKT level was slightly higher than that in the sham group, emphasizing the protective role of OM-LV20 via the BDNF/AKT signaling pathway.

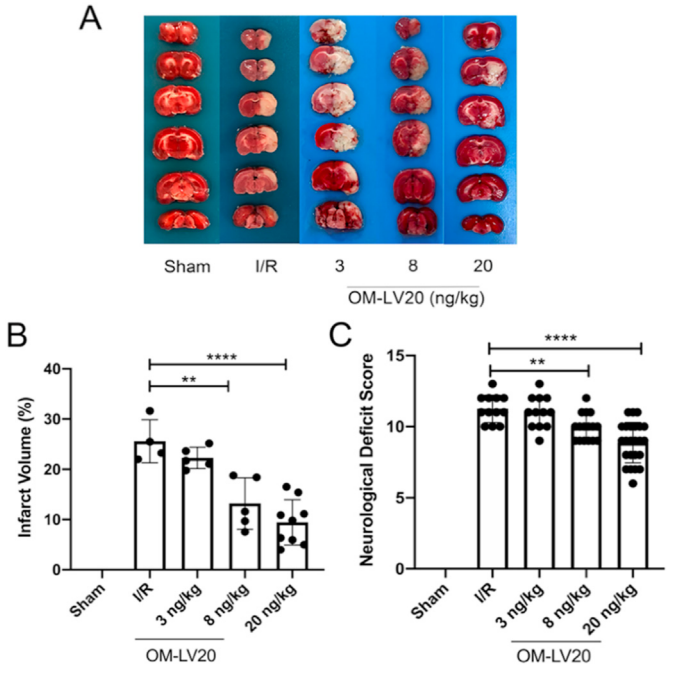

Fig. 2. OM-LV20 administration improved neurological deficits and reduced infarct volume in rats. A. Representative images of TTC-stained brain slices after MCAO. B. Quantified infarct volume in each group (4 rats in I/R group; 5 rats in 3 ng/kg group; 5 rats in 8 ng/kg group; 9 rats in 20 ng/kg group). C. OM-LV20 improved mNSS score after I/R in rats. Data are means ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.5. OM-LV20 pretreatment regulated TPH1, PAC1R and cAMP levels in rat brains

The TPH1 was the most significantly up-regulated gene in the OM-LV20 group compared with the I/R group (Fig. S1). To verify the change of TPH1 level, protein expressions were tested. In I/R samples, the TPH1 level was decreased significantly, and OM-LV20 pretreatment increased the protein level in both 8 and 20 ng/kg groups (Fig. 4F). In Fig. 4G and H, the mRNA level of pituitary adenylate cyclase-activating polypeptide receptor (PAC1R) was reduced in the I/R group, whereas the cAMP level was increased. After OM-LV20 (20 ng/kg) pretreatment, the cAMP level decreased, but the PAC1R level was higher than that in both I/R group and sham group. These results demonstrated that PAC1R could be significantly increased by the peptide.

4. Discussion

How to treat stroke and improve outcomes, especially IS nerve injury, are critical research areas currently. In this study, we demonstrated the neuroprotective effects of OM-LV20, a peptide derived from amphibian skin secretions, by using cerebral I/R rat models and the underlying mechanisms were initially explored.

Firstly, the stability of OM-LV20 in rat plasma showed a half-life of 2.843 h (Fig. 1F), and it was the reason to establish the I/R model 1 h after OM-LV20 application on day 7, to ensure the peptide could enter through the MCAO-damaged blood-brain barrier. Then mNSS score and TTC staining were used to evaluate the degree of neurological damage and infarct area caused by I/R. After operation, rats showed obvious neurological dysfunctions and formed 25.58% ± 3.7% cerebral infarction area, whereas OM-LV20 pretreatment significantly reduced the infarction formation and improved behavioral abnormalities (Fig. 2). In Nissl staining results, a number of neurons in rat brain died after MCAO, while in the 20 ng/kg group, the survival rate of neurons was 83.65% ± 7.4% in cortex and 89.74% ± 6.3% in hippocampus (Fig. 3B, D). These results suggested that OM-LV20 might play a role in resisting neuronal death against I/R brain injury.

The activation of the MAPK and BDNF/AKT pathways, which are known to relate to neuroprotection, is examined as a hint of our RNA-seq results (Fig. S1). The ERK family are considered to be related to cell proliferation, and the JNK and p38 families are related to cell apoptosis, when IS occurs, the phosphorylated MAPK levels will increase, hence reducing p-MAPK levels is deemed to have neuroprotective significances [21,22]. As shown in Fig. 4A, B and C, OM-LV20 application reduced the phosphorylated MAPK levels caused by I/R. In the 3 ng/kg group, only the p-ERK1/2 level was decreased, but levels of p-JNK and p-p38 showed no statistical significance compared with that in the I/R group, indicating that OM-LV20 might exert the neuroprotective effects by enhancing cell proliferation that was closely related with the activation of the ERK 1/2 signaling pathway. BDNF/AKT has been reported to play an important role in regulating the survival of neurons [23]. In this study, OM-LV20 pretreatment activated the BDNF/AKT signaling pathway (Fig. 4D and E), and according to the significant neuroprotective effects of maintaining the survival of neurons (Fig. 3B and D), OM-LV20 might activate the BDNF/AKT and MAPK pathway to protect neurons against death, and finally played the role of neuroprotection against I/R injury.

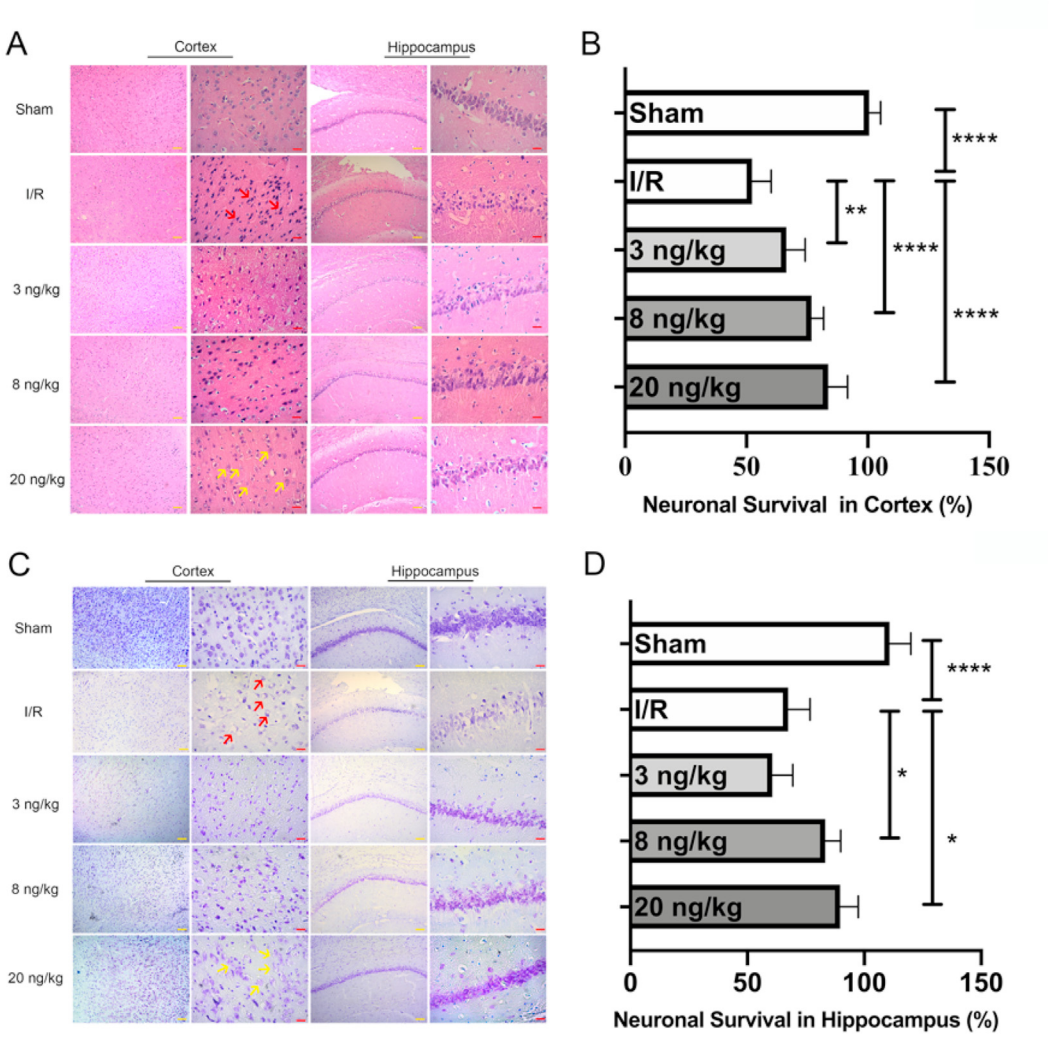

Fig. 3. OM-LV20 administration has a protective effect on neuronal survival in rats based on H&E and Nissl staining. A, C. H&E and Nissl staining of cortex and hippocampus. B, D. Histogram of survival ratio of neurons in cortex and hippocampus. Scale bars are 100 μm (yellow) and 75 μm (red). Cells are highlighted by arrows. Bars represent means ± SEM from six different sections (two visual fields were randomly selected in a total of 15 rats). *P < 0.05, **P < 0.01, ****P < 0.0001. Control group was considered as 100% and data were normalized accordingly.

PAC1R as a member of the G protein-coupled receptor family is a specific receptor of PACAP [24]. It has been reported that the activation of PAC1R can regulate the downstream second messenger cAMP level, then promote the neurite after rat IS injury [25]. In this study, however, we found that the peptide led to a significantly higher level of PAC1R in the rat brain tissue compared with that in the I/R group and sham group (Fig. 4H), this was also consistent with the transcriptome results that OM-LV20 pretreatment up-regulated PAC1R (Fig. S1). But, whether OM-LV20 is an agonist of PAC1R requires further exploration. Interestingly, the level of cAMP in the I/R rat brain was significantly enhanced, and OM-LV20 application decreased the cAMP level (Fig. 4G). Although it was reported that the PAC1R increase can elevate the cAMP level [26], in this research, the PAC1R level was increased but the cAMP level decreased in the OM-LV20 group. The reason may be that the cAMP level could be regulated by a variety of receptors and PAC1R is only one of them. It also seemed that OM-LV20 might affect other receptors or signaling pathways, then finally affect the cAMP level. All these results indicated that the neuroprotective mechanism of OM-LV20 needed to be further explored.

The normal amine metabolism in brain tissue could be destroyed after IS [27]. TPH1, as a rate-limiting enzyme and key enzyme for the 5-hydroxytryptamine (5-HT) synthesis of monoamine neurotransmitter, was known related to the post-stroke depression release and development [28]. In this study, OM-LV20 elevated the TPH1 level, which could help maintain the brain amine homeostasis (Fig. 4F), suggesting that the peptide might also have an interference effect on post-stroke depression. Although previous studies have been reported the decrease of the TPH1 level after IS [20], whether TPH1 is a key target for the IS treatment needs to be further explored.

Compared with other neuroprotective peptides, OM-LV20 exhibited series of desirable characteristics, i.e., the amino acid sequence of OM-LV20 (20 aa) is much shorter than Hi1a (76 aa), PcTx1 (40 aa) and Exendin-4 (39 aa); furthermore, in brain-injury models, OM-LV20 (20 ng/kg, i.p.) showed stronger neuroprotective effect than Exendin-4 (0.05 μg/g, i.p.). Although PcTx1 and Hi1a showed much stronger neuroprotective effects than OM-LV20 (1 ng/kg and 2 ng/kg, ICV), OM-LV20 could be preferentially administered by intraperitoneal injection.

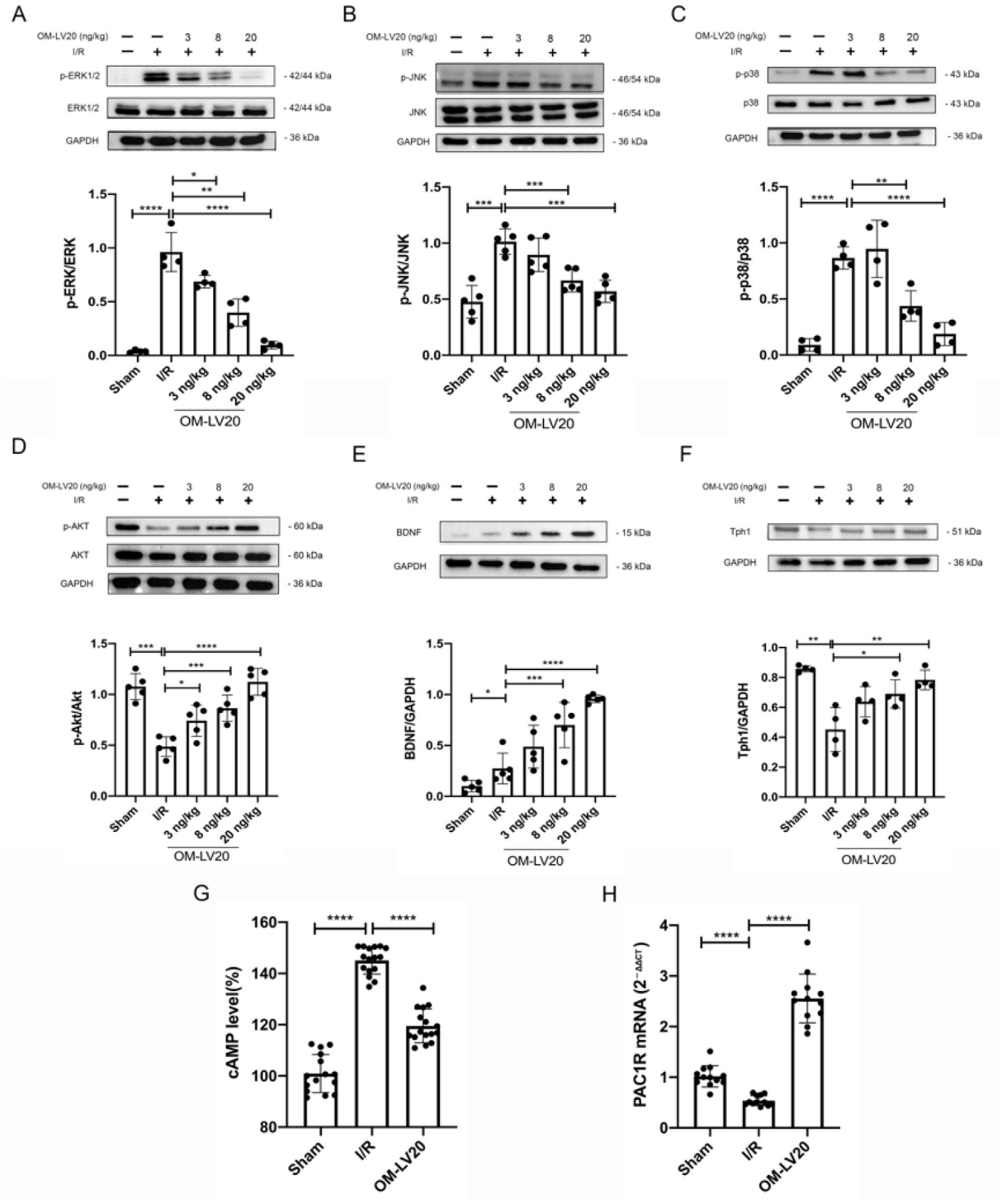

Fig. 4. Neuroprotective mechanism of OM-LV20 in I/R rats. Total 23 rats were used in the western blotting analysis; 11 rats were used for ELISA, and another 11 rats were used for rt-qPCR. OM-LV20 administration reversed the phosphorylation of ERK1/2 (A), JNK (B) and p38 (C) compared with the I/R group. And also increased the expression of p-AKT (D), BDNF (E) and TPH1 (F). G. OM-LV20 administration reversed the abnormal increase caused by I/R. H. OM-LV20 markedly up-regulated the PAC1R level. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. ****P < 0.0001. The control group was considered as 100% and data were normalized accordingly.

In conclusion, this study showed that intraperitoneal administration of OM-LV20 could significantly reduce the infarct area formation, improve behavioral abnormalities, and protect cortical and hippocampal neurons from death in I/R rats. Moreover, the underlying molecular mechanism was partly involved with the regulation of the MAPK and BDNF/AKT signaling pathways, as well as TPH1, cAMP, and PAC1R levels. To the best of our knowledge, this study is the first time to report the neuroprotective effects of an amphibian skin secretion-derived peptide in I/R rats, thus highlighting OM-LV20 as a promising drug candidate for the development of novel anti-stroke therapies.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.12.053.

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