Published in final edited form as:

CNS Neurol Disord Drug Targets. 2013 May 1; 12(3): 360–370.

TrkB receptor agonist 7,8 Dihydroxyflavone triggers profound gender-dependent neuroprotection in mice after perinatal hypoxia and ischemia

Kutluay Uluç^{1,2}, Pinar Kendigelen^{3,4}, Emin Fidan^{2,3}, Louise Zhang², Vishal Chanana², Douglas Kintner^{2,3}, Erinç Aktüre^{1,3}, Chihwa Song⁵, Keqiang Ye⁶, Dandan Sun⁷, Peter Ferrazzano^{2,3}, and Pelin Cengiz^{2,3}

¹Dept. of Neurological Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI

²Dept. of Waisman Center, University of Wisconsin School of Medicine and Public Health, Madison, WI

³Dept. of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, WI

⁴Dept. of Anesthesiology and Critical Care, Afüin State Hospital, Kahramanmaraş, Turkey

⁵Dept. of Medical Physics, University of Wisconsin School of Medicine and Public Health, Madison, WI

⁶Dept. of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA

⁷Department of Neurology, University of Pittsburgh, Pittsburgh, PA.

Abstract

In this study, we investigated the effects of a bioactive high-affinity TrkB receptor agonist 7,8-dihydroxyflavone (7,8 DHF) on neonatal brain injury in female and male mice after hypoxia ischemia (HI). HI was induced by exposure of postnatal day 9 (P9) mice to 10% O₂ for 50 minutes at 37°C after unilateral ligation of the left common carotid artery. Animals were randomly assigned to HI-vehicle control group [phosphate buffered saline (PBS), intraperitoneally (i.p.)] or HI + 7,8 DHF-treated groups (5 mg/kg in PBS, i.p at 10 min, 24 h, or with subsequent daily injections up to 7 days after HI). The HI-vehicle control mice exhibited neuronal degeneration in the ipsilateral hippocampus and cortex with increased Fluoro-Jade C positive staining and loss of microtubule associated protein 2 expression. In contrast, the 7,8 DHF-treated mice showed less hippocampal neurodegeneration and astrogliosis, with more profound effects in female than in male mice. Moreover, 7,8 DHF-treated mice improved motor learning and spatial learning at P30-60 compared to the HI-vehicle control mice. Diffusion tensor imaging of *ex-vivo* brain tissues at P90 after HI revealed less reduction of fractional anisotropy values in the ipsilateral corpus callosum of 7,8 DHF-treated brains, which was accompanied with better preserved myelin basic protein expression and CA1 hippocampal structure. Taken together, these findings strongly suggest that TrkB agonist 7,8 DHF is protective against HI-mediated hippocampal neuronal death, white matter injury, and improves neurological function, with a more profound response in female than in male mice.

Keywords

tyrosine kinase B (TrkB); 7,8 dihydroxyflavone (7,8 DHF); brain derived neurotrophic factor (BDNF); rotarod test; Morris water maze test; fractional anisotropy

INTRODUCTION

Hypoxia ischemia (HI) related brain injury is an important health problem among neonates. Most term neonates who require neonatal intensive care have hypoxic ischemic insult at birth [1]. One of every three neonates with HI develops chronic morbidity including cerebral palsy, mental retardation, learning disabilities or epilepsy, while the other third dies [2, 3, 4]. Although hypothermia has been shown to improve the neurological outcome of term neonates after moderate HI, many patients (term neonates after 6 hours of life and pre-term neonates) do not qualify for hypothermia therapy [5]. Therefore, developing new therapies and/or exploring a pharmacological therapy as an adjunct to hypothermia therapy are the focus of HI related brain injury research.

The role of brain derived neurotrophic factor (BDNF) has been extensively studied after stroke. BDNF belongs to the neurotrophin family and plays an important role in brain development, neuronal survival, morphogenesis and plasticity by activating tyrosine kinase B receptor (TrkB) [6, 7]. Activation of TrkB by BDNF results in the phosphorylation of several tyrosine residues and stimulation of important signaling pathways such as MAPK, PI3K and phospholipase C- γ [6]. Up-regulation of BDNF and TrkB mRNAs has been shown in the peri-infarct areas 24 h after HI in postnatal day (P) 7 rats [8]. It has also been demonstrated that intracerebroventricular (ICV) injection of BDNF post-hypoxia results in robust phosphorylation of the TrkB receptor in the hippocampus and reduces infarct volume by 55% in neonatal rats following HI [9]. BDNF increases hippocampal neuronal survival at 4 weeks and improves spatial learning at 20-30 days following perinatal HI [10]. BDNF has been shown to have variety of potent neurobiological effects that promote growth and development including enhancement of glutamate-mediated transmission, modulation of NMDA receptors, decreased hyperpolarization in CA1 neurons, facilitation of memory, increased dendritic spine and spine synapse number and promotion of dentate gyrus neurogenesis [11]. However, while BDNF is generally implicated in neuroprotection after ischemia, there are reports that are suggesting the exacerbation of damage and increasing seizure susceptibility by altering free radical handling or modifying glutamate receptor expression [12, 13].

Unfortunately, the poor bioavailability of BDNF limits its translation to therapeutic applications. Its plasma half life is short and penetration through blood brain barrier is slow [14]. Clinical trials using BDNF for treatment of amyotrophic lateral sclerosis, Huntington's Disease, human immunodeficiency virus related neuropathy and other neurological diseases have been universally disappointing [15]. Efforts have been made to find non-peptide, small molecule ligands capable of activating TrkB signaling with a high potency, specificity and better bioavailability [16, 17]. Recently, Jang and colleagues identified a selective TrkB agonist, 7,8 dihydroxyflavone (7,8 DHF), a member of the flavonoid family that crosses the blood brain barrier when administered systemically [17]. 7,8 DHF reduces neuronal apoptosis, inhibits kainic acid-induced toxicity in cultured neurons and caspase-3 activation in an *in vivo* mouse model of Parkinson disease [17]. 7,8 DHF also decreases infarct volumes after 48 h of permanent middle cerebral artery occlusion (MCAO) in adult male mice [17]. Treatment of the *Mecp2* mutant mouse model of Rett's disease with 7,8 DHF improves survival, delays body weight loss, and enhances voluntary locomotor distance

[18]. However, whether 7,8 DHF has any neuroprotective effects following perinatal HI remains unknown.

In this study, we subjected neonatal mice to HI and examined the protective effects of administration of 7,8 DHF after HI. Our data indicate that 7,8 DHF treatment decreases neuronal degeneration, gliosis, and white matter damage, while improving long-term learning and memory in female mice more than the male mice after perinatal HI injury.

MATERIALS AND METHODS

Materials

Microtubule associated protein 2 (MAP2) antibody, polyclonal anti-neurofilament (NF) antibody, goat serum and DPX mounting medium were from Sigma (St. Louis, MO). Glial fibrillary acidic protein (GFAP) antibody was from Dako Inc. (Carpinteria, CA). Monoclonal anti-myelin basic protein (MBP) antibodies were from Millipore (Billerica, MA). Goat anti-mouse Alexa Fluor 488-conjugated IgG, goat anti-rabbit Alexa Fluor 546-conjugated IgG and To-Pro-3 Iodide were from Invitrogen (Carlsbad, CA). Fluoro-Jade C (FJ-C) was from Histo-Chem Inc. (Jefferson, AK). Rabbit polyclonal anti-phospho-TrkB (Try515) and anti-TrkB antibodies were from Abcam (Cambridge, MA). 7,8 Dihydroxyflavone (7,8 DHF) was developed in Dr. K. Ye's laboratory (Emory University, Atlanta, GA).

Animal use

All procedures on animals were carried out in adherence with NIH Guide for the Care and Use of Laboratory Animals and subject to review by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

Induction of neonatal HI

Postnatal day (P) 9 C57BL/6J mice were anesthetized with isoflurane (3% for induction, 1.5% for maintenance) in 2:1 nitrous oxide/oxygen. The body temperature of the animal was maintained at 37°C using a heating pad. Under a surgical microscope, a midline skin incision was made and the muscle that overlies the trachea was visualized. The left common carotid artery was electrically cauterized and cut as described in our previous study [19]. The incision was infused with 0.5% bupivacaine and sutured with a 6.0 prolene suture. Animals were returned to their dams and monitored continuously for a 2 h recovery period. To induce ipsilateral ischemic injury, the animals were placed in a hypoxia chamber (BioSpherix Ltd, Redfield, NY) equilibrated with 10% O₂ and 90% N₂ at 37°C for 50 min as described in our previous study [19]. After HI, animals were monitored continuously for 30 min and then checked every 30 min for 2 h and then daily until sacrificed. This is a well-characterized model of neonatal HI and results in reproducible brain injury ipsilateral to the carotid ligation. In this model, unilateral ligation of carotid artery alone does not induce ischemic injury due to collateral circulation from the contralateral side through Circle of Willis. Only subsequent exposure to hypoxia results in hemispheric ischemia due to preferential decrease of blood flow to the hemisphere ipsilateral to the carotid artery ligation [20].

Drug administration

The animals (male and female) were randomly divided into HI-vehicle control and HI + 7,8 DHF treatment groups. The HI + 7,8 DHF-treated groups received the initial dose of 7,8 DHF (5 mg/kg, ip.) at 10 min after HI and subsequent daily dose(s) for one or 6 days after HI. The HI-vehicle control groups received an equal volume of phosphate buffered saline (PBS) at the same time points.

Brain tissue preparation

At day 3 (P12) or day 81 (P90) after HI, animals were anesthetized with isoflurane as described above. Animals were transcardially perfused with 4% paraformaldehyde and decapitated. After post-fixation of the brains in 4% paraformaldehyde overnight, brains were stored in a 30% sucrose/PBS solution for 48 h and sectioned (35 or 70 μm thickness) on a freezing sliding microtome (Leica SM2000R, Buffalo Grove, IL). The brain sections were then cryoprotected in an antifreeze solution for storage at -20°C .

FJ-C staining and quantification

Mounted brain sections (70 μm) were dried on a slide warmer at 50°C for 30 min. The following steps were performed in the dark. Sections were treated with 0.06 % KMnO_4 for 15 min. After a brief rinse in ddH_2O , the sections were stained with 0.001 % FJ-C in 1% acetic acid for 25 min on a shaker. Sections were rinsed 3×1 min in ddH_2O , and followed by air-drying on the slide warmer at 50°C for 8 min. Slides were then rinsed in CitriSolv (Decon Laboratories, King of Prussia, PA) and mounted with DPX mounting medium. FJ-C stained images were taken using a Nikon TE300 epifluorescence microscope equipped with a FITC filter, as described before [19]. In a blind manner, the total number of FJ-C positive cells in the ipsilateral CA1 region of the hippocampus was determined in each slice using Metamorph image analysis software (Molecular Devices, Downingtown, PA). The area covering the entire CA1 region of two brain sections (210 μm apart) per brain was analyzed. A neurological injury score for the ipsilateral cortex CA1 was calculated as follows: 0: no FJ-C positive cells, 1: 1-500, 2: 501-1000, 3: 1001-1500, 4: >1500 FJ-C positive cells, as described before [19]. To create whole brain images, a mosaic of FJ-C stained images was obtained using an epifluorescence microscope (Zeiss Axioplan, 2.5X objective lens,) equipped with a motorized stage and compiled with StereoInvestigator software (MBF Biosciences, Williston, VT).

Double immunofluorescence staining

After rinsing with tris-buffered saline (TBS), brain sections (35 μm) were incubated with TBS^{++} (0.1% Triton X-100 and 3% goat serum in 0.1 M TBS) for 30 min at 37°C . Sections were double-stained with a monoclonal antibody against MAP2 (1:150) and rabbit polyclonal antibody against GFAP (1:100), and monoclonal antibody against MBP (1:50) and rabbit polyclonal antibody against NF (1:160) for 1 h at 37°C and then overnight at 4°C . After washing with TBS (3×10 min), brain sections were incubated for 1 h at 37°C with goat anti-mouse Alexa Fluor 488-conjugated IgG (for MAP2 and MBP, 1:200) and goat anti-rabbit Alexa Fluor 546-conjugated IgG (for GFAP and NF, 1:200). Following rinsing with TBS, sections were incubated in To-Pro-3 Iodide (1:1500 in TBS^{++}) for 15 min and mounted on slides. Slides were imaged with a Leica DMIRE 2 inverted confocal microscope and Leica microscope software (Leica Inc, Buffalo Grove, IL).

Immunoblotting

To investigate the expression of total TrkB and phosphorylated TrkB (p-TrkB) at 72 h (P12) following HI, the contralateral and ipsilateral hippocampi were harvested and pooled from 5 brains. The tissues were homogenized and centrifuged at 2,200 g for 5 min at 4°C . The protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL). The protein samples (100 μg) and pre-stained molecular mass markers in a SDS buffer were electrophoretically separated on 8% SDS gels. The resolved proteins were electrophoretically transferred to a nitrocellulose membrane. After incubation in 5% nonfat dry milk in TBS for 1 h, the blots were probed with the anti-TrkB antibody (1:500) or anti-p-TrkB antibody (1:500) overnight at 4°C . After rinsing with TBS, the blots were incubated

with goat anti-rabbit horseradish peroxidase-conjugated secondary IgG (1:2000) for 1 h. Bound antibodies were visualized using the enhanced chemiluminescence assay.

Accelerating rotarod test

The accelerating rotarod test examined balance and motor coordination, as well as skill learning. The accelerating rotarod test was performed when the HI-vehicle control and 7,8 DHF treated mice reached P30 (21 days post-HI). After mice were placed onto a horizontal rotating rod at 4 rpm (Model ENV-575M, Med associates, St. Alban, CT), the rotarod was accelerated from 4 to 40 rpm over the course of a 5 min trial, as described before [19]. The time the mouse spent on the rotating rod until it fell off, or the latency to the first complete revolution was recorded in a single test. The mouse was then returned to the home cage. Mice were tested for 4 trials per day for 3 consecutive days. The mice rested in their home cages for 20 min - 1 h between each trial. Average time on the rotating rod across days was presented.

Hidden Morris Water Maze (MWM) Test

Spatial learning was assessed by the MWM test at P60 (51 days post-HI) on HI-vehicle control and 7,8 DHF-treated mice. Mice were trained to locate a hidden escape platform in a circular pool full of water, as described previously [19]. The pool was equipped with the clear acrylic hidden escape platform and a Panasonic Digital CCTV (Model WV-BP334) camera and a Piccolo Diligent video capture board plus Noldus Instruments EthoVision XT software (Leesburg, VA). The pool was virtually divided into equal quadrants with four virtual points, west, north, east, and south. The escape platform was placed in the center of the NW quadrant (Q1), 26 cm from the pool wall at the 45° diagonal from center.

Mice were transported from the housing room to the testing room, and were allowed to acclimate to the room for 30 min before training or testing. A brief preliminary training was conducted with each mouse to ensure it could swim and climb onto the platform. Each mouse was trained for 8 trials (2 blocks) per day for 4 days (a total of 32 trials). Between each trial, mice were allowed to rest on the platform for 10-15 sec. Trials lasted 1 min, or until the mouse found the platform. If the mouse did not find the platform, it was placed on the platform for 10-15 sec by the researcher. Latency to reach the platform was automatically tracked and recorded. After trial 32, each animal was given a probe trial, during which the platform was removed from Q1 and each animal was allowed 60 sec to search the platform in the pool. The amount of time that each animal spent in the training quadrant (Q1) and the other quadrants (Q2-4) was recorded.

Diffusion Tensor Magnetic Resonance Imaging (DTI) of *ex vivo* brains

The HI-vehicle control and 7,8 DHF-treated mice at P90 (81 days post-HI) were anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde and decapitated. After post-fixation of the brains in 4% paraformaldehyde overnight, brains were stored in a 30% sucrose/PBS solution for 48 h and rinsed with PBS for 24 h. DTI was then performed on *ex vivo* brain specimens, using a multislice spin echo sequence to acquire 3 reference and 30 non-collinear diffusion weighted image volumes with the following parameters: TE/TR = 22/2000 ms, nex=4, matrix size = 192×192 reconstructed to 256×256, field of view = 30×30 mm, 25 slices, slice thickness = 0.5mm, b-value = 1100 s/mm². Fractional Anisotropy (FA) maps were created using Matlab based software developed in-house. For DTI analysis, binary masks of corpus callosum (CC) were manually drawn on axial slices well within the margins of CC in order to reduce partial volume effects. Masks were then applied to the FA maps, and mean FA values were calculated for the entire volume of either contralateral or ipsilateral CC.

Statistical analysis

Values are expressed as the mean $\pm$ SD or $\pm$ SE. Statistical analysis was performed using an ANOVA (with a Bonferroni post-test) in the case of multiple comparisons (SigmaStat, Systat Software, Point Richmond, CA). For comparisons of mean ipsilateral and contralateral FA values, a paired t-test was used. For behavioral testing, repeated-measures analysis of variance was performed. P-values smaller than or equal to 0.05 were considered statistically significant.

RESULTS

TrkB agonist 7,8 DHF reduced acute neuronal injury in the hippocampus of female mice after perinatal HI

To investigate whether TrkB activity plays a role in neuroprotection after HI, the effect of TrkB agonist 7,8 DHF was examined in both male and female animals 72 h (P12) after HI. Brain injury was assessed using FJ-C staining, a method that detects degenerative cells [21]. As shown in **Figure 1A**, severe hippocampal injury was visible in the ipsilateral hemisphere of the HI-vehicle control brain, as reflected by the ipsilateral hippocampal and cortical atrophy (**arrow**). FJ-C positive cells were detected in the ipsilateral hippocampus in CA1, CA2, and CA3 regions as well as in the cortex (**Figure 1A, arrow**). In contrast, the HI + 7,8 DHF-treated brain exhibited preserved ipsilateral hippocampal morphology (**Figure 1A, arrowhead**) and fewer FJ-C positive cells in the pyramidal neuronal layers of CA1, CA2, and CA3 (**Figure 1A, arrowhead**). **Figure 1B** shows summarized data on the neurological injury scoring in the HI-vehicle control and the HI + 7,8 DHF-treated groups. The neurological injury score in the HI + 7,8 DHF-treated male mice was not statistically significant from the male HI controls. Interestingly, the neurological injury scoring in the ipsilateral hippocampus of the HI + 7,8 DHF-treated female animals was significantly less than the HI-vehicle control female mice (**Figure 1B**). The HI + 7,8 DHF-mediated neuroprotection remained significant in the mixed gender groups. This initial study implies that female mice may respond more effectively to 7,8 DHF after HI.

To address this issue, we investigated whether 7,8 DHF treatment triggers any preferential activation of TrkB receptors in the hippocampus of male and female mice at 72 h following perinatal HI. Hippocampi of the HI-vehicle control and the 7,8 DHF-treated mice were probed for p-TrkB and total-TrkB by immunoblotting assays. **Figure 1C** shows that p-TrkB expression was low in the contralateral hippocampus tissues of both male and female mice. HI resulted in an increase of p-TrkB expression in the ipsilateral hippocampus. Interestingly, the HI + 7,8 DHF-treated female mice exhibited more pronounced elevation in p-TrkB expression in the ipsilateral hippocampi (**Figure 1C**). As shown in **Figure 1C**, 7,8 DHF treatment resulted in a small but significant elevation of p-TrkB/t-TrkB expression in the contralateral male hippocampus ($p < 0.05$). A similar trend occurred in the female contralateral hippocampus, but with larger variation (0.46 ± 0.45 non-treated versus 2.05 ± 1.35 treated, $p > 0.05$).

TrkB agonist 7,8 DHF significantly attenuated acute loss of MAP2 expression and reactive astrocytosis formation after perinatal HI

We further investigated hippocampal neuronal degeneration and gliosis by examining changes in the immunoreactivity of MAP2 and GFAP at 72 h (P12). The contralateral hippocampus of the HI-vehicle control (male) and HI + 7,8 DHF-treated (male) mice exhibited abundant MAP2 expression (**Figure 2a and 2a'**). The hippocampal regions, such as stratum pyramidale (SP) and stratum radiatum (SR), show the well-organized structure of cell body and dendrites (**Figure 2a and 2a', arrow**). The ipsilateral hippocampus of the HI-vehicle control brain shows an apparent loss of pyramidal neurons and MAP2 expression,

accompanied by disorganization of the SR in the ipsilateral hippocampus (**Figure 2b, arrowhead**). In the HI + 7,8 DHF-treated brain, the loss of MAP2 expression in the ipsilateral hippocampus was attenuated (**Figure 2b', arrow**). Structural organization of dendrites was preserved in the SR of the ipsilateral hippocampus of the HI + 7,8 DHF-treated brains (**Figure 2b', arrow**). Similarly, the 7,8 DHF-treated brains displayed less GFAP expression after HI (**Figure 2d', open arrowhead**), in contrast to the increased GFAP expression in the HI-vehicle control brains (**Figure 2d, open arrowhead**).

TrkB agonist 7,8 DHF improved motor learning of mice at P30 following perinatal HI

To examine whether the 7,8 DHF-mediated neuroprotection in the neonatal mice is long lasting we evaluated motor learning in the HI-vehicle control and the HI + 7,8 DHF-treated animals when they reached P30 (21 days post HI). As shown in **Figure 3A**, the time spent on the accelerating rotarod increased over the 3 day trial period in the naive combined (female and male mice) control animals demonstrating motor learning in these animals ($p < 0.05$). Interestingly, the combined HI + 7,8 DHF-treated mice also showed improved performance on the accelerating rotarod over the 3 days in a manner similar to the naive controls (**Figure 3A**, $p < 0.05$). In contrast, the combined HI-vehicle control animals failed to show motor learning between day 1-3 (**Figure 3A**, $p > 0.05$). Additional information was revealed when the performance of the female and male mice on the rotarod was compared. Both female (**Figure 3B**) and male HI + 7,8 DHF-treated mice (**Figure 3C**) increased their time on the rotarod significantly by trial day 3 ($p < 0.05$). However, the male HI + 7,8 DHF-treated mice showed an improvement at trial day 2 (**Figure 3C**, $p < 0.05$). These data suggest that 7,8 DHF is effective in improving motor learning in both male and female mice after HI.

TrkB agonist 7,8 DHF improved spatial learning of mice at P60 following perinatal HI

We further investigated whether the HI + 7,8 DHF-treated mice show different spatial learning and memory than the HI-vehicle control mice. The hidden MWM test probe trial was performed in these mice when they reached P60 (51 days after HI). The naive control animals (combined male and female data) show spatial memory and learning in the probe trial, as evidenced by the increased time spent exploring the training quadrant (Q1) relative to the other quadrants (**Figure 4A**, $p < 0.05$). However, the combined HI-vehicle control animals demonstrated a decrease in spatial learning and memory deficits because they did not exhibit significant preference for the training quadrant (Q1) in the probe trial test. In contrast, in the combined HI + 7,8 DHF-treated animals a clear preference in exploring the training quadrant (Q1) was preserved. Gender analysis of the data show that 7,8 DHF is clearly more effective in improving spatial memory and learning in the female mice than the male mice (**Figure 4B**), which is consistent with its neuroprotective effects in the female mice. We further evaluated MAP2 expression and reactive astrogliosis formation in female mice at P90 (81 days following HI). Compared to the loss of MAP2 and increased expression of GFAP seen in HI-vehicle control brains (**Figure 4C**, white and yellow arrowhead), TrkB agonist 7,8 DHF attenuated both loss of MAP2 expression (**Figure 4C**, white arrow), and gliosis (**Figure 4C**, yellow arrow). Taken together, TrkB agonist 7,8 DHF has sustained neuroprotective effects against neurodegeneration at P90, which may play an important role in reducing neurological deficits and improving spatial memory and learning of these animals.

TrkB agonist 7,8 DHF attenuated white matter damage of mice at P90 after HI

We further investigated effects of 7,8 DHF on reducing the white matter injury in these animals and its potential contribution to the neurobehavioral outcome. White matter injury was first quantified using DTI and FA mapping at P90 (81 days after HI). The HI-vehicle

brain showed a decrease in FA in the ipsilateral CC (**Figure 5A, B**) and a moderate decrease in FA in the ipsilateral internal capsule (**Figure 5A**). The HI + 7,8 DHF-treated brain demonstrated preserved FA in the ipsilateral CC (**Figure 5A, B**). Additional gender analysis of the data revealed that the female HI-vehicle control mice had significant FA reductions in the ipsilateral CC ($p < 0.001$, **Figure 5C**) while the male HI-vehicle control mice only demonstrated a trend towards decreased FA in the ipsilateral CC (**Figure 5D** $p = 0.075$). Interestingly, no significant reduction of FA was found in the HI + 7,8 DHF-treated mice (male or female), suggesting 7,8 DHF preserves ipsilateral corpus collosum FA after HI. To confirm this finding, we evaluated the expression of MBP and NF in the ipsilateral and contralateral CC of the same brains by immunohistochemical staining at P90. As shown in **Figure 5E**, the ipsilateral CC of the HI-vehicle control brain (female) expressed less MBP as well as less NF immunostaining (arrowhead). Interestingly, such a reduction of MBP and NF immunoreactive signals was absent in the HI + 7,8 DHF-treated brain (**Figure 5F**, arrow and yellow arrowhead). These findings clearly suggest that the HI + 7,8 DHF-treated mice also exhibit less sustained white matter injury after HI, which may contribute to improved neurological function in these mice.

DISCUSSION

Sustained neuroprotection achieved by administration of TrkB agonist 7,8 DHF after perinatal HI

In this study, we found that administration of TrkB receptor agonist 7,8 DHF in P9 mice after HI resulted in an initial decrease in hippocampal neurodegeneration, MAP2 loss, and reactive astrogliosis formation at 3 days after HI. Our findings are complimentary to the report that ICV injection of recombinant human BDNF led to less hippocampal injury at 7 days after HI [9]. Early neuroprotective effects of 7,8 DHF has been reported in adult mice after transient MCAO. Administration of 7,8 DHF (5 mg/kg) in adult mice at 2 h prior to the induction of MCAO reduced infarct volume by 50% and apoptosis at 48 h after MCAO [17].

The significance of our findings lies on the long lasting efficacy of the TrkB receptor agonist 7,8 DHF. Administration of 7,8 DHF in P9 mice during 10 min - 6 days after HI is able to provide sustained neuroprotection in both hippocampus and white matter when these mice reached P60-P90. Moreover, these mice exhibited improved accelerating rotarod motor learning and spatial learning and memory at 1-3 months of age after the perinatal HI. In order to identify the effects of delayed therapy that starts later than 10 min after HI, further studies are warranted. The cellular and molecular mechanisms underlying the 7,8 DHF-mediated effects are not well known. BDNF injections (prior to perinatal HI induction) have neuroprotective effects on spatial memory retention in rats assessed by the MWM test at P20-30 [10]. In addition to its neurotropic effects, BDNF regulates the synaptic plasticity in various brain regions including hippocampus [22] which involves BDNF-mediated activation of PI3-K and MAPK signaling pathways [22]. Hippocampus-dependent learning and neurogenesis are directly related to each other [23] and hippocampal neurogenesis plays a specific role in hippocampus-dependent learning [24]. In the light of these findings, 7,8 DHF could improve neurological functions of HI-treated mice via neuroprotection or altering neurogenesis and plasticity in hippocampus, striatum, and cortex and/or white matter. In adult animal models of MCAO, excitotoxicity and Parkinson disease, therapeutic benefits were achieved by using 7,8 DHF in male mice [17]. However, both the therapeutic window and the possible mechanisms involved after 7,8-DHF treatment need to be investigated in future studies in neonatal mice after perinatal HI. Hence, the potential therapeutic application of 7,8 DHF after perinatal HI depends on the results of the future studies in small and large animal models in different genders.

Administration of TrkB agonist 7,8 DHF decreases long-term white matter degeneration after perinatal HI

We have investigated the effects of 7,8 DHF administration on the white matter injury in mice when they reached P90 (81 days after perinatal HI). When the FA values of the contralateral and ipsilateral CC were assessed using DTI and FA mapping, we found that HI resulted in significant decrease in the ipsilateral CC. In contrast, such a change was not detected in the 7,8 DHF-treated mice. We also obtained evidence for 7,8 DHF-mediated protection against white matter injury in the ipsilateral CC of P90 mice when assessed by double immunofluorescence staining of NF and MBP expressions. These results suggest that 7,8 DHF has sustained protective effects in white matter against HI injury. It has been reported that in ibotenate-induced neonatal excitotoxic brain lesion model, ICV administration of BDNF reduced white matter lesions by 60% in P5 mice [25]. A recent *in vitro* study showed that BDNF promotes oligodendrocyte myelination through ERK-mediated signaling transduction which is activated in mature oligodendrocytes of CC [26]. In light of these findings, it is possible that 7,8 DHF could protect against HI-mediated white matter injury via promoting oligodendrocyte myelination.

Differential effects of TrkB agonist 7,8 DHF in female and male mice after perinatal HI

In this study, we observed that treatment with TrkB receptor agonist 7,8 DHF protects hippocampal pyramidal neurons and improves long-term spatial learning significantly more in females than males after perinatal HI. In P12 mice, neurological injury scores were significantly lower in the HI + 7,8 DHF-treated females, compared to the HI-vehicle treated females, while the 7,8 DHF-mediated effects in the male mice did not reach statistical significance. Long-term spatial learning assessed with MWM test probe trial showed that the HI+7,8 DHF-treated females performed significantly better than control females at P90. Arteni and colleagues reported that perinatal HI does not result in any gender differences in the MWM probe trial in P90 rats [27] that is similar to our finding of no significant gender differences in the HI-vehicle controls. To our knowledge, the current study is the first to show a gender difference in hippocampal learning in response to 7,8 DHF administration after perinatal HI. In addition, as shown in Figure 3, we did not see any differences in striatum-dependent motor learning between the genders, males seemed to learn as well as the females. Therefore, the pooled data from both genders were displayed. However, more detailed biochemical studies about the TrkB activation in male and female striatum and hippocampus should be done in future studies. These future studies may reveal the differential changes of the TrkB signaling pathway in male and female brains at specific tissue locations and in specific cell types.

The mechanisms responsible for these gender differences are not apparent. Phosphorylation of TrkB is associated with recruitment of pro-survival signaling pathways that lead to long-term neuroprotection and increased learning. Three subtypes of the TrkB receptors have been identified, a full-length possessing a tyrosine kinase domain and two variants of truncated TrkB receptor (TrkB-T1 and TRKB-T2) lacking this domain [28]. Fryer and colleagues showed that TrkB receptors are detected in the earliest stages of embryonic life, and increase rapidly after birth. Phosphorylation of the tyrosine domain results in downstream activation of the MAPK/ERK, PI3K and PLC γ signaling pathways. While the pro-survival effect of ERK signaling is controversial, it seems clear the downstream effectors of PI3K (Akt) and PLC γ (TRPC receptors) support survival of neurons [6]. TrkB receptors play a major role in neurogenesis through the ligands BDNF and NT-4/5, and as such are extremely important in the early development of immature brains [8].

Immediately after the HI injury, there appears to be a slight increase in TrkB expression, indicating that TrkB receptors may play a role in early protection of the neurons with HI

injury [8]. However, this initial increase is only a temporary response. TrkB receptors themselves are actually quite vulnerable to HI injuries. In the infarct regions following HI injury, there is a permanent reduction of TrkB receptors after the injury [29]. In our study, HI treatment alone induced a small elevation of p-TrkB expression in both male and female mice. However, treatment of TrkB agonist 7,8 DHF significantly increased the levels of p-TrkB in the ipsilateral hippocampus of both male and female mice. These results suggest that the 7,8 DHF-mediated neuroprotection and improved motor and spatial learning and memory could be via activation of TrkB receptor and the subsequent cascade of TrkB receptor-mediated signaling pathways. For instance, BDNF plays a fundamental role in neuronal plasticity through modulation of neurotransmitter release and synaptic proteins secondary to MAPK/ERK activation [6]. In addition, the function of postsynaptic glutamate receptors is regulated by BDNF via IP₃ receptor and TRPC-dependent Ca²⁺ signaling [6]. It remains unknown whether 7,8-DHF-mediated neuroprotection is via the classical TrkB down-streaming pathways (MAPK/ERK, PI3K and PLC γ signaling pathways) in vivo after neonatal HI. Hippocampal neurons in culture were shown to be protected from apoptosis after phosphorylation of the TrkB by 7,8 DHF administration. In these neurons, 7,8 DHF stimulated Akt and Erk phosphorylation [17]. In the current study, we did observe significant up-regulation of p-TrkB expression in female mice following 7,8-DHF treatment. Further studies are needed to investigate possible differential changes of the downstream signaling in male and female mice after TrkB activation after HI.

While gender has been shown to have no significant influence on TrkB expression, it has been proposed that estrogen enhances BDNF functionality via the TrkB receptors [30, 31]. However, a recent study showed that no significant correlations could be made between the serum estradiol and BDNF-TrkB expression during adolescence in female mice [32]. Conversely, the same study showed that TrkB phosphorylation in the brains of developing adolescent female mice correlated with uterine weights and that TrkB phosphorylation decreased with ovariectomy suggesting that other uterine hormones may be involved with regulation of TrkB signaling. Taken together, future studies are needed to investigate the relationship between female and male endocrinology and the profound 7,8 DHF-mediated protection in female mice after HI. Alternatively, differential changes of TrkB receptors and its signaling pathways in male and female brains, in different cell types (neurons, glia, etc), or in different brain regions, could contribute to the differential effects of 7,8 DHF after HI.

CONCLUSION

In this study, we subjected neonatal mice to HI and examined the neuroprotective effects of a TrkB agonist 7,8 DHF after HI. We report here that 7,8 DHF treatment increased phosphorylation of TrkB receptor in the ipsilateral hippocampus, decreased hippocampal neuronal degeneration, astrogliosis, as well as white matter damage at P12-P90. The 7,8 DHF-treated mice exhibited improved motor learning and long-term spatial learning and memory after HI, with a more profound response in female than in male mice. Therefore, this study strongly suggests that TrkB receptors and its signaling transduction pathways are important in protection against immature brain injury after HI. There are potential differential responses of TrkB receptor pathways in male and female brains after HI.

Acknowledgments

University of Wisconsin Department of Pediatrics Research & Development Grant (Cengiz P), 9U54TR000021 from the Clinical and Translational Science Award program of NCATS (Cengiz P & Ferrazzano P), NIH grants RO1NS38118 and RO1NS48216 (Sun D), NIH grant NIH RO1 DC010204 (Ye K) and NIH P30 HD03352 (Waisman Center).

Abbreviations

HI	Hypoxia ischemia
P	postnatal day
BDNF	brain-derived neurotropic factor
TrkB	tyrosine kinase B receptor
p-TrkB	phosphorylated TrkB
7,8 DHF	7,8 Dihydroxyflavone
MAP2	microtubule associated protein 2
NF	neurofilament
MBP	myelin basic protein
GFAP	glial fibrillary acidic protein
FJ-C	Fluoro-Jade C
TBS	tris-buffered saline
PBS	phosphate buffered saline
SP	stratum pyramidale
SR	stratum radiatum

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Figure 1. A,B. Evaluation of hippocampal neuronal damage after HI with FJ-C staining
FJ-C staining was performed to assess HI-mediated brain damage in vehicle-control and 7,8 DHF-treated animals at 72 h following HI. The 7,8 DHF-treated animals received an initial dose of 7,8 DHF (5 mg/kg, ip) 10 min after HI and subsequently at 24 and 48 h post HI. The vehicle control animals received an equal volume of PBS at the same time points. **A.** FJ-C staining of whole brain sections (**Upper panels**). Enlargements of dashed boxes containing ipsilateral hippocampus are shown in **Lower panels**. **Arrow:** Severe neuronal damage in hippocampus and cortex. **Arrowhead:** Reduced neuronal damage in hippocampus. **B.** Summary of neurological damage scores based on FJ-C staining. Data are mean $\pm$ S.D. n=5 male and n = 6 female. A score was given as: 0: no FJ-C positive cells, 1: 1-500, 2:

501-1000, 3: 1001-1500, 4: >1500 FJ-C positive cells. * $p < 0.05$ vs. vehicle control. **C.** 7,8 DHF-induced increase in p-TrkB expression in the ipsilateral hippocampus of female mice. Pooled contralateral and ipsilateral hippocampi in male and female mice were probed for total TrkB (t-TrkB) and phosphorylated TrkB (p-TrkB) proteins at 72 h after HI. **Upper panel:** Representative blots of t-TrkB and p-TrkB of male and female hippocampi with or without 7,8 DHF treatment 72 hr following HI. **Lower panel:** Summary figure of the p-TrkB/t-TrkB ratio in the hippocampus 72 hr following HI. The ratio is normalized to the value for the contralateral hippocampus in untreated males. $n = 3$, * $p < 0.05$ vs. ipsilateral female untreated hippocampus; # $p < 0.05$ vs ipsilateral male treated hippocampus.

Figure 2. Changes in MAP2 and GFAP immunoreactivity in the ipsilateral hippocampus following HI

Changes of MAP2 and GFAP expression in HI control (male) and HI + 7,8 DHF (male) in the ipsilateral hippocampus mouse brain were examined at 72 h. Specific hippocampal regions were indicated, SP: stratum pyramidale; SR: stratum radiatum. **a, b, a', b'**: MAP2 (green) immunolabeling in the CA1 regions. **Arrow**: undamaged neuronal projections. **Arrowhead**: damaged neuronal projections. **c, d, c', d'**: GFAP (red) immunolabeling in the CA1 regions. **Open Arrowhead**: GFAP⁺ reactive astrocytes. **e, f, e', f'**: ToPro-3 Iodide (blue) nuclear staining. **g, h, g', h'**: overlay images. Images were representative from n = 11 in each group.

Figure 3. 7,8 DHF improved the outcome in rotarod motor learning test after HI

The average time of male and female mice spent on the rotarod were recorded for four trials per day for 3 consecutive days at P40 after HI. **A.** Results in control naïve group, the HI-vehicle control group, and the HI 7,8 DHF-treated group. **B.** Effects of 7,8 DHF on the accelerating rotarod motor learning test in female mice. **C.** Effects of 7,8 DHF on the accelerating rotarod motor learning test in male mice. Data are mean $\pm$ SEM (n = 4-5 male, n = 4 female). * p < 0.05 vs. day 1 in each group.

Figure 4. A,B. The Hidden MWM test and hippocampal MAP2 and GFAP immunoreactivity
 The platform latency on the hidden Water Maze was analyzed in the naïve control, HI-vehicle control and HI + 7,8 DHF animals at P60 after HI. **A.** The Hidden MWM test probe trial results in three groups. Q1 was target quadrant. **B.** The comparison of the results of the Hidden MWM test probe trial in the female mice (**left panel**) and the male mice (**right panel**). Data are mean $\pm$ SEM ($n = 7$ male, $n = 7$ female). * $p < 0.05$ vs Q2-Q4 multivariate analysis. **C.** Overlay images of Changes of MAP2 and GFAP in the ipsilateral hippocampus in the CA1 regions at P90 in mice subjected to HI or HI + 7,8 DFA. **Arrow:** Undamaged neuronal projections. **Arrowhead:** Damaged neuronal projections. **Yellow Arrowhead:**

GFAP⁺ reactive astrocytes. Images were taken at 40x magnification and were representative from n = 6 in each group.

Figure 5. Effects of 7,8 DHF on fractional anisotropy and MBP/NF immunoreactivity in the corpus callosum following HI

Diffusion tensor MRI was performed on *ex-vivo* tissues of the HI-vehicle control and the HI + 7,8 DHF mice at P90 (81 days after HI). **A.** Representative fractional anisotropy (FA) map of the HI-vehicle-control (female) and the HI+7,8 DHF-treated brains (female). **Dotted line:** delineates ROI for the corpus callosum (CC). **Arrow:** Reduced FA in ipsilateral CC. **Arrowhead:** Reduced FA in internal capsule. **Yellow Arrow:** Preserved FA in 7,8 DHF ipsilateral CC. **B.** Summary of the ipsilateral and contralateral CC FA values in the combined male and female mice determined from FA maps. **C-D.** Changes of FA values in female (**C**) and male (**D**) mice. Data are mean $\pm$ SEM ($n = 7$ male, $n = 6-7$ female). * $p < 0.05$ vs. contralateral. **E.** Overlay images of MBP and NF immunoreactivity in the contralateral (CL) and ipsilateral (IL) CC were examined in the same *ex-vivo* brains imaged via DTI. **Arrow:** abundant MBP and NF expression in contralateral CC. **Arrowhead:** reduced MBP and NF expression in ipsilateral CC of HI mice. **Yellow Arrow:** preserved MBP and NF expression in ipsilateral CC of HI + DHF mice. Scale Bar: 25 μ m. Images were taken at 40x magnification and were representative (female brains) from $n = 4$ in each group.