Chemerin reverses neurological impairments and ameliorates neuronal apoptosis through ChemR23/CAMKK2/AMPK pathway in neonatal hypoxic–ischemic encephalopathy

Yixin Zhang, Ningbo Xu, Yan Ding, Desislava Met Doycheva, Yiting Zhang, Qian Li, Jerry Flores, Mina Haghighiabaneh, Jiping Tang and John H. Zhang

Abstract
Hypoxic–ischemic encephalopathy (HIE) is a devastating neurological event that contributes to the prolonged neurodevelopmental consequences in infants. Therapeutic strategies focused on attenuating neuronal apoptosis in the penumbra appears to be promising. Given the increasingly recognized neuroprotective roles of adipokines in HIE, we investigated the potential anti-apoptotic roles of a novel member of adipokines, Chemerin, in an experimental model of HIE. In the present study, 10-day-old rat pups underwent right common carotid artery ligation followed by 2.5 h hypoxia. At 1 h post hypoxia, pups were intranasally administered with human recombinant chemerin (rh-chemerin). Here, we showed that rh-chemerin prevented the neuronal apoptosis and degeneration as evidenced by the decreased expression of the pro-apoptotic markers, cleaved caspase 3 and Bax, as well as the numbers of Fluoro-Jade C and TUNEL-positive neurons. Furthermore, rh-Chemerin reversed neurological and morphological impairments induced by hypoxia–ischemia in neonatal rats at 24 h and 4 weeks after HIE. In addition, chemerin-mediated neuronal survival correlated with the elevation of chemerin receptor 23 (chemR23), phosphorylated calmodulin-dependent protein kinase kinase 2 (CAMKK2), as well as phosphorylated adenosine monophosphate-activated protein kinase (AMPK). Specific inhibition of chemR23, CAMKK2, and AMPK abolished the anti-apoptotic effects of rh-chemerin at 24 h after HIE, demonstrating that rh-chemerin ameliorated neuronal apoptosis partially via activating chemR23/CAMKK2/AMPK signaling pathway. Neuronal apoptosis is a well-established contributing factor of pathological changes and the neurological impairment after HIE. These results revealed mechanisms of neuroprotection by rh-chemerin, and indicated that activation of chemR23 might be harnessed to protect from neuronal apoptosis in HIE.

Introduction
Hypoxic–ischemic encephalopathy (HIE) is a leading cause of morbidity and mortality in infants, and perinatal brain damage leads to life-long neurodevelopmental consequences, such as cerebral palsy, cognitive deficits, and mental retardation. Several factors have been proposed to be involved in neonates with ischemic brain injury, such as oxidative stress, inflammation, apoptosis...
and necrosis. Among these factors, accumulating data suggest that apoptotic mechanisms play a fundamental role in the pathogenesis of ischemic brain injury in neonatal rodents.

Adipokines, also known as adipocyte-derived secretory factors, have been well documented in metabolic diseases. Of note, recent data provided a new sight into the neuroprotective action of adipokines in ischemic stroke. Intrastriatal administration of exogenous adipokines ameliorated neuronal apoptosis and decreased infarction size in rats with cerebral ischemic injury, while in vivo knockdown of endogenous adipokines exacerbated the ischemic stroke outcomes, indicating that adipokines might be a promising treatment strategy for HIE-induced neuronal apoptosis.

Chemerin, a new member of the adipokine family, is synthesized as a 163-amino acid precursor, secreted by various tissues, including liver, spleen, and immune cells, and subsequently cleaved to form the 18 kDa active chemerin. Chemerin functions through chemerin receptor 23 (chemR23), which is expressed on selected types of cells such as macrophages, dendritic cells, and neurons in the dorsal root ganglion, spinal cord, and retina. Chemerin has been considered as a potent endogenous anti-inflammatory mediator in zymosan-induced peritonitis, and its effects primarily depend on chemR23. Recently, activation of chemR23 after myocardial ischemia–reperfusion injury showed an obvious suppression of cardiomyocytic apoptosis. Furthermore, chemerin-15 peptide could significantly reduce the number of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive cells, suppress generation of reaction oxygen species (ROS), and then decrease infarct size in the cardiac tissue. However, there is still lack of evidence indicating the anti-apoptotic role of chemerin in HIE. Moreover, chemerin also has affinity to another two receptors, G protein-coupled receptor 1 (GPR1) and chemokine receptor-like 2 (CCRL2). Whether these two receptors have function in HIE remains unexplored.

In vitro studies showed that treatment with chemerin activated multiple kinases, that might contribute to its diverse biological functions. Adenosine monophosphate-activated protein kinase (AMPK) is an important homeostatic regulator to maintain neuronal energy balance under metabolic stresses, such as ischemia, hypoxia, or glucose deprivation. Mounting evidence has demonstrated that AMPK activation protects neurons from apoptosis under ischemia conditions. The well-known direct upstream activators of AMPK include cardiac tissues liver kinase B1 (LKB1), Ca2+/calmodulin-dependent protein kinase 2 (CAMKK2) and transforming growth factor-β-activated kinase 1. A recent study by Anderson et al. showed that the CAMKK2 was abundantly expressed in the cortex and hippocampus. Deleting CAMKK2 gene in mice enhanced infarct sizes and edema formation than wild-type controls in an experimental model of middle cerebral artery occlusion. As one of the major downstream regulators of AMPK, nuclear factor erythroid 2-related factor 2 (Nrf2) also acts as a transcription factor to regulate expressions of antioxidant genes, thus decreasing apoptosis and accumulation of ROS. Upregulation of Nrf2 protects astrocytes from cell death in an in vitro model of ischemia–reperfusion. Nrf2 over-expression in osteoarthritis chondrocytes suppressed the interleukin-1β-induced production of caspase 3/8/9. Of note, in vivo and in vitro studies reported that administration of chemerin potentiated the phosphorylation of AMPK and CAMKK2 in various cell types. However, to date, the role of chemerin/chemR23 and their functional mechanism in HIE-induced neuronal apoptosis remain to be established.

We first induced HIE model by exposing P10 rat pups who had been subjected to right common carotid ligation in a hypoxic chamber for 2.5 h. In the present study, we observed a robust induction of endogenous chemerin and chemR23 expression 12 h after HIE. Intranasal administration of human recombinant chemerin (rh-chemerin) at 1 h post HIE could attenuate neuronal apoptosis and oxidative stress, improve neurological function, and alleviate morphological impairment, whereas chemR23 knockdown reversed these neuroprotective effects of rh-chemerin. Mechanistically, rh-chemerin-mediated anti-apoptosis was regulated by the activation of the chemR23/CAMKK2/AMPK/Nrf2 pathway. Collectively, chemerin plays a critical role in neuronal cell protection in response to HIE, offering a novel therapeutic target for HIE treatment.

**Results**

**Expression levels of endogenous chemerin and chemR23 were upregulated after HIE**

We performed an experimental HIE model induced by ligation of right common carotid artery followed by 2.5 h hypoxia. Double immunofluorescence labeling detected a robust increase in chemerin and chemR23 expression in cortical neurons around the peri-infarct area (ipsilateral cortex) compared to Sham pups and the contralateral control following HIE (Fig. 1a, Supplementary Fig. 1). Our western blot analysis of ipsilateral brain homogenates demonstrated that the chemerin (Fig. 1b, c) and chemR23 (Fig. 1b, d) were significantly upregulated in a time-dependent manner and peaked 72 h after HIE.

Considering chemerin has two other receptors, we then evaluated the expression of endogenous GPR1 and CCRL2 following HIE. Western blot results revealed that GPR1 (Fig. 1b, d) and CCRL2 (Fig. 1b, e) expression levels...
Fig. 1 Expression of endogenous chemerin and chemerin receptor 23 (chemR23) were upregulated after hypoxic-ischemic encephalopathy (HIE). a Immunofluorescence staining showing an increased in expression of chemerin (red, upper line) and chemR23 (red, lower line) on neurons (green) in ipsilateral cortex of vehicle-treated animals compared with sham and further increased expression of chemerin and chemR23 after human recombinant chemerin (rh-chemerin) treatment (4′,6-diamidino-2-phenylindole (DAPI), blue). Arrows indicate chemerin or chemR23 colocalized with neuron (arrowhead part was amplified). Scale bar = 50 µm, n = 3 for each group. b Representative western blots showing temporal expression of endogenous chemerin, chemR23, G protein-coupled receptor 1 (GPR1), and chemokine receptor-like 2 (CCRL2) in the brain after HIE. c–f Densitometric quantification of protein expression of chemerin (c), chemR23 (d), GPR1 (e), and CCRL2 (f). Chemerin expression levels significantly increased from 12 h reaching peak at 72 h post HIE. ChemR23 expression levels significantly increased from 24 h, reached highest at the 72 h after HIE. GPR1 and CCRL2 expression levels were comparable between pups with and without HIE at five time points. Data were normalized for actin expression. Data are mean ± SD. Analysis of variance (ANOVA), Tukey; *p < 0.05 compared with sham, n = 6/group.
were slightly increased after HIE, but did not reach statistical significance. Thus, chemR23 was selected as the major target for chemerin after HIE.

**Rh-chemerin treatment improved short-term neurological deficits and reduced infarct area after HIE**

We then used righting reflex and negative geotaxis to evaluate the effects of rh-chemerin on the neurological outcomes in pups after HIE. It was observed that the vehicle group exhibited marked neurological deficits, and these deficits were significantly attenuated by rh-chemerin treatment (Fig. 2c, d). In addition, rh-chemerin (3 μg/kg, 9 μg/kg, or 27 μg/kg) significantly reversed the HIE-induced body weight loss, and body weight of rh-chemerin (9 μg/kg)-treated pups was comparable to that of Sham pups (Fig. 2e). Vehicle-treated pups with HIE showed a significant infarct volume compared to Sham pups (Fig. 2a, b). Compared with the vehicle group, rh-chemerin treatment (3 μg/kg, 9 μg/kg or 27 μg/kg) significantly reduced the infarct volume and the 9 μg/kg rh-chemerin exerted the optimal effect (Fig. 2a, b). In these experiments, rh-chemerin-treated pups did not show abnormal behavior. Collectively, these results indicated that post-HIE rh-chemerin treatment provided a neuroprotective effect, and 9 μg/kg of rh-chemerin was therefore selected for further experiments.

**Rh-chemerin administration suppressed HIE-induced neuronal apoptosis via chemR23**

Since neurons have high metabolic demand, they are particularly at risk for programmed cell death during the ischemic and hypoxic event. Thus, we evaluated neuronal degeneration and apoptosis by performing Fluoro-Jade C staining, and TUNEL assay and NeuN dual immunofluorescence at 24 h post HIE. We found that the number of Fluoro-Jade C+ cells was markedly reduced in rh-chemerin-treated pups than in HIE pups (Fig. 3a, c). Furthermore, chemR23 small interfering RNA (siRNA) reversed rh-chemerin-mediated protective effect as shown by the increased Fluoro-Jade C+ cells (Fig. 3a, c). TUNEL staining confirmed similar results (Fig. 3a, d). Additionally, we observed that Fluoro-Jade C signals seemed to be greater in the ipsilateral hippocampus than the contralateral one (Supplementary Fig. 2). In contrast to pups with HIE, TUNEL and Fluoro-Jade C+ neurons were scarce in the Sham group.

Previous study showed that chemerin-15 peptide treatment significantly suppressed activation of caspase 3 in the ischemic myocardium. Thus, we investigated whether rh-chemerin administration could suppress the expression of the pro-apoptotic markers, activated caspase 3 and Bax, primarily through chemR23. Western blot results confirmed that rh-chemerin significantly down-regulated cleaved caspase 3 (Fig. 3e, h) and Bax (Fig. 3e, g). In vivo knockdown of chemR23, which decreased chemR23 expression by approximately twofold (Fig. 3e, f), had significantly inducible regulation of cleaved caspase 3 and Bax. Thus, our results suggested that rh-chemerin exerted its neuroprotective role in HIE-induced cerebral injury, at least in part, through suppressing neuronal cell death. Therefore, upregulation of chemR23 via rh-chemerin may render neurons resistant to programmed cell death following HIE.

**Rh-chemerin administration attenuated production of ROS following HIE**

ROS is known to be potent initiators of neuronal apoptosis in HIE. To investigate whether rh-chemerin suppresses neuronal apoptosis involving the inhibition of ROS, we evaluated superoxide anion by DHE staining, and levels of free radical adducts by 4-HNE staining. Immunolabeling of coronal brain sections revealed that intensified dihydroethidium (DHE) staining in the ipsilateral cortex of pups with HIE, and rh-chemerin significantly attenuated superoxide anion production in pups with HIE compared with vehicle-treated pups and chemR23 siRNA group (Fig. 4a, b). Similar results were observed in 4-HNE staining, which showed that rh-chemerin suppressed the generation of free radical adducts in pups with HIE, as evidenced by fewer 4-hydroxynonenal (4-HNE)+ neurons compared with vehicle-treated pups and chemR23 siRNA group (Fig. 4a, c).

**Rh-chemerin administration improved long-term neurological function and brain morphology at 4 weeks post HIE**

As for the brain atrophy, HIE elicited significant brain weight and tissue loss; however, rh-chemerin ameliorated histological injury at 4 weeks after HIE (Fig. 5a–c). Vehicle-treated animals performed markedly worse compared with Sham animals in water maze test, and rh-chemerin improved cognitive function in spatial learning and memorizing compared with vehicle animals, as demonstrated by less swim distance to find the platform (Fig. 5d), less escape latency (Fig. 5e), and more time spent in target quadrant during the probe test (Fig. 5f). In the meantime, no significant changes were observed in velocity among the three groups (Fig. 5g). Treatment with rh-chemerin also significantly improved sensorimotor function in foot-fault and rotarod tests. Rh-chemerin significantly reduced total foot-faults, especially in the left lateral, compared with vehicle pups (Fig. 5h). Moreover, rh-chemerin significantly increased rotarod latency at both of the 5 rpm and 10 rpm acceleration compared with vehicle animals (Fig. 5i).
Rh-chemerin administration ameliorated HIE-induced brain injury via chemR23/CAMKK2/AMPK activation

On the basis of the observation that rh-chemerin decreased the production of cleaved caspase 3 in pups with HIE, we proposed that chemerin functioned as an upstream factor in suppressing neuronal apoptotic signaling after HIE. Considering that AMPK is activated by a number of upstream stress-related activators, primarily CAMKK2 in neurons, we investigated whether AMPK and CAMKK2 were modulated by chemerin upon ischemic and hypoxic insults. Although HIE induced upregulation of p-CAMKK2 and p-AMPK, as well as Nrf2 (a major downstream transcription factor of AMPK) (Fig. 6a–d), the expression of cleaved caspase 3 and Bax remained high (Fig. 3d, f, g). Notably, treatment with rh-chemerin further enhanced the CAMKK2 and AMPK phosphorylation, as well as Nrf2 expression after knockdown of chemR23 in vivo (Fig. 6a–d). These data indicated that HIE-induced endogenous p-CAMKK2, p-AMPK and Nrf2 were insufficient to exert anti-apoptotic effects, and that upregulation of chemR23 via rh-chemerin administration could significantly attenuate induction of pro-apoptotic proteins. Together, CAMKK2/AMPK/Nrf2 pathway might be the potential downstream mediator of chemerin/chemR23 signaling after HIE.

To further test whether the chemR23/CAMKK2/AMPK/Nrf2 signaling protected neonatal brain from HIE, specific chemR23 (Alpha-NETA)/CAMKK2 (STO-609)/AMPK (Dorsomorphin) inhibitors were delivered along with rh-chemerin. ChemR23 expression in the ipsilateral hemisphere was unaffected at 24 h after HIE in 2-(α-Naphthoyl) ethyltrimethylammonium iodide (Alpha-NETA), 7-Oxo-7H-benzimidazo[2,1-a]benz[de]isoquinoline-3-carboxylic acid, acetate salt (STO-609), and Dorsomorphin-treated pups when compared with rh-chemerin-treated pups (Fig. 6e, f). The phosphorylation of CAMKK2 was significantly reduced at 24 h after treatment with Alpha-NETA.
Fig. 3 (See legend on next page.)
and STO-609, while there was no significant difference between Dorsomorphin- and rh-chemerin-treated pups, further supporting CAMKK2 was a downstream kinase of chemR23 in HIE (Fig. 6e, g). Administration of Dorsomorphin significantly reduced phosphorylation of AMPK (Fig. 6e, h) and the expression of Nrf2 (Fig. 6e, i), whereas Dorsomorphin exerted no significant effect on either the expression of chemR23 (Fig. 6e, f) or phosphorylation of CAMKK2 (Fig. 6e, g). Administration of Alpha-NETA, STO-609 and Dorsomorphin all significantly increased the expression of cleaved caspase 3 and Bax compared with rh-chemerin+/dimethyl sulfoxide (DMSO)-treated pups (Fig. 6e, j, k).

Triphenyltetrazolium chloride (TTC) staining confirmed that silencing chemR23 or pharmacological inhibition of chemR23/CAMKK2/AMPK pathway reversed the neuroprotective effects of rh-chemerin after HIE, as seen from the significant increase in infarct volume (Fig. 7a, b). Furthermore, rh-chemerin treatment significantly maintained body weight after HIE (Fig. 7c). However, all four interventions with Alpha-NETA, STO-609, and Dorsomorphin, as well as chemR23 siRNA reversed those effects when compared with rh-chemerin, rh-chemerin+/Scramble siRNA group or rh-chemerin +DMSO group (Fig. 7c). Collectively, chemR23/CAMKK2/AMPK inhibition exacerbated production of pro-apoptotic proteins and enlarged infarct area after neonatal stroke, indicating a key role of chemR23/CAMKK2/AMPK signaling in protecting neonatal brain from HIE-induced neuronal apoptosis.

**Discussion**

The mechanism underlying HIE-induced brain damage has not been well elucidated. Thus, little clinical progress has been made in the development of therapeutic strategies. Routine therapies used to protect affected premature infants are symptom supportive and have unwanted adverse effects. Neuronal apoptosis has been demonstrated as a primary pathophysiological process post HIE. Therefore, therapeutic strategies that augment endogenous anti-apoptotic mechanisms could be a safe approach to reducing neurological impairment in HIE patients.

The present study characterized the previously unidentified but essential roles of chemerin in attenuating HIE-induced neuronal apoptosis. First, neuronal chemerin and chemR23 were robustly upregulated during the subacute phase of HIE. Second, intranasal administration of rh-chemerin attenuated HIE-induced deterioration of histological and behavioral outcomes. Third, rh-chemerin suppressed neuronal apoptosis and production of ROS after HIE. Finally, rh-chemerin decreased infarct volume and generation of apoptotic markers by activating the chemR23/CAMKK2/AMPK signaling pathway. To the best of our knowledge, this study was the first to demonstrate that chemerin played a crucial role in reducing post-stroke neuronal death. Furthermore, we showed that rh-chemerin facilitated upregulation of endogenous chemerin and chemR23, and that in vivo knockdown of chemR23 resulted in a marked elevation in infarct size and expression of apoptotic proteins. Together, these studies suggested the possibility of chemerin to limit brain injury in premature infants with HIE.

Previous study found that cleaved caspase 3-positive neurons were scarcely found in Sham-operated controls and in the contralateral hemisphere of HIE pups, but was dramatically induced in the ipsilateral hemisphere of HIE pups. These data indicated that an ischemic event might irreversibly damage, while penumbra has the potential to recover unless effectively treated. Thus, penumbral tissues could be the therapeutic target for clinical intervention. Three natural receptors for chemerin have been detected in vivo, chemR23, GPR1, and CCR12. Recent study showed that under ischemic condition, CCR12...
expression was significantly increased in mouse brain slices; however, they did not detect the changes of chemR23 or GPR1 expressions.\textsuperscript{43} Our experiments in a HIE rat model revealed that endogenous chemerin and chemR23, rather than GPR1 and CCRL2, expressions were elevated in the ipsilateral hemisphere after HIE injury. Double immunofluorescence data demonstrated that chemerin and chemR23 were colocalized on neurons in the peri-infarct regions, while little expressions were observed in the contralateral hemisphere. Experiments using rh-chemerin treatment supported that neurons expressing chemerin and chemR23 in the peri-infarct area might critically modulate neuronal apoptosis under brain ischemic conditions. In this context, we hypothesized that activation of chemerin/chemR23 signaling pathway in the peri-infarct area might exert anti-apoptotic role following cerebral ischemia.

To examine whether chemerin could indeed have neuroprotective roles, we investigated the influence of rh-chemerin on the brain damage and neurological functions observed at 24 h and 28 days following HIE insult. First, we evaluated the most effective dose of rh-chemerin,

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\caption{Human recombinant chemerin (rh-chemerin) administration attenuated oxidative stress at 24 h post hypoxic-ischemic encephalopathy (HIE). (A) Representative images of dihydroethidium (DHE, red, upper line) and 4-hydroxynonenal (4-HNE, red, lower line; NeuN, green)-positive staining in the ipsilateral cortex at 24 h after HIE. Data showed that there were greater DHE signals and more positively 4-HNE-stained neurons observed in vehicle group compared with sham, whereas rh-chemerin treatment markedly decreased these positively stained cells. However, chemerin receptor 23 (chemR23) small interfering RNA (siRNA) reversed rh-chemerin effects by showing increase in the number of positive stained cells. Scale bar = 100 µm; *p < 0.05 compared with sham, \#p < 0.05 compared with HIE + vehicle, &p < 0.05 compared with HIE + rh-chemerin + Scramble siRNA, n = 6/group. Data are mean ± SD. Analysis of variance (ANOVA), Tukey.}
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Fig. 5 Human recombinant chemerin (rh-chemerin) administration improved long-term neurological function and brain morphology at 4 weeks post hypoxic-ischemic encephalopathy (HIE). a Representative images of Nissl-stained brain sections showing tissue loss in ipsilateral hemisphere at 4 weeks after HIE. Quantification showing rh-chemerin significantly increased the ratio of ipsilateral/contralateral regions (b) and reduced the percent of tissue loss (c) when compared with vehicle (all samples in Nissl staining were from the same animals which were killed after long-term neurobehavioral tests). d–g rh-chemerin treatment group showed significant improvement in spatial memory in terms of the less swim distance to find the platform (d), less escape latency (e), and more time in the target quadrant (f) when compared with vehicle. Rh-chemerin treatment group significantly improved motor function as demonstrated by foot-fault (h) and rotarod (i) tests; *p < 0.05 compared with sham, #p < 0.05 compared with vehicle, n = 6/group. Data are mean ± SD. Analysis of variance (ANOVA), Tukey.
which was determined by the infarct area and neurological function at 24 h after HIE. To eliminate the potential systemic effects of exogenous chemerin, three doses of rh-chemerin were delivered intranasally at 1 h post HIE. Three doses significantly reduced infarct volume and attenuated developmental delay at 24 h post HIE, with the medium dose of rh-chemerin conferring optimal effects. Thus, medium dose of rh-chemerin was chosen for subsequent experiments. Similar improvement in cognitive and sensorimotor function after treatment with rh-chemerin was observed in long-term neurobehavioral studies (4 weeks after HIE). Neuronal apoptosis has been demonstrated as one of the major factors that contributes to the neurological dysfunction. Then, we observed that rh-chemerin significantly reduced apoptosis as measured by TUNEL and Fluoro-Jade C staining. Western blot analysis confirmed that pro-apoptotic markers, such as cleaved caspase 3 and Bax, were significantly reduced.

Prolonged reduction of blood and oxygen supply in neonatal brains results in cellular energy deficiency, and that ultimately leads to cell death. AMPK is a heterotrimeric protein kinase expressed abundantly on neurons, where it plays a crucial role in maintaining cellular energy metabolism. AMPK could be activated by its
upstream kinases, LKB1 and CAMKK2. A previous study showed that expression of p-AMPK was not affected in cortical neurons upon cortex-specific deletion of LKB1. Pharmacological and genetic inhibition of CAMKK2 worsened outcomes in mice with middle cerebral artery occlusion. In addition, activation of CAMKK2 potentiated phosphorylation of AMPK, and significantly reduced brain infarct volume in rats with ischemia. Thus, we depicted chemerin/chemR23 as a critical anti-apoptotic modulator that acted on the upstream CAMKK2/AMPK signaling pathway. We observed that the phosphorylation of CAMKK2 and AMPK, as well as Nrf2, followed a similar trend as the expression of chemR23 after treatment with rh-chemerin. Knockdown of chemR23 significantly reduced p-CAMKK2, p-AMPK, and Nrf2, further supporting the hypothesis that chemR23 is required to activate the CAMKK2/AMPK/Nrf2 pathway. Consistent with the pro-apoptotic effects of genetic chemR23 ablation, pharmacological ablation of chemR23/CAMKK2/AMPK resulted in increased volumetric stroke lesions and expression of pro-apoptotic proteins. In summary, we demonstrated that CAMKK2/AMPK were anti-apoptotic targets of the chemerin/chemR23 pathway.

In this study, we focused on the effects of chemerin on neuronal apoptosis in HIE. Previous studies showed that chemerin and chemR23 also expressed on astrocytes and microglia, and the effects of chemerin on astrocytes or microglia warrant further investigation. Moreover, previous studies reported that chemerin exerts multiple

Fig. 7 Rh-chemerin suppressed apoptosis via the chemR23/CAMKK2/AMPK signaling pathway at 24 h post HIE. Effects of specific inhibiting chemerin receptor 23 (chemR23), Ca2+/calmodulin-dependent protein kinase 2 (CAMKK2) and adenosine monophosphate-activated protein kinase (AMPK) with human recombinant chemerin (rh-chemerin) administration on infarct volume (a, b) and body weight (c) at 24 h post hypoxic–ischemic encephalopathy (HIE). a, b The infarct volume was significantly increased in all 4 intervention groups with chemR23 small interfering RNA (siRNA), Alpha-NETA, STO-609, and Dorsomorphin when compared with rh-chemerin+Scramble siRNA or rh-chemerin+DMSO group. Alpha-NETA 2-(α-Naphthoyl)ethyltrimethylammonium iodide, STO-609 7-Oxo-7H-benzimidazo[2,1-a]benz[de]isoquinoline-3-carboxylic acid, acetate salt. c rh-chemerin significantly preserved body weight when compared with vehicle; however, all 4 intervention groups with chemR23 siRNA, Alpha-NETA, STO-609, and Dorsomorphin showed to significantly reverse those effects when compared with rh-chemerin+Scramble siRNA or rh-chemerin+DMSO group. *p < 0.05 vs sham, #p < 0.05 vs HIE+vehicle, &p < 0.05 vs HIE+rh-chemerin+Scramble siRNA, @p < 0.05 vs HIE+rh-chemerin+DMSO, n = 6/group.

Data are mean ± SD. Analysis of variance (ANOVA), Tukey

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protective properties, such as anti-inflammation and anti-apoptosis, via chemR23 in different cell types. In the present study, we only focused on the neuroprotective effects of chemerin on neuronal apoptosis after HIE. Hence, whether chemerin/chemR23 interfered with inflammation or oxidative stress-mediated neuronal apoptosis during HIE remains to be determined.

In conclusion, administration of rh-chemerin after HIE could improve neurological outcomes, reduce infarct volume, and suppress neuronal apoptosis and oxidative stress in neonatal rats. Such neuroprotection by rh-chemerin was most likely mediated through chemR23/CAMKK2/AMPK signaling pathway. Therefore, rh-chemerin might be a promising treatment strategy for ameliorating neuron death in infants with HIE.

Materials and methods

Animals

Animals procedures were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The study protocol has been approved by Institutional Animal Care and Use Committee of Loma Linda University. A total of 204 10-day-old (P10) unsexed Sprague–Dawley neonatal pups (weight = 14–20 g, Harlan, Livermore, CA) were randomly subjected to either Sham (n = 30) or HIE surgery (n = 174). All pups were kept in a facility with controlled temperature and 12 h light/dark cycle, and given ad libitum access to water and food.

All experiments were done in a blinded fashion, one researcher (not blinded) assigned pups into different groups and administrated drugs to pups throughout the study. Neurobehavioral tests, TTC staining, western blot, immunofluorescence staining, DHE fluorescence, Fluoro-Jade C, TUNEL staining, and Nissl’s staining were performed and analyzed by two investigators who were blinded to the experimental group.

Neonatal hypoxia–ischemia brain injury rat model

P10 rat pups were subjected to HIE as previously described. Briefly, pups were subjected to right common carotid ligation followed by a recovery period (1 h) and 2.5 h of hypoxia in a chamber with 8% O₂ and 92% N₂ at 36 °C resulting in a severe unilateral injury. Sham animals were subjected to exposure of right common carotid artery without ligation, and without exposure to hypoxic conditions. After hypoxic exposure, all rat pups were returned to their mothers.

Experimental design and animal groups

Experiment 1

The temporal expression of endogenous chemerin, chemR23, GPR1, and CCRL2 post HIE were evaluated. The groups included Sham and HIE group at 6 h, 12 h, 24 h, and 72 h following the injury. Whole brain samples were collected for immunofluorescence staining to evaluate the cellular localization of chemerin and chemR23. Right hemisphere samples were collected for western blot to evaluate the expression of the proteins at various time points.

Experiment 2

The neuroprotective effects of exogenous rh-chemerin treatment for HIE were evaluated. Three doses of rh-chemerin (3 µg/kg, 9 µg/kg, and 27 µg/kg) were tested. The groups included Sham, HIE+Vehicle, and HIE+rh-chemerin (3 dosages). Rat pups were randomly injected rh-chemerin or 0.9% of NaCl solution (NS) as vehicle via intranasal route 1 h following HIE induction. Rat pups were subjected to neurobehavioral tests 24 h post HIE, and then killed to collect whole brain samples for TTC staining to evaluate infarct area, for Fluoro-Jade C and TUNEL staining to evaluate the neuronal apoptosis, and for 4-HNE and DHE fluorescence staining to evaluate free radical adducts and superoxide levels in situ.

Experiment 3

Long-term effects of rh-chemerin treatment were assessed. The groups included Sham, HIE+Vehicle (NS), and HIE+rh-chemerin. Neurobehavior tests were performed 4 weeks post HIE and whole brain samples were collected for Nissl staining to measure brain tissue loss.

Experiment 4

The roles of chemR23, CAMKK2, and AMPK in anti-apoptotic effect of rh-chemerin were assessed. The groups included Sham, HIE+Vehicle (NS), HIE+rh-chemerin, HIE+rh-chemerin+Scramble siRNA, HIE+rh-chemerin+chemR23 siRNA, HIE+rh-chemerin+DMSO, HIE+rh-chemerin+alpha-NETA, HIE+rh-chemerin + STO-609, HIE + rh-chemerin + Dorsomorphin. The siRNA for chemR23 or Scramble siRNA (both from Life Technologies) was injected via intracerebroventricular route 24 h prior to HIE. Alpha-NETA (chemR23 specific inhibitor, 10 mg/kg), STO-609 (CAMKK2 specific inhibitor, 10 mg/kg), or Dorsomorphin (AMPK specific inhibitor, 10 mg/kg) were dissolved in DMSO, diluted to 4% DMSO in NS, and administered intraperitoneally 30 min following HIE induction. Neurobehavioral tests were performed 24 h post HIE, and then whole brain samples were collected for TTC staining to evaluate infarct area and right hemisphere samples for western blot to measure apoptotic marker levels (cleaved caspase 3 and Bax).

Drug administration

Intranasal administration was performed as described previously. A total volume of 6 µl was delivered into the bilateral nares in 1 day. Pups were randomly assigned to
receive rh-chemerin (3 μg/kg/day, 9 μg/kg/day or 27 μg/kg/day) or NS at 1 h post HIE and then once daily for 3 (short-term study) or 7 (long-term study) consecutive days. The dosage and treatment regimen were based on a previous study59. Rat-derived chemR23 siRNA (0.5 nmol/2 μl, Life Technologies) or Scramble siRNA (2 μl, Life Technologies) was delivered via intracerebroventricular injection (1.5 mm anterior, 1.5 mm lateral to the Bregma, and 1.7 mm deep on the ipsilateral hemisphere) at 24 h prior to HIE induction.

Neurobehavioral tests

Neurobehavioral tests were performed by two blinded investigators in an unbiased setup, as previously reported21,50. Short-term neurological tests, including righting reflex and negative geotaxis tests, were performed at 24 h post HIE. Long-term neurological tests, including water maze, rotarod, and foot-fault, were performed at 4 weeks post HIE (shown in supplementary material).

Infarct volume measurement

At 24 h post HIE, pup brains were cut into 2 mm coronal sections and a total of five sections were prepared as described previously21,54,55. The sections were incubated with TTC staining solution for 5 min at room temperature before being washed by phosphate-buffered saline (PBS), and then brain slices were fixed in 10% formalin overnight. The sections were imaged, and the volume of the infarct area was quantified with ImageJ 4.0 (Media Cybernetics). The percentage of infarct volume = [(contralateral hemisphere – non-lesioned ipsilateral hemisphere)/2 × contralateral hemisphere] × 100%.

Western blot analysis

The lysates from frozen tissues were loaded onto an 10–12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels56,57. After the electrophoresis for 90 min at 100 V, proteins were transferred onto 0.2 μm or 0.45 μm nitrocellulose membranes at 80–120 V for 120 min. The membranes were blocked for 2 h at room temperature in TBST (Tris-buffered saline with Tween-20) containing 5% non-fat milk, followed by overnight incubation at 4 °C with the following primary antibodies (detailed information in supplementary material): anti-chemerin, anti-chemR23, anti-GPR1, anti-CCRL2, anti-CAMKK2, anti-Phospho-CAMKK2 (Ser511), anti-Phospho-AMPKα (Thr172), anti-AMPKα, anti-Nrf2, anti-cleaved caspase 3, anti-caspase 3, and anti-Bax. The same membranes were probed with actin as internal loading controls. Next, the membranes were incubated with secondary antibodies (Santa Cruz Biotechnology) for 2 h at room temperature. Bands were visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences) and quantified through ImageJ 4.0 (Media Cybernetics).

Histological analysis

The 8–10 μm-thick brain slices for double immunofluorescence21,58, DHE57, Fluoro-Jade C21 and TUNEL21 staining were prepared as described previously. The slides were observed and imaged by fluorescence microscopy using the Zeiss LSM Image Browser (Carl Zeiss MicroImaging, Jena, Germany) equipped with LASX software.

Immunofluorescence staining

The 8 μm-thick brain slices were incubated with anti-chemerin, anti-chemR2, anti-neuronal nuclei, or anti-4-HNE at 4 °C overnight. Then, the sections were incubated in the appropriate secondary antibodies for 1 h. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). The number of 4-HNE+ neurons per field of view (FOV) was quantified manually in the peri-ischemic regions of vehicle and rh-chemerin treated pups with HIE, and compared with that in respective regions of Sham group. Six sections per pup (each section with 3 randomly chosen images (Fig. 3b)) over a microscopic field of 20× were averaged and expressed as positive neuronal nuclei/total neuronal nuclei per FOV, as described previously50. FOV = 2.3 × 106 μm3.

DHE fluorescence

Each slice was incubated with 10 μM DHE for 30 min at 37 °C in the dark. After incubation, the sections were washed with PBS (pH 7.4). ImageJ 4.0 was used to analyze relative fluorescence intensity of DHE staining as described previously57.

Fluoro-Jade C staining

The 10 μm-thick brain slides were immersed in 1% sodium hydroxide solution for 5 min, then transferred into 70% ethanol for 2 min and rinsed with distilled water for 2 min. After incubation in 0.06% potassium permanganate solution for 10 min, sections were stained with 0.0001% solution of Fluoro-Jade C (Millipore) for 10 min followed by dehydration in a slide incubator at 50 °C for 5 min and xylene for 1 min. Then, a coverslip with DPX mounting media (Sigma-Aldrich) was placed. The counting of Fluoro-Jade C+ cells was the same as that in the Immunofluorescence staining section.

TUNEL staining

For NeuN and TUNEL co-staining, the sections were first stained with NeuN staining (green), followed by TUNEL staining (red) with In Situ Apoptosis Detection Kit (Roche), according to the manufacturer’s protocol. The counting of TUNEL+ neurons was the same as that
in the Immunofluorescence staining section, and the data were expressed as positive neuronal nuclei/total neuronal nuclei.

Brain weight loss and Nissl staining
The ipsilateral and contralateral hemispheres were separated by a midline incision, and weighed on a highly precise balance (sensitivity ± 0.001 g) respectively. As described previously, the percentage of brain weight loss = (weight of the ipsilateral hemisphere/weight of the contralateral hemisphere) × 100%.[21] The 16 µm-thick brain sections were consecutively dehydrated in 95 and 70% ethanol for 2 min, then rinsed in distilled water for 10 s. Sections were then stained with 0.5% cresyl violet 70% ethanol for 2 min, then rinsed in distilled water for 2 min (two times, respectively) before DPX mounting media. The sections were imaged by microscope (Olympus-BX51) equipped with MagnaFire SP 2.1B software (Olympus). Brain tissue loss was measured with ImageJ 4.0. The percentage of brain tissue loss = [(contralateral hemisphere – ipsilateral hemisphere)/contralateral hemisphere] × 100%.[4,21,57]

Statistical analysis
Data were presented as mean ± SD. Statistical analysis was conducted with SPSS v.21.0 (IBM Corp., USA) and GraphPad Prism 6 software (GraphPad Software). The appropriate parametric test was applied (Student’s t-test or one-way ANOVA) followed by the post hoc Tukey’s or Newman–Keuls test if necessary. A p value less than 0.05 was defined statistically significant.

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Author details
1 Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China. 2 Department of Physiology and Pharmacology, Basic Sciences, School of Medicine, Loma Linda University, Loma Linda, CA 92354, USA. 3 Departments of Anesthesiology, Neurosurgery and Neurology, Loma Linda University School of Medicine, Loma Linda, CA 92354, USA.

Authors’ contributions
The conception and design of these experiments were made by Y.X.Z., N.X., Y.D., D.M.D., J.T. and J.H.Z. Y.X.Z., N.X., Y.D., Y.T.Z., Q.L., J.F., M.H., J.T. and J.H.Z. collected and analyzed the data. Drafting the article was done by Y.X.Z. Critically revising the article was done by all the authors (Y.X.Z., N.X., Y.D., D.M.D., Y.T.Z., Q.L., J.F., M.H., J.T. and J.H.Z.). Approval of the final version of the manuscript on behalf of all authors was done by Y.X.Z. and J.H.Z.

Conflict of interest
The authors declare that they have no conflict of interest.

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