Systemic 7,8-Dihydroxyflavone Treatment Protects Immature Retinas Against Hypoxic-Ischemic Injury via Müller Glia Regeneration and MAPK/ERK Activation

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PURPOSE. Perinatal hypoxic-ischemic (HI) injury causes significant damages in the immature retina. The brain-derived neurotrophic factor is well known for its neuroprotective role but has limited clinical applications. A selective agonist of tyrosine kinase receptor B, 7,8-dihydroxyflavone (DHF), is a powerful therapeutic tool, when administered systemically. However, it remains unclear whether DHF treatment can protect the immature retinas against HI injury.

METHODS. Postnatal (P) day 7 rat pups were intraperitoneally injected with DHF or vehicle 2 hours before and 18 hours after being subjected to HI injury. The outcomes were assessed at various timepoints after injury by electroretinography and histologic examinations. Neurogenesis was assessed by double-labeling of retinal sections with 5-bromo-2'-deoxyuridine and different neuronal markers.

RESULTS. At P8, 24-hours postinjury, brain-derived neurotrophic factor mRNA levels in the retina decreased significantly. DHF treatment partially protected immature retinas at both histologic and functional levels between P14 and P30 but did not prevent apoptosis, inflammation, or damage of the blood-retinal barrier (BRB) at P8. On the other hand, DHF treatment promoted the survival of proliferating inner retinal cells, including Müller glia, and enhanced their transdifferentiation to bipolar cells at P17. Moreover, DHF treatment rescued the levels of extracellular signal-regulated kinase (ERK) phosphorylation, which were significantly decreased after injury. The neuroprotective effects of DHF were markedly eliminated by inhibition of ERK phosphorylation.

CONCLUSIONS. Early systemic DHF treatment has neuroprotective effects against HI injury in immature retinas, possibly via promoting neurogenesis through the tyrosine kinase receptor B/ERK signaling pathway.

Keywords: hypoxic-ischemic, immature retinal injury, TrkB agonist, DHF, ERK inhibitor
It is estimated that 1.15 million infants develop hypoxic-ischemic (HI) encephalopathy every year worldwide. Up to 60% of these cases result in death or in severe disabilities, including mental retardation, epilepsy, and cerebral palsy. Specifically, the prevalence of visual impairments, such as strabismus, ocular motor apraxia, nystagmus, optic atrophy, restriction of the visual field, defective color vision, and reduced grating acuity range from 66% to 94% in these children. The treatment and long-term care of the affected children consist of a huge burden and cost to the family, professionals, and society. The above-mentioned visual impairments in those brain-damaged infants have been mainly attributed to occipital lesions in the brain. However, we have previously demonstrated that HI insult can also cause significant histologic and functional damages to the immature retina, including hypoxic stress, apoptosis, and neuroinflammation. Therefore, it is important to implement a neuroprotective strategy that is beneficial for both the retina and the brain.

Brain-derived neurotrophic factor (BDNF) is known to mediate neuronal survival, differentiation, and neurogenesis, mainly by activating the tyrosine kinase receptor B (TrkB), which in turn stimulates various signaling cascades, such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways. However, clinical trials using recombinant BDNF have been disappointingly unsuccessful, presumably because of the poor delivery and short half-life of the protein and other limitations. A selective TrkB agonist, 7,8-dihydroxyflavone (DHF), can cross the blood-brain barrier when administered systemically and has been demonstrated to be a powerful therapeutic tool in animal models of excitotoxicity, Alzheimer’s, and Parkinson disease. An in vitro study showed that, in addition to stimulating neuritogenesis, DHF also protects retinal ganglion cells (RGCs) against excitotoxicity and oxidative injury. However, it remains unclear whether systemic DHF treatment can protect the immature retina against HI injury.

**目的**：周産期缺血缺氧會造成未成熟視網膜顯著傷害。腦源性神經營養因子（BDNF）以其神經保護作用而廣為人知，但其臨床應用有限。7,8-二氫基黃酮（DHF）是酪氨酸激酶受體B（TrkB）的選擇性興奮劑，已知當全身給藥時是強有力的治療工具。然而，DHF治療是否能保護未成熟視網膜免受缺血缺氧損傷尚不清楚。

**方法**：出生後第7天（P7）的鼠幼仔在受到缺血缺氧損傷之前2小時和之後18小時腹膜內注射DHF，或賦形劑作為對照。在損傷後不同時間點，利用視網膜電位檢查和組織學檢查評估DHF的治療效果。利用5-溴-2'-脫氧尿苷（BrdU）和不同的神經元標記對視網膜切片進行多重染色來評估神經細胞是否再生。

**結果**：在損傷後24小時，即出生後第8天（P8），視網膜BDNF mRNA的表現顯著下降。自出生後第14天（P14）至第30天（P30），DHF治療可在未成熟視網膜組織和功能上達到顯著但部分的保護效果，但是無法阻止出生後第8天（P8）時的細胞凋亡，發炎反應，及視網膜屏障的損傷。另一方面，DHF治療促進了包括Müller神經膠質細胞內的視網膜細胞存活並增生，並且在出生後第17天（P17）促使其轉分化成為極細胞。此外，缺血缺氧損傷後明顯下降的細胞外信號調節激酶（ERK）磷酸化程度，可在DHF治療後而顯著增加。同時，DHF的神經保護效果會因抑制ERK磷酸化而被顯著消除。

**結論**：早期全身性DHF治療對未成熟視網膜遭受缺血缺氧損傷時具有神經保護作用，而此保護的可能機轉為藉由TrkB/ERK信號傳導途徑促進神經再生。

**關鍵詞**：缺血缺氧損傷，未成熟視網膜傷害，酪氨酸激酶受體B（TrkB）選擇性興奮劑，7,8-二氫基黃酮（DHF），細胞外信號調節激酶（ERK）抑制劑
Many nonmammalian vertebrates have the remarkable ability to replace neurons, which are lost after retinal damage.15 However, the ciliary marginal zone, which exists in amphibians and is analogous to the stem cell zones in the brain, is not present in the mammalian retina. In fish, the initially quiescent Müller glia (MG) responds to damage by reentering the cell cycle and dedifferentiating to form multipotent progenitors.16 Instead, the mammalian MG normally responds to damage by becoming reactive and undergoing gliosis.16 In rodents, MG can form new neurons, such as photoreceptors, but only in very limited numbers.17 These responses are mediated by growth factors produced by retinal cells, such as the basic fibroblast growth factor and BDNF10 and their enhancement can be an exciting strategy for retinal repair.19 Here, we investigate whether DHF treatment can promote the proliferation and transdifferentiation of MG or other specific neuron subtypes and restore the function of the immature retina after HI injury.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Care Committee of the Chang Gung Memorial Hospital in Kaohstung and is in agreement with the ARVO Statement for the Use of Animals in Research and Vision Research. Ten to 12 Sprague-Dawley rat pups per dam were used. The pups were housed in a temperature- and humidity-controlled colony room and were kept in a 12/12-hour light/dark schedule. The pups were weaned at postnatal (P) day 21, and then housed in groups of 4 to 5 per cage.

HI Eye Injury

On P7, animals were anesthetized with 1% to 2% isoflurane, followed by permanent ligation of the right common carotid artery with 5-0 surgical silk. After surgery, the pups were returned to the dam for a 1-hour recuperation period before HI. The animals were then placed in airtight 500-mL containers showing extensive GFAP immunoreactivity extending from the cornea with 0.5% methyl cellulose (Omni Vision, Zürich, Switzerland). A reference electrode was inserted to the subcutaneous area of the forehead, and a ground electrode was inserted to subcutaneous tissue on the back. The luminance of the stimulus was 3 cd/s/m², with a duration of 10 ms. Scotopic 0-dB ERGs were recorded with a standard white flash and a dark background. Twenty responses, elicited by identical flashes and applied at 10-second intervals, were averaged.

Immunohistochemical Staining and Quantification

Paraffin sections were dewaxed, hydrated through graded concentrations of alcohol, and placed in phosphate-buffered saline. Cryosections were prepared after fixation in 4% paraformaldehyde and dehydration in a sucrose gradient. After antigen unmasking and blocking of nonspecific sites, the sections were incubated overnight at 4°C with primary antibodies against immunoglobulin G (IgG; 1:100; Millipore, Temecula, CA, USA), ED1 (1:100; Biosourse, Camarillo, CA, USA), antiligal fibrillary acidic protein (GFAP; 1:200; Millipore), and BrdU (1:100; Novocasta, Newcastle upon Tyne, UK) and subsequently with secondary antibodies for 60 minutes at room temperature. The immunoreactivity of IgG was evaluated at a 200× magnification by calculating the integrated optical density with the ImagePro Plus 6.0 software. The number of ED1 cells was counted in an area of 400 × 100 μm at 200× magnification. GFAP immunoreactivity was quantified by assigning different grades: grade 1, immunoreactivity in the nerve fiber layer (NFL) and around vessels; grade 2, immunoreactivity in the NFL in an outward tentacle-like pattern, extending toward the inner nuclear layer (INL); grade 3, showing occasional and grade 4, showing extensive GFAP immunoreactivity extending from the NFL to the outer nuclear layer (ONL).

BrdU™ cells were counted on flat-mounted retinas in four central areas near the optic disc and four peripheral areas near the ora serrata (area size, 400 × 200 μm) at 200×. For double labeling, cryosections were incubated with anti-BrdU and primary antibodies against protein NeuN (for RGCs, 1:50; Millipore), ionized calcium-binding adapter molecule 1 (Iba-1, for microglia; 1:200; Wako, Osaka, Japan), recoverin (for cone and rod cells; 1:200; Millipore), SOX9 (for MG and progenitors, 1:200; Millipore), calbindin (for horizontal cells, 1:100; Cell Signaling Technology), and Chx10 (for mature bipolar cells and progenitors, 1:50; Exaltra, Shirley, MA, USA). Double immunopositive cells were counted (400 × 100 μm) at 200× by using a fluorescence microscope (Nikon).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

The ApopTag fluorescent direct in situ apoptosis detection kit (Millipore) was used, and samples were counterstained with a microscope (Nikon, Tokyo, Japan). The extent of retinal damage was quantified by two independent observers, in two nonoverlapping visual fields (250 × 250 μm) within central areas (100 μm from the optic disc).
Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Retinas were dissected and ground with a mortar and pestle in liquid nitrogen under RNase-free conditions. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was amplified by PCR by using the following gene-specific primers: BDNF, 5'-AGCTTCATTCTGAGAGACG-3' (forward) and 5'-GATGGTGATGTTCCCTCGGTA-3' (reverse); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-TGTAACGGAATTGGCCGTA-3' (forward) and 5'-GATGGTGATGTTCCCTCGGTA-3' (reverse). PCR amplification products were separated in 2% agarose (Lonza, Rockland, ME, USA) gels with ethidium bromide (0.5 mg/L) in Tris-borate-ethylenediaminetetraacetic acid buffer. Gel bands were quantified by densitometry; data were normalized against GAPDH (Millipore) expression, and the ratio of gene expression in the treated eyes to the sham controls was calculated. Quantitative PCR was performed on an ABI 7500 sequence-detector system (Applied Biosystems, Grand Island, NY, USA) in 50-μL reaction volumes by using a TransStart Green qPCR SuperMix Kit (Catalog no. AQ101-01; TransGen Biotech, Beijing, China). The 2^ΔΔCt method was used to determine the relative mRNA fold changes. Assays were performed at least 3 times.

Western Blotting

Retinas were homogenized in cold lysis buffer, and 40-μg samples were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk, incubated with primary antibodies and horseradish-conjugated secondary antibodies, and the signal was visualized with chemiluminescence. The following primary antibodies were used: anti-TrkB (1:2000; Abcam, Cambridge, UK), anti-phosphorylated (p) TrkB (1:1000; Millipore), anti-ERK (1:1000; Cell Signaling Technology), anti-β-actin (1:1000; Abcam), anti-p-ERK (1:1000; Millipore), and anti-p/Akt (1:1000; Cell Signaling Technology). After densitometric analysis, data were normalized against GAPDH (Millipore) or tubulin (Millipore) and the ratio of protein expression in the treated eyes to the sham controls was calculated.

Statistics

Statistical analyses were performed by using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA) and 1-way or 2-way ANOVA. Values were considered significant at P < 0.05 and data were presented as mean ± standard error of the mean.

RESULTS

Systemic DHF Treatment Activates TrkB Without Increasing Total TrkB and BDNF Levels in Immature Retinas After HI Injury

The BDNF mRNA levels were elevated in the contralateral retina 12 to 18 hours post-HI injury. In contrast, in the ipsilateral retina, BDNF mRNA decreased as early as 6 hours after injury, reached the lowest level after 24 hours (P < 0.05), and then returned to the basal level 48 hours after injury (Fig. 1A). At P10, the BDNF mRNA level was lower in HI-injured retinas than sham controls and there was no obvious difference between DHF- and DMSO-treated groups (Fig. 1B). TrkB phosphorylation, assessed by Western blotting, was significantly increased in DHF-treated retinas compared with DMSO-treated and sham control groups at 24 hours after HI. However, there were no differences in the Trk protein expression between the controls and the treated HI groups (Fig. 1C; P < 0.05).

Systemic DHF Treatment Provides Long-Term Protection Against HI Injury at Pathologic Level, but Not in the Early Stages of Injury

In agreement with the ERG signals, the RGC number and the thickness of the inner plexiform layer (IPL) and INL, but not the outer plexiform layer or the ONL, were markedly decreased in HI-injured rats at P14 and P30. However, compared to DMSO, treatment with DHF significantly increased RGC count and the IPL and INL thickness at P14 (P < 0.05) and P30 (P < 0.01). There were no significant differences between the DHF-treated and sham control group at P14 and P30. In contrast, there were no significant differences between the DHF- and DMSO-treated groups early (24 hours) after HI injury (Figs. 2B, 2C).

Systemic DHF Treatment Does Not Reduce Early Apoptosis, Neuroinflammation, and BRB Damage, but Decreased Late Gliosis

Twenty-four hours post-HI injury, TUNEL+ cells were significantly increased in the IPL and INL of the DHF- (P < 0.01) and DMSO-treated groups (P < 0.001) than that of the sham controls. ED1+ cells were significantly more abundant in these layers in the DHF- and DMSO-treated groups (P < 0.01) than in sham controls. There was also prominent extravasation, seen by IgG staining in the DHF- and DMSO-treated groups. However, there were no significant differences between the treated groups in the number of TUNEL+ or ED1+ cells and in the IgG immunoreactivity early after HI injury. In contrast, gliosis, evident by GFAP immunostaining, was quite strong and extensive throughout all retinal layers in the DMSO- but not in the DHF-treated and sham control groups at P30. The semiquantitative grading of GFAP immunoreactivity showed that this was significantly higher in the DMSO- than the DHF-treated (P < 0.01) or sham control (P < 0.05) groups. No significant difference was found between DHF-treated and sham control groups (Fig. 3).
Systemic DHF Treatment Enhances MG Survival and Bipolar Cell Differentiation After HI Injury

At P10, the total number of BrdU$^+$ cells in flat-mount retinas was higher in the HI-injured than in the sham control rats ($P < 0.001$). However, there were no significant differences upon DHF or DMSO treatment. In contrast, the DHF-treated group had significantly more BrdU$^+$ cells than the DMSO-treated and control groups at P17 ($P < 0.001$; Fig. 4). Most of these BrdU$^+$ cells were localized in the INL on cryosections at P17. Few cells were coimmunostained with NeuN, in the RGC layer, and recoverin, in the photoreceptors of the ONL (Fig. 5A). Further double labeling showed that most of the BrdU$^+$ cells in the INL were MG (SOX9$^+$) or bipolar cells (Chx10$^+$), whereas very few were horizontal cells (calbindin$^+$) or microglia (Iba1$^+$). The percentage of the different cell types in the total BrdU$^+$ cell population was not different between the HI-injured and sham control group. However, the total number of BrdU$^+$/SOX9$^+$ and BrdU$^+$/Chx10$^+$ cells was significantly higher in DHF-treated group than the DMSO-treated group (Fig. 5B; $P < 0.05$).

ERK Activation Mediates the Long-Term Neuroprotective Effects of DHF

Three major signaling pathways known to mediate TrkB receptor activation$^{21}$ were examined 24-hours post-HI injury. We did not detect any significant differences in the phosphorylation levels of p38 or Akt between the HI-injured and sham controls. In contrast, in the DMSO-treated group, we found a marked decrease in pERK levels, whereas DHF-treatment led to high levels of ERK activation. There were no significant differences between the DHF-treated group and sham controls (Fig. 6A; $P < 0.05$).

To inhibit the ERK pathway, we injected the DHF-treated rats with the ERK inhibitor, PD98059, after HI injury. The ERG showed that PD98059 treatment significantly decreased the amplitude of b- but not of a-wave at P30 (Fig. 6B; $P < 0.01$). In agreement, there was a significant decrease in the RGC number ($P < 0.05$) and the thickness of IPL ($P < 0.01$) and INL ($P < 0.01$; Fig. 6C). There were no differences between the DHF-treated HI-injured group and the sham controls.

DISCUSSION

The results of the present study indicate that early systemic treatment with DHF leads to long-term neuroprotective effects against HI injury in the immature retina. In contrast to previous studies, DHF treatment did not attenuate the apoptosis, inflammation, BRB damage, and cell loss in the inner retina (RGC layer, IPL, and INL) at early stages (24 hours) after HI injury. We also found that the long-term protective effects of DHF were related to decreased gliosis and increased neuro-
FIGURE 2. Systemic DHF treatment protects against HI injury in both functional and histologic levels. (A) At P14, P22, and P30, the electroretinogram analysis showed significantly decreased amplitude of the b-wave, but not the a-wave, in HI-injured compared with sham-operated rats. However, the b-wave amplitude was significantly higher in the DHF- than the DMSO-treated pups. At P8, (B) representative retinal histologic sections and (C) group data showed both DHF- and DMSO-treated HI-injured pups had significantly fewer RGCs and thinner IPL than the sham controls. No significant differences between the DHF- and DMSO-treated groups were observed. At P14 and P30, the DHF-treated group had higher RGC numbers and thicker IPL and INL than the DMSO-treated group. No significant differences were seen between the DHF-treated group and sham controls. Scale bars: 100 μm; n = 6 to 12 per group in each timepoint. *P < 0.05, **P < 0.01, ***P < 0.001.
FIGURE 3. Systemic DHF treatment does not reduce early apoptosis, neuroinflammation, and BRB damage, but decreases gliosis. (A) Twenty-four hours after HI injury, the number of TUNEL+ and ED1+ cells, as well as the extent of extravasation (IgG staining), were much higher in the IPL and INL of both DMSO- and DHF-treated groups than of the sham control group. At P30, GFAP immunostaining was very dense in all retinal layers in DMSO-treated, but not in the DHF-treated or sham control group. (B) No significant differences between DHF- and DMSO-treated groups were found in the number of TUNEL+ or ED1+ cells and IgG immunoreactivity 24 h post-HI injury. At P30, the DMSO-treated group had significantly higher GFAP grades than the DHF-treated and sham control groups. No significant difference in GFAP immunoreactivity was found between DHF-treated and sham control groups. Scale bars: 100 µm; n = 6 to 10 per group in each time point. *P < 0.05, **P < 0.01, ***P < 0.001.
Most BrdU+ cells were detected in the inner retinal layers and were positive for MG or bipolar cell markers. These results suggest that in this injury model, TrkB activation via DHF can promote the conversion of proliferating retinal cells in the INL to MG, bipolar cells, and to a lesser extent, to other retinal neurons. Furthermore, the TrkB-mediated MAPK/ERK pathway seems to be critically involved in the neuroprotection and regeneration processes after HI injury of the immature retina.

Considerable research on the retina has mainly focused on neurons. However, glial cells are known to regulate retinal homeostasis and support neuronal function by maintaining retinal architecture and providing trophic support. More recently, MG, the main type of glia in the retina, has been recognized as a form of late stage progenitor cell type, which acquires certain specialized glial functions, but does not irreversibly differentiate. MG has also been shown to proliferate and produce neurons in several retinal injury models, albeit to a limited extent. The regeneration of retinal neurons, such as bipolar or amacrine cells, by MG proliferation, can be enhanced by treatment with retinoid acid or growth factors in adult rodents. We found significantly more BrdU+ cells in the retinas of HI-injured rats, indicating the high potential for cell proliferation after injury. Furthermore, we showed that DHF treatment can prolong the survival of proliferating MG and promote the differentiation of mainly bipolar cells, a fact which contributes to long-term neuroprotection in both an anatomic and functional level.

MG transdifferentiation after retinal injury in mammals is considered to be mediated by the Wnt, Notch, and Shh signaling pathways. Daily injections of neurotrophins have been shown to stimulate MG in producing new neurons in the absence of injury. In agreement, BDNF does not stimulate MG proliferation or the expression of neuronal markers in the degenerating retina of TrkBGFAP knockout mice. These results demonstrate that glial BDNF signaling has important roles in neuronal survival and regeneration and, particularly, in the conversion of MG into neurons. Activation of the TrkB receptor by BDNF is known to stimulate important signaling pathways, including the MAPK/ERK, PI3K/Akt, phospholipase Cγ, and Janus kinase (JAK) /signal transducer and activator of transcription (STAT). In RGCs, the Akt and ERK signaling pathways were enhanced through the activation of TrkB by DHF. BDNF promotes neurite growth in the major pelvic ganglion of rats and differentiation of neural stem cells by activating the JAK/STAT pathway, but it is not clear whether JAK/STAT is directly acted on by BDNF in the nervous system.
FIGURE 5. Systemic DHF treatment enhances MG survival and bipolar cell differentiation. (A) Immunostained retinal cryosections from DHF-treated pups show that most of the BrdU<sup>+</sup> cells were localized in the INL at P17. Few BrdU<sup>+</sup> cells costained for NeuN (RGCs), recoverin (photoreceptors in the ONL), and Iba1 (microglia, arrows). (B) At P17, double labeling shows that most of the BrdU<sup>+</sup> cells were MG (SOX9<sup>+</sup>, arrows) and bipolar cells (Chx10<sup>+</sup>, arrowheads) and few were horizontal cells (calbindin<sup>+</sup>). The percentages of different cell types over the total BrdU<sup>+</sup> population were not different between the HI-injured and sham-operated pups. However, the total count of BrdU<sup>+</sup>/SOX9<sup>+</sup> cells and BrdU<sup>+</sup>/Chx10<sup>+</sup> cells was significantly higher in the DHF-treated group than the DMSO-treated group. Scale bars: 100 μm; 50 μm in inset; n = 5 to 10 per group. *P < 0.05, ***P < 0.001.
FIGURE 6. ERK activation mediates the neuroprotective effects of DHF. (A) At 24-h post-HI injury, Western blot analysis showed that there were no significant differences in pp38 and pAkt levels between the HI-injured and sham-operated rats. The DHF-treated group had higher levels of pERK than the DMSO-treated group. There were no significant differences between the DHF-treated and sham control groups; n = 4 per group. (B) Inhibition of ERK phosphorylation, with PD98059 after HI injury significantly decreased the amplitude of the b-, but not the a-wave in the electroretinograms of DHF-treated rats at P30. (C) Histologic analysis also showed that PD98059 significantly reduced the number of RGCs and the thickness of the IPL and INL of the DHF-treated rats at P30. There were no differences between the DHF-treated and sham control groups. Scale bars: 100 μm; n = 12 to 20 per group. *P < 0.05, **P < 0.01, ***P < 0.001.
In our experiment, DHF treatment increased the retinal levels of pERK but not pAKT at 24 hours post-HI and promoted the proliferation of retinal cells mainly in the INL at P17. Our data indicate that ERK activation is pivotal for the long-term neuroprotective effects of DHF. It is thus possible that the MAPK/ERK signaling mediates the effects of neurotrophins on MG proliferation and transdifferentiation. The relationship between TrkB and the JAK/STAT pathway is not clear and remains to be explored in our model.

Many studies have demonstrated the neuroprotective effects of BDNF/TrkB activation. In models of brain injury, BDNF treatment decreases early neuronal death by reducing apoptosis and inflammation. Moreover, intravitreous injection of BDNF reduces early apoptosis and microglial activation after retinal injury in adult rats. Although there is an upregulation of BDNF expression after neuronal damage in adult rodents, the amount of BDNF is not sufficient to promote recovery. Hence, exogenous supplementation with BDNF could be beneficial in that regard. In our injury model, BDNF mRNA levels in the retina significantly decreased 6 to 24 hours after HI injury. Systemic DHF treatment 2 hours before and 18 hours after HI injury stimulated TrkB activation, without increasing the expression of BDNF to preserve the integrity of the retina at P14 and P30. Nonetheless, DHF treatment did not provide complete protection, as demonstrated by the ERG on P14 and P30 and the INL thickness on P30. Further cocktail therapy, such as combining VEGE DHF, and hypothermia treatment, may be a better strategy for neonatal neuronal injury.

Most investigations have focused on promoting brain recovery after HI injury in neonates, although this injury can also cause systemic damages, including retinal damage. Therefore, future therapeutic strategies for treating HI insults should take into account the retina in addition to the brain. Furthermore, it has been shown that BDNF enhances RGC survival and function when injected both to the eyes and central visual pathway than only to the eyes. These data imply that systemic treatment is necessary for achieving functional recovery after HI insult, such as therapy using hypothymia. In the current study, we show for the first time that systemic treatment with DHF, which can cross the BRB, protects the immature retina against HI injury via enhancing neurogenesis. This systemic-delivery strategy might prove promising toward the aim of replacing a variety of neuronal subtypes after perinatal HI injuries.

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