ORIGINAL CONTRIBUTION

Oxytocin Improves Intracerebral Hemorrhage Outcomes by Suppressing Neuronal Pyroptosis and Mitochondrial Fission

Miaoxian Yang, MD*; Shuixiang Deng, MD*; Junliang Jiang, MD; Mi Tian, MD; Lei Xiao, PhD; Ye Gong, MD

BACKGROUND: Intracerebral hemorrhage (ICH) causes severe sensorimotor dysfunction and cognitive decline which are aggravated by secondary brain injury, yet there are no effective management to alleviate these outcomes. Pyroptosis is strongly related to neuroinflammation, which plays a crucial role in the pathophysiological processes of secondary brain injury after ICH. OXT (oxytocin), as a pleiotropic neuropeptide, has multiple functions including anti-inflammation and antioxidation. This study aims to investigate the role of OXT in improving ICH outcomes and the underlying mechanisms.

METHODS: C57BL/6 mice were used to establish the ICH model by autologous blood injection. OXT was administered intranasally (0.2 μg/g) after ICH. Combing behavioral tests, Western blot, immunofluorescence staining, electron microscopy, and pharmacological approaches, we evaluated the effect of intranasal OXT application on neurological outcomes after ICH and explored the underlying mechanism.

RESULTS: Endogenous OXT level was decreased, whereas OXTR (oxytocin receptor) expression was increased after ICH. OXT treatment improved the short-term and long-term neurological functions and alleviated neuronal pyroptosis and neuroinflammation. In addition, OXT reduced excessive mitochondrial fission and mitochondrial-derived oxidative stress 3 days after ICH. OXT decreased the expression of pyroptotic and proinflammatory factors including NLRP3 (NOD-like receptor protein 3), ASC (apoptosis-associated speck-like protein containing a CARD), GSDMD (gasdermin D), caspase-1, IL (interleukin)-1β, and IL-18 and increased the expression of p-PKA (phospho-protein kinase A) and p-DRP1 (S637; DRP1 [dynamin-related protein 1] phosphorylation at Ser637). OXT-induced neuroprotective effects were blocked by either OXTR inhibitor or PKA inhibitor.

CONCLUSIONS: Intranasal application of OXT can ameliorate neurological deficits and alleviate neural pyroptosis, inflammation, and excessive mitochondrial fission via OXTR/p-PKA/DRP1 signaling pathway after ICH. Thus, OXT administration may be a potential therapeutic strategy to improve the prognosis of ICH.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: cerebral hemorrhage • mitochondrial dynamics • neuron • oxytocin • pyroptosis
to improve the neurofunctional outcomes of ICH. Therefore, exploring the pathological mechanisms of ICH and seeking effective treatments are urgently needed.

ICH induces both primary and secondary brain injury. Following the hematoma mass effect in the first few days, the cascade of secondary brain injury occurs and develops over days to weeks, which contributes to poor prognosis. Pyroptosis and neuronal inflammation play a crucial role in the pathophysiological processes of secondary brain injury. Pyroptosis is a novel type of programmed necrosis that is strongly related to neural inflammation. DAMPs (damage-associated molecular patterns) released after ICH triggers the activation of NLRP3 (NOD-like receptor protein 3), which is involved in regulating mitochondrial fission. However, the influence of OXT on the mitochondrial fission remains unclear.

In this study, we investigated the role of OXT in alleviating pyroptosis, neuroinflammation, and cognitive decline in ICH mice. Our study not only uncovers that intranasal oxytocin alleviates the secondary brain injury by reducing excessive mitochondrial fission through p-PKA/DRP1 (dynamin-related protein 1) pathway after ICH but also suggests that targeting oxytocin signal may be a potential therapeutic strategy to improve ICH prognosis.

### METHODS

This study fulfills the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0. See Supplemental Material for detailed information on methods. The data are available from the corresponding author upon reasonable request.

#### Animals

Adult male (7–9 weeks) and female (9–11 weeks) C57BL/6J mice, 21 to 26 g, were purchased from Zhejiang Vital River Laboratory Animal Technology Co, Ltd. All experimental procedures were approved by the Animal Care and Use Committee of Fudan University.

#### ICH Model and Drug Administration

Experimental ICH model was established by stereotactic-guided injection of 35 μL autologous whole blood to right striatum as previously described. OXT were intranasally
administered with 4 doses (0.2 μg/g at 2 hours, 1, 2, and 3 days after ICH).20 L-368, 899 (5 μg/g) and H89 (1 μg/g) were administered intraperitoneally before OXT administration.25,26

Behavioral Tests
We performed modified Garcia score test, pole test, and forelimb placement test 3 days after ICH to assess the sensorimotor function.22 Rotarod test, novel object recognition, and 3-chamber test were performed 14 days after ICH to evaluate the functions of motor coordination and cognition.25,26

Immunofluorescence, Morphological, and Molecular Analyses
The changes of OXT signal, mitochondrial fission, and pyroptotic pathway were assessed by ELISA, Western blot, immunofluorescence, Fluoro-Jade C staining, and transmission electron microscopy as previously described and manufacturer’s instructions.19,22,27

Statistical Analysis
Statistical analysis was conducted by GraphPad Prism 9. Data were exhibited as mean±SE. P<0.05 was considered statistically significant.

RESULTS
Endogenous OXT Concentration and OXTR Expression Are Changed After ICH
Though OXT level and OXTR expression were reported to be changed in subarachnoid hemorrhage and peri-infarct of vascular dementia,28,29 whether and how oxytocin signals are varied in ICH remains unclear. ELISA was performed to assess the OXT level in the ipsilateral (right) corpus striatum of male mice in sham, 1-day, and 3-day groups. OXT concentration was significantly decreased on day 1 and 3 days after ICH (Figure 1A). In ipsilateral striatum of male mice, the OXTR expression was measured by Western blot. Compared with the sham group, OXTR expression was significantly increased at 12 hours, 1, 2, 3, 5, and 7 days, and then recovered to the level as sham group 14 days after ICH (Figure 1B). Similar changes of OXT concentration and OXTR expression in striatum after ICH were also observed in the female mice (Figure 1C). Therefore, endogenous oxytocin signals are disturbed in the ICH.

Intranasal OXT Treatment Recovers OXT Level in Striatum and Alleviates Long-Term Neurological Deficits
Since endogenous OXT was reduced after ICH (Figure 1A and 1C), we studied whether exogenous OXT application could recover this reduction and improve neurological deficits. We observed that, in both male and female mice, OXT level was decreased in striatum and hypothalamus 3 days after ICH and intranasal OXT treatment recovered OXT level in the striatum, but the OXT level in the hypothalamus did not recover (Figure 1D). Then, we used rotarod test, novel object recognition, and 3-chamber test to evaluate whether the recovered OXT level in striatum could promote the mouse motion, cognition, memory, and sociability.21 In the rotarod test, mice in the ICH+PBS group had significantly shorter falling latency compared with the sham group 14 days after ICH, and intranasal administration of OXT significantly improved the motor function (Figure 1E). Learning and memory abilities were assessed by the novel object recognition 14 days after ICH.26 Compared with the sham group, mice in the ICH+PBS group spent less time exploring the novel object, and OXT-treated ICH mice recovered the novel object exploring time and had a higher discrimination index (P<0.05, Figure 1E). The 3-chamber test was exerted to evaluate cognition in the form of general sociability.26 Mice in sham group spent more time in the chamber containing a novel mouse, while ICH mice spent similar time in social and nonsocial chambers (Figure 1E). OXT administration tended to increase the social time of ICH mice, though it is not significant (Figure 1E). Consistent with the male mice, OXT treatment also recovered the falling latency and the novel object exploring time of the female ICH mice (Figure STA). These results indicate that intranasal OXT administration is sufficient to increase the OXT level in striatum and improve the motor function and cognitive memory of ICH mice.

OXT Mitigates Short-Term Behavioral Deficits and Neuronal Pyroptosis by Activating OXTR
In addition to the long-term behavioral deficits, ICH also induced short-term sensorimotor impairments, which can be evaluated by the modified Garcia score test, pole test, and forelimb placement test 3 days after ICH. Both male and female mice in the ICH+PBS group experienced sensory and motor impairments compared with the sham group, which can be efficiently recovered by OXT treatment (Figure 2A; Figure S1B).

Since ICH induced similar OXT signal changes in male and female mice and OXT treatment had consistent neuroprotective effects in both genders, we explored the mechanism of OXT only using male mice. OXT mainly activates OXTR to play diverse functions.29 We investigated whether the effects of OXT on behavioral deficits are mediated by activating OXTR, and we found that preadministration of OXTR inhibitor L-368, 899 blocked the OXT-induced improvement in short-term behaviors (P<0.05, Figure 2A).

In the ventilator-induced lung injury model, OXT alleviated the pyroptosis of lung tissue through NLRP3-medi- ated pathways.15 Given the neuroprotective effects of OXT and obvious pyroptosis in perihematomal region of ICH mice,5 we evaluated the levels of pyroptosis-associated
Figure 1. The changes of OXT (oxytocin) and OXTR (OXT receptor) after intracerebral hemorrhage (ICH) and the neuroprotective effects of OXT treatment on long-term neurobehavior.

A, Comparison of OXT level in ipsilateral striatum of male mice. n=6 mice/group. B, Quantitative analyses and representative Western blot bands of OXTR expression in striatum of male mice after ICH. n=6 mice/group. C, Comparison of OXT and OXTR level in ipsilateral striatum of female mice. n=6 mice/group. D, Comparison of OXT level in striatum and hypothalamus of male and female mice 3 days after ICH. n=6 mice/group. E, Rotarod test, novel object recognition test, and 3-chamber sociality test 14 days after ICH. The representative mouse trajectory of novel object recognition test during test section. Red circle indicated novel object and blue circle indicated familiar object. n=10 mice/group. *P<0.05, **P<0.01, ***P<0.001 vs sham group; #P<0.05, ###P<0.001.

**Figure 1.** The changes of OXT (oxytocin) and OXTR (OXT receptor) after intracerebral hemorrhage (ICH) and the neuroprotective effects of OXT treatment on long-term neurobehavior.
Figure 2. OXT (oxytocin) mitigates short-term neurological deficits and neuronal pyroptosis by activating OXTR (oxytocin receptor). A, Summary about modified Garcia score test, pole test, and forelimb placement of male mice 3 days after intracerebral hemorrhage (ICH). B, Representative Western blot (WB) bands and C, quantitative analyses of ASC (apoptosis-associated speck-like protein containing a CARD) and NLRP3 (NOD-like receptor protein 3) in ipsilateral corpus striatum of mice 3 days after ICH. D, Quantitative analysis of caspase-1–positive neurons and GSDMD (gasdermin D)–positive neurons in the perihematomal area 3 days after ICH. E, Representative images of colocalization of caspase-1 (red) with neuron (neuronal nuclear [NeuN], green) in the perihematomal area. Scale bar=50 μm. F, Representative images of GSDMD (red) colocalized with neuron (NeuN, green). Scale bar=25 μm. n=6 mice/group. DAPI indicates 4′,6-diamidino 2-phenylindole; and IF, immunofluorescence. *P<0.05, **P<0.01, ***P<0.001 vs sham group; #, &P<0.05, ##, &&P<0.01, ###, &&&P<0.001.
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Oxytocin decreases the formation of pyroptotic pores and mitochondria-derived oxidative stress by activating OXTR

During pyroptosis, the release of inflammatory cytokines depends on the formation of pyroptotic pores on the neuronal membrane. We utilized transmission electron microscopy to detect the neuronal membrane after ICH.

As shown in Figure 4A, the formation of neuronal membrane pores in the ipsilateral striatum was obvious in the ICH+PBS group 3 days after ICH, while OXT administration mitigated this trend via activating OXTR.

Apart from the neuronal pore, mitochondrial morphological changes were also observed. Long tubular or short virgulate mitochondria with clear cristae ridges were exhibited in the sham group, but the neuronal mitochondria in the ICH+PBS group exhibited small globular mitochondria with marked swelling structure, collapse cristae, and disruption of membranes (Figure 4A). In the ICH+OXT group, mild mitochondrial edema with prominent cristae ridges and prolonged structure was detected, whereas in the L-368, 899-treated group, internal structural disorder, and swelling global structure were observed (Figure 4A).

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Figure 3. OXT (oxytocin) reduces neuroinflammation and neuronal degeneration.

A, Representative Western blot (WB) bands and quantitative analyses of IL-18 (green) in ipsilateral striatum of mice 3 days after intracerebral hemorrhage (ICH). B, Representative microphotographs and relative fluorescence intensity of IL-1β (green) in perihematomal region 3 days after ICH (n=6 mice/group). C, Representative images and quantitative analysis of colocalization of microglia (Iba-1 [ionized calcium-binding adaptor molecule 1], green) with TNF-α (tumor necrosis factor-α; red) in the perihematomal area 3 days after ICH. D, Representative images and quantitative analysis of Fluoro-Jade C (FJC)–positive degenerating neurons in the perihematomal area 3 days after ICH. Scale bar=50 μm. n=6 mice/group. DAPI indicates 4′,6-diamidino 2-phenylindole. **P<0.01, ***P<0.001 vs sham group; #, &P<0.05, ##, &&P<0.01, ###, &&&P<0.001.
mice, and OXT treatment rescued the DRP1 phosphorylation at Ser637, which was reversed by L-368, 899 (Figure 5B). Total DRP1 protein level remained unchanged in different conditions (Figure 5B). Additionally, p-PKA level was increased in the ICH+OXT group, but not in the ICH+OXT+L-368, 899 group, compared with the ICH+PBS group (Figure 5B). To confirm these findings, we performed immunostaining to detect the subcellular localization of DRP1. Consistent with the changes in p-PKA and p-DRP1 (S637), increased DRP1 was localized to the mitochondria 3 days after ICH (Figure 5C).

PKA Inhibitor Blocks the Neuroprotective Effects of OXT in Neural Pyroptosis, Mitochondrial Fission, and Behaviors After ICH

To further investigate the necessity of the p-PKA signaling pathway in OXT-mediated neuroprotective effects, we selectively inhibited p-PKA by H89. OXT treatment reduced the colocalization of DRP1 with TOMM20 (translocase of outer mitochondrial membrane 20), and L-368, 899 reversed these changes (Figure 5C).
Figure 5. OXT (oxytocin) inhibits excessive mitochondrial fission by p-PKA (phospho-protein kinase A)/DRP1 (dynamin-related protein 1) pathway.

A, Mitochondria were labeled by TOMM20 (translocase of outer mitochondrial membrane 20) staining (green). Imaris and ImageJ software were used to achieve 3D visualization and visual comparison of mitochondria. The boxed area in the left micrographs were enlarged on the right oblong micrographs. The mitochondrial morphology changes were quantified by the aspect ratio. B, Representative Western blot bands and quantitative analyses of p-DRP1 (S637; DRP1 [dynamin-related protein 1] phosphorylation at Ser637), DRP1, and p-PKA in ipsilateral striatum 3 days after intracerebral hemorrhage (ICH). C, Representative images and quantitative analysis of DRP1 colocalized with TOMM20 in the perihematomal area. Scale bar=5 μm. n=6 mice/group. DAPI indicates 4′,6-diamidino 2-phenylindole. ***P<0.001 vs sham group; #P<0.05, ##, &&P<0.01, ###, &&&P<0.001.
Figure 6. PKA (protein kinase A) inhibitor, H89, blocks the neuroprotective effects of OXT (oxytocin).

A. Representative Western blot (WB) bands and quantitative analyses of p-PKA (phospho-protein kinase A), NLRP1 (NOD-like receptor protein 1), ASC (apoptosis-associated speck-like protein containing a CARD), IL (interleukin)-18, p-DRP1 (S637; DRP1 [dynamin-related protein 1] phosphorylation at Ser637), and DRP1. n=6 mice/group. B. Electron photomicrographs of mitochondria. Blue arrows: normal mitochondrial structure; green arrows: fragmented mitochondria. Scale bar=1 μm. n=2 mice/group. C. Modified Garcia score test, pole test, and forelimb placement were performed 3 days after intracerebral hemorrhage (ICH). n=6 mice/group. DMS indicates dimethyl sulfoxide. *P<0.05, **P<0.01, ***P<0.001 vs sham group; #, &P<0.05, ##, &&P<0.01, ###, &&&P<0.001.
the ICH+OXT+dimethyl sulfoxide (DMSO) group, H89 application before OXT treatment decreased the p-PKA level and increased the expressions of pyroptotic factors NLRP3, ASC, and proinflammatory cytokine IL-18 three days after ICH (Figure 6A). Swelling mitochondria with collapsed cristae and disrupted membranes were exhibited in the ICH+PBS group, and OXT protected the mitochondria from deformation, which was abolished by H89 (Figure 6B). Additionally, H89 also abolished the OXT-induced short-term behavioral improvements 3 days after ICH (Figure 6C). Hence, the p-PKA signaling pathway is required for OXT to improve the ICH outcomes.

DISCUSSION

This study proves that OXT can alleviate neural pyroptosis, inflammation, and behavioral impairment via OXTR/p-PKA/DRP1 pathway after ICH. We found that OXT level was decreased, while OXTR expression was increased after ICH in both male and female mice. Intranasal OXT administration recovered the OXT concentration in striatum after ICH. OXT improved short- and long-term neurological deficits and suppressed neuronal pyroptosis, inflammation, and microglia activation via activating OXTR. OXT reduced excessive mitochondrial fission and mitochondrial ROS via activating p-PKA and increasing the DRP1 phosphorylation at Ser637. Hence, our study uncovers the neuroprotective role of OXT in ICH and suggests the potential clinical translation of OXT in treating ICH.

Endogenous OXT level is decreased in several neurological diseases including aneurysmal subarachnoid hemorrhage, autism, and Huntington disease. Patients with poor outcome after subarachnoid hemorrhage had lower OXT level in cerebrospinal fluid than patients with good outcome. Consistent with these literatures, we detected a notable decrease in OXT level in the ipsilateral striatum after ICH. In contrast, OXTR was reported to be upregulated in the peri-infarct space of postmortem frontal cortex samples from vascular dementia patients. Similarly in our study, OXTR expression was increased at 12 hours to 7 days and then decreased 14 days after ICH. Given the established roles of OXTR signaling in anti-inflammatory and antioxidant responses, we speculate that the increase of OXTR expression may be a tissue-protective response to the ICH damage. Nevertheless, the increase of OXTR, in the condition of OXT decline, may not be sufficient to achieve the neuroprotection.

Intranasal delivery of OXT has been reported to be successful in nervous system deposition with prominent advantages including noninvasion, speediness, high efficiency, and safety. In this study, we found that intranasal OXT administration at 2 hours, 1, 2, and 3 days after ICH can recover the OXT concentration in striatum, but OXT level in hypothalamus remained decreased compared to the sham group. Given the short half-life of OXT, we suggested that intranasal OXT treatment may stimulate the endogenous release of OXT in striatum. Then, we further explored the role of intranasal OXT delivery in improving ICH outcomes.

Pyroptosis is considered a whistle-blower to release injury alarms and amplify the proinflammatory signals. The canonical pyroptosis pathway is induced by an NLRP3 inflammasome sensor that can detect the DAMPs released after ICH. NLRP3 binds adaptor protein ASC and subsequently activates caspase-1. Caspase-1 enables the maturation of the pro–IL-1β and pro–IL-18 and the activation of GSDMD. N-GSDMD forms pores on the plasma membrane, causing the release of IL-1β and IL-18. We found obvious neuronal pyroptosis 3 days after ICH, as shown by the increased level of NLRP3, ASC, cleaved caspase-1, N-GSDMD, IL-1β, and IL-18, more caspase-1–positive and GSDMD-positive neurons, and pyroptotic membrane pores. The existence of GSDMD pores and ASC accumulation may extend the scope and duration of the pyroptosis and inflammation. We also observed significant activation of pro-inflammatory microglia labeled with TNF-α in the perihematoma area. Thus, targeting neuronal pyroptosis and inflammation is a potential strategy to improve the ICH prognosis. We demonstrate that OXT significantly inhibits the release of pyroptotic factors and proinflammatory cytokines, alleviates the activation of proinflammatory microglia, and reduces the degenerating neurons after ICH. Furthermore, OXT improves the sensorimotor function and recognition memory of ICH mice. OXT did not significantly improve 3-chamber social recognition test. One possible explanation is that the current OXT administration strategy is not sufficient to improve the social memory function. A study in rat traumatic brain injury demonstrated that intranasal OXT dose-dependently increased social recognition. Further study is required to optimize the OXT dosage to better improve ICH outcomes.

It is well established that most biological effects of OXT are mediated by OXTR. Consistently, we found that L-368, 899 application abolished the OXT-mediated beneficial effects in improving ICH outcomes, which implies that OXT exerts neuroprotective effects via OXTR pathway. In some neoplastic cells, OXT has been found to activate the p-PKA pathway, a nonconventional OXT signaling. We also observed that OXT treatment increased the level of p-PKA after ICH, which was blocked by L-368, 899. It was reported that p-PKA catalyzed the mitochondrial fission executioner, DRP1, phosphorylation at Ser637 to confine mitochondrial fragmentation. Excessive mitochondrial fission in the perihematoma tissue of ICH mice exacerbated mitochondrial ROS release, neuroinflammation, and neuronal pyroptosis. Given that OXT can protect mitochondrial activity against oxygen-glucose deprivation, we hypothesize that, via activating p-PKA, OXT may reduce excessive mitochondrial fission and maintain the homeostasis of
mitochondrial network. Through Western blot, immunofluorescence, and transmission electron microscopy, we found that OXT protects against mitochondrial disturbance, evidenced by the increased p-DRP1 (S637) level, decreased mitochondria-DRP1 colocalization, lessened mitochondrial ROS, and intact mitochondrial structure. Inhibiting p-PKA by H89 abolished these OXT-mediated effects, suggesting that p-PKA pathway is essential for OXT to improve the ICH outcomes.

This study has some limitations. First, we cannot rule out the contribution of noncanonical pyroptotic pathways like NLRP1 inflammasome and activated caspase-4/5/11 in ICH. Second, we only evaluated the neuroprotective effects of OXT on neural pyroptosis and inflammation. Further research should be undertaken to explore the other pathological processes including brain edema, ferroptosis, and calcium overload, etc. Lastly, we only used adult mice to assess the effects of OXT, ignoring the age-specific differences in ICH and oxytocin function.12,19

CONCLUSIONS

In summary, this study demonstrates that OXT administration can ameliorate neurobehavioral impairments, alleviate neural pyroptosis, inflammation, and reduce excessive mitochondrial fission partly through OXTR/p-PKA/DRP1 signaling pathway after ICH. Thus, OXT may be a promising therapeutic approach to improve the prognosis of patients with ICH.

ARTICLE INFORMATION

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Dr Yang and Deng performed the experiments, data analysis, and article writing. Drs Jiang and Tian assisted in experiment performance and data analysis. Drs Xiao and Gong designed and directed the whole process. The graphic abstract is created with BioRender.com by Dr Yang and we have been granted a license to use it. All authors approved the final article.

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DISCLOSURES

None.

SUPPLEMENTAL MATERIAL

Supplemental Methods

Tables S1

Figure S1

ARRIVE Guidelines Checklist

REFERENCES


