Sex differences in Hippocampal Memory and Learning following Neonatal Brain Injury: Is There a Role for Estrogen Receptor- α ?

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Keywords

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Abstract

Neonatal encephalopathy due to hypoxia-ischemia (HI) leads to severe, life-long morbidities in thousands of neonates born in the USA and worldwide each year. Varying capacities of long-term episodic memory, verbal working memory, and learning can present without cerebral palsy and have been associated with the severity of neonatal encephalopathy sustained at birth. Among children who sustain a moderate degree of HI at birth, girls have larger hippocampal volumes compared to boys. Clinical studies indicate that female neonatal brains are more resistant to the effects of neonatal HI, resulting in better long-term cognitive outcomes compared to males with comparable brain injury. Our most recent mechanistic studies have addressed the origins and cellular basis of sex differences in hippocampal neuroprotection following neonatal HI-related brain injury and implicate estrogen receptor- α (ER α) in the neurotrophin receptor-mediated hippocampal neuroprotection in female mice. This review

summarizes the recent findings on ER α -dependent, neurotrophin-mediated hippocampal neuroprotection and weighs the evidence that this mechanism plays an important role in preservation of long-term memory and learning following HI in females.

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Introduction

Neonatal encephalopathy due to hypoxia-ischemia (HI) leads to severe, life-long morbidities in thousands of neonates born in the US and worldwide each year [1, 2]. The severe morbidities following neonatal encephalopathy include but are not limited to cerebral palsy (CP), epilepsy, cognitive impairment, and memory deficits [2]. Interestingly, cognitive impairments can take place in the absence of motor deficits seen in patients with cerebral palsy [3]. In fact, varying capacities of long-term episodic memory, verbal working memory, and learning can present without CP and have been associated with the severity of neonatal encephalopathy sustained at birth [4].

Dila Zafer and Nur Aycan contributed equally to this work.

Hippocampal Impairment following HI in Human Neonates

Several studies in humans have implicated hippocampal injury in episodic memory deficits following neonatal HI. In fact, bilateral severe hippocampal atrophy determined by quantitative MRI imaging studies is a common finding among survivors of neonatal HI [5]. Hippocampal injury in school age children results in visuospatial memory deficits, leading to difficulties in acquiring new information, learning at a slower pace, and a need for repeated exposure to new information in school age children [6]. Interestingly, in a cohort of children who sustained a moderate degree of HI at birth and underwent MRI at 9–10 years of life, girls had a greater hippocampal volume compared to boys [3]. In the same study, although the intelligence quotient was not found to be different between the girls and the boys, visuospatial long-term memory deficits correlated with hippocampal volumes, indicating better outcomes in girls [3].

Is Hippocampal Neuroprotection following HI Greater in Female versus Male Human Neonates?

Clinical studies indicate that female neonatal brains are more resistant to the effects of neonatal HI, resulting in better long-term cognitive outcomes as compared to males with comparable brain injury [7–14]. The relative resistance of the neonatal female brain to HI suggests that some sex-specific mechanisms afford females greater neuroprotection and/or facilitate recovery [7]. The molecular and cellular mechanisms that mediate female-biased hippocampal neuroprotection in neonates are not well understood, representing a critical knowledge gap that currently limits the development of treatment strategies that could be based on amplification of these neuroprotective responses. Our most recent mechanistic studies aim to address the origins and cellular basis of sex differences in hippocampal neuroprotection following neonatal HI-related brain injury and implicate estrogen receptor- α (ER α) in neurotrophin receptor-mediated hippocampal neuroprotection in females.

This review summarizes the recent findings on ER α -dependent, neurotrophin-mediated hippocampal neuroprotection and weighs the evidence that this mechanism plays an important role in preserving long-term memory and learning following HI in females. Elucidation of the mechanisms underlying female-specific neuroprotective responses that may be lacking in male brains may provide the foundational insights necessary for future development of novel sex-specific therapies targeting the hippocampus [15].

Neurotrophin Receptors Mediate Female-Specific Hippocampal Neuroprotection following HI

Neurotrophins are growth factors that regulate the development and maintenance of the central nervous system. Much attention has been focused on putative roles of neurotrophins and their receptors in neuroprotection and recovery from injury following adult stroke and neonatal HI [11, 12, 16]. The most extensively studied endogenous neurotrophin, i.e., brain-derived neurotrophic factor (BDNF), has been shown to bind and induce phosphorylation of tyrosine kinase B receptors (TrkB) in hippocampal cells, reduce the infarct volume by 55% [17], and improve visuospatial learning following neonatal HI [18]. While these endogenous neurotrophins contribute to hippocampal neuroprotection after ischemia, the clinical utility of neurotrophin therapies has been limited by their susceptibility to enzymatic degradation and poor bioavailability compounded by concerns that their nonspecific affinity for other receptors such as the p75 receptor may exacerbate neuronal damage [19, 20]. Accordingly, clinical trials of neurotrophins, including BDNF, as therapy for various neurological diseases have been universally disappointing [21, 22]. Efforts have therefore been made to find nonpeptide, small molecule ligands capable of specifically activating TrkB signaling with a high specificity and potency and improved bioavailability that might serve as an alternative to BDNF [23]. Recently, a selective and potent TrkB agonist, i.e., 7,8-dihydroxyflavone (7,8-DHF), was identified to cross the blood-brain barrier with a high bioavailability and phosphorylate TrkB [16, 24, 25]. Early neuroprotective effects of 7,8-DHF as a TrkB agonist have been reported in adult male mice after transient middle cerebral artery occlusion (MCAO) and in hippocampal neurons [16]. We have similarly found that 7,8-DHF exerts a profound early and late neuroprotective effect in neonatal mice following *in vivo* HI by phosphorylating the full-length TrkB [11]. However, this protection was confined to female mice only, while male mice derived no benefit from 7,8-DHF treatment [11, 12]. Thus, 7,8-DHF fully mimics the effects of BDNF as a selective and potent TrkB agonist and reveals a sex difference in neurotrophin receptor-mediated neuroprotection following neonatal HI. This neuroprotection appears to be due to the significantly increased phosphorylation of TrkB we found in the hippocampus of 7,8-DHF-treated female mice [11, 12].

Female-Specific Hippocampal Neuroprotection following Neonatal HI Requires ER α

We have contributed to a growing body of literature demonstrating the importance of estrogen receptor signal-

ing in response to cerebral ischemia [12, 26–28]. The role of estrogen receptors such as ER α and ER β in the mammalian hippocampus has been studied in neonatal and adult rodent models of cerebral ischemia [12, 29, 30]. Following focal ischemia in adult rodents, the expression of ER α and ER β showed different patterns, with upregulation of ER α expression early after injury followed by a later increase in ER β expression [29]. In a study which established the neuroprotective effects of estradiol after focal ischemia, deletion of ER α abolished the protective actions of estradiol in all regions of the brain, whereas ablation of ER β had no effect on neuroprotection by estradiol. Similarly, neuroprotective effects of estradiol in ovariectomized adult female rodents following MCAO have been found to be dependent on ER α but not on ER β [29], and selective deletion of neuronal ER α prevents estradiol-dependent neuroprotection after MCAO. Thus, ER α is critical in mediating the protective effects of physiologic levels of estradiol following cerebral ischemia in adult female rodents [28, 30]. Additionally, ER α expression is differentially increased in adult females compared to adult males in ischemic cortical brain regions after MCAO [31]. Interestingly, following neonatal HI, the expression of ER α mRNA and protein but not ER β is increased in the female hippocampus compared to corresponding sham mice [12].

Both ER α and ER β have been shown to play an important role in hippocampal-dependent memory and learning [32]. However, the regional distribution and time course of expression differ between ER α and ER β depending on the age at injury (neonatal, adult, or postmenopausal), suggesting that they have different functions under different physiological and pathological conditions. For instance, periodic treatment of ovariectomized young and senescent female rodents with an ER β agonist following MCAO decreased blood-brain barrier breakdown and cell death, resulting in improved hippocampal-dependent spatial memory and learning [33–35]. In contrast, following neonatal HI in mice, the expression of hippocampal ER α , but not ER β , increases in the female hippocampus and it is associated with decreased apoptosis [12]. This region-specific sexually differential ER α upregulation was not observed in the diencephalon and forebrain of neonatal rats 4 days following injury [36]. Thus, attention must be paid to the region-, age-, sex- and model-specific mechanisms by which ER α activation confers hippocampal neuroprotection following cerebral ischemia.

Estradiol is one of the most commonly studied ligands of the ER in the hippocampus, and measurements of estradiol content have not revealed any sex differences over the developmental life span of a rodent [37]. Despite the

absence of sex differences during normal brain development, recent studies conducted in zebra finch have implicated estradiol synthesized in reactive astrocytes as the locally produced ligand for estrogen receptors following brain injury [38, 39]. Sex differences seen following brain injury in zebra finch were attributed to rapid astrocytic aromatase induction leading to estradiol production in female zebra finch brains. This rapid aromatization to estradiol leads to decreased neuronal inflammation and increased neuronal survival in females but not in males [40]. Following neonatal HI, the estradiol content did not increase or differ between the sexes in the diencephalon and forebrain over the course of 4 days following injury [36]. Administration of estradiol as a neuroprotective strategy following neonatal HI has revealed conflicting and inconclusive results [41, 42]. Most recently, exogenous estradiol administration was shown to increase long-term neurogenesis and improve behavioral outcomes in both males and females but did not improve hippocampal atrophy after an ischemic injury [43]. Taken together, the time course and cellular origins of the hippocampal ER response to neonatal HI in male and female neonates need to be studied further.

Female-Specific Responsiveness to TrkB Agonist following Neonatal HI Requires ER α

We recently demonstrated that in vivo HI induces a sex-specific increase in TrkB phosphorylation in the female, but not the male, neonatal hippocampus [11, 12]. Additionally, treatment with the TrkB agonist 7,8-DHF enhances this HI-induced hippocampal TrkB phosphorylation only in females [12] and results in a profound improvement in hippocampal neuronal survival, again only in females [11]. This rapid enhancement in hippocampal TrkB phosphorylation following HI also results in long-term improvement in hippocampus-dependent visuospatial memory in only young adult female mice. We went on to investigate the role of ER α in TrkB-mediated neuroprotection and found that the female-specific TrkB phosphorylation and decreased apoptosis after 7,8-DHF treatment was eliminated in ER α -null mutant mice [12]. Thus, for the first time following neonatal HI, we showed that the female-specific responsiveness to TrkB agonist therapy in hippocampal neuroprotection is dependent upon ER α expression. In particular, important mechanistic questions remain to be answered, including whether nuclear or membrane ER α is responsible for ER-mediated responses to ischemia and whether ER α activation occurs in a ligand-dependent or a ligand-independent way leading to TrkB phosphorylation (Fig. 1).

Fig. 1. Working hypothesis. HI induces upregulation of the ER α in the female hippocampus (1). This upregulation might be influenced by neonatal hormonal environment (T) and/or epigenetic regulation of the ER α gene promoter regions. The membrane-initiated, nongenomic ER α signaling (2) may occur in a ligand-dependent or ligand-independent way (3); phosphorylation of SFK (4) leads to TrkB receptor phosphorylation (5). The addition of TrkB agonist (7,8-dihydroxyflavone) increases the phosphorylation of TrkB, which in turn activates downstream signaling pathways, resulting in decreased apoptosis (6) and neuroprotection (7).

Neurotrophin-Mediated Neuroprotection Is Dependent on ER α : What Are the Intracellular Signaling Pathways for ER α -TrkB Crosstalk?

There is increasing evidence that rapid nongenomic signaling via membrane-localized extranuclear estrogen receptors may contribute to neuroprotection in the brain [44]. A link between ER α expression and TrkB signaling is suggested by the finding that estradiol treatment induces phosphorylation of TrkB in the adult mouse hippocampi, and this response is absent in ER α -null mutant mice [45]. Estradiol rapidly activates membrane-associated ER α coupled to intracellular signaling via Src family kinases (SFK) [46]. SFK are a family of nonreceptor tyrosine kinases that have been implicated in regulation of diverse physiological and pathological processes in the nervous system as well as other organs, and they have been shown to augment the phosphorylation of TrkB receptors [46, 47]. We recently showed that HI and 7,8-DHF treatment induces Src phosphorylation in an ER α -dependent manner [12]. A physical association of estrogen receptors with nerve growth factor receptor and Src has also been described [48–50].

Recent evidence suggests that the interplay of ER α with growth factor receptor pathways influences tumor growth in certain cancers [46]. In these cancers, activation of SFK was proposed to mediate crosstalk between ER α and TrkB [46]. Once activated by ER α , SFK phosphorylate the cytoplasmic tyrosine residues of TrkB^{Y705} leading to activation of cell survival pathways such as mitogen-activated protein kinase (MAPK) pathway [46, 49,

50]. Interestingly, both TrkB and ER α activate similar downstream survival pathways, such as the Ras-MAPK pathways [16, 51, 52]. In cultured cortical neuronal cells, administration of BDNF increased the phosphorylation of TrkB resulting in activation of the MAPK pathway and neuroprotection following in vitro hypoxia [52]. Similarly, in hippocampal neurons following in vitro HI, phosphorylation of TrkB via 7,8-DHF administration, in the absence of BDNF, resulted in activation of the MAPK pathway and decreased apoptosis [16]. Following neonatal HI in rodents, phosphorylation of TrkB via intracerebroventricular administration of BDNF decreased the caspase 3-mediated apoptosis and increased the phosphorylation of MAPK-ERK1/2 in cortical and hippocampal neurons. These beneficial effects of BDNF administration were blocked by inhibition of the MAPK-ERK1/2 pathway but not the PI3-kinase pathway [53]. Similarly, estradiol acts via estrogen receptors, growth factor receptors, and MAPK/ERK signaling to promote hippocampal neuronal survival following global ischemia [54, 55]. Activation of the tyrosine kinase and Src represents one of the initial steps in ER α -mediated cell signaling and neuroprotection [56]. However, the intracellular mechanisms by which ER α signaling influences and activates the membrane-localized TrkB in the neonate hippocampi are currently unknown. It is possible that the ER α signaling in this context occurs mainly through rapid nongenomic, nonclassical signaling via ER α complexed with caveolar proteins at the plasma membrane [57, 58]. We have reported that: (1) BDNF expression is not increased in the

female hippocampus following neonatal HI, effectively ruling out an altered ligand production as a source of neuroprotection [12]; (2) unlike ER α upregulation, ER β is not upregulated [12]; (3) and phosphorylated Src^{Y418} expression is upregulated only in female neonatal mice hippocampi following HI and 7,8-DHF treatment in an ER α -dependent manner [12]. Taken together, these findings support the hypothesis that coupling of non-genomic, membrane-initiated ER α signaling to TrkB phosphorylation in neonatal hippocampi following HI might be mediated by Src, ultimately resulting in increased neuronal survival (Fig. 1). However, the functional interplay of ER α , Src, and TrkB signaling is not completely understood. It remains to be determined whether membrane-localized ER α is integral to TrkB-mediated neuroprotection and whether Src mediates this crosstalk in the hippocampus following HI.

Neonatal HI Induces ER α Expression in Female, but Not Male, Hippocampi: Are Epigenetic Mechanisms Involved?

ER α is equally expressed at baseline in male and female rat [59–61] and sham-operated mouse [12] hippocampi. Recent studies have provided evidence that upregulation of ER α in response to brain injury or an environmental stimulus, such as maternal grooming, may be mediated by epigenetic alterations to the ER α gene promoter [62]. We and others have demonstrated that cerebral ischemia induces upregulation of ER α mRNA and protein expression in the ischemic brain regions of females only [12, 31].

Upregulation of ER α in response to brain injury may be mediated by epigenetic alterations to the ER α gene promoter in a sex-specific manner. This was most clearly demonstrated by the finding that DNA methylation of the ER α promoter is decreased in the cerebral cortex following ischemia only in adult female rodents, likely resulting in upregulation of the ER α [31]. This data suggests that there might be some epigenetic modifications playing a role in upregulation of ER α after a significant stress such as brain injury. To determine whether sex-specific ER α upregulation is mediated by HI-induced DNA demethylation, and hence decreased repression of the ER α promoter, we investigated mRNA expression levels of a panel of DNA methylating and demethylating enzymes in the hippocampus of male and female neonatal mice after HI. We did not observe any sex differences on day 1 or day 3 after HI among this panel of enzymes that are involved in DNA methylation or demethylation including tet1, tet2, dnmt1, dnmt3a, and dnmt3b (un-

publ. data) [63]. Interestingly, only the demethylating DNA repair enzyme, Gadd45b mRNA, and protein expression were significantly upregulated in the female hippocampus compared to the male hippocampus 1 day after HI. This is consistent with the finding from other studies that in P7 rats Gadd45b responds to DNA damage as part of the DNA repair response [64] and that Gadd45b is expressed in sublethally injured neurons in the infarct and peri-infarct regions after MCAO [64]. However, further studies are needed to fill the knowledge gap in the epigenetic mechanisms that may play a role in the upregulation of ER α in neonatal female hippocampi and identify what mechanism is deficient in male mice to improve the neurological outcomes following neonatal HI (Fig. 1).

HI Induces Sex-Dependent ER α Expression and Neuroprotection: Does the Perinatal Hormone Environment Program Subsequent ER α Expression in Response to HI?

Classic studies of sex differences in brain structure and function in rodents have demonstrated that the perinatal hormone environment – characterized by a surge in testosterone (T) in males and the absence of a T surge in females – programs the development of some neuronal circuitries that support sex-specific behaviors and physiological functions in adulthood [65]. The perinatal hormonal environment is characterized by 2 surges of T in male mice in utero at E13 and at birth 0–4 h postnatally, while females demonstrate low T levels throughout the embryonic and perinatal period [66, 67]. While the embryonic T surge is responsible for gonadal development, this male-specific perinatal T surge at birth has been linked to masculinization of neuronal morphology and behavior [13], and many sex differences in response to environmental or somatic stimuli have been shown to be established by the organizational effects of T, or its absence, in the perinatal environment [65]. These sex differences in perinatal hormonal environment have been proposed to underlie the clinical observation that male infants suffer more severe cognitive deficits, language delays, and learning disorders than girls with comparable brain injury [13].

The effects of T following neonatal HI have been studied. T exposure at birth results in worsened neurologic outcomes following subsequent HI in newborn female rodents [13]. These female newborn pups that are exposed to T at birth showed worsening of the brain injury and impaired behavioral outcome after HI, effectively equalizing the brain injury to that seen in males [13]. The

role of the perinatal hormone environment in programming sex-specific ER α expression and hippocampal neuroprotection following HI needs to be further assessed in order to better understand the effect of embryonic and perinatal T exposure in males.

Conclusion

Studies that directly address the origins and cellular basis of sex differences in neuroprotection following neonatal encephalopathy due to HI will help guide further research into sex-specific therapies for developmental brain injury [15]. Discovering the cellular mechanisms by which ER α confers female-specific hippocampal neuro-

protection after HI will provide new information needed to develop novel therapeutic approaches in both sexes. In addition, these findings may have broad application to other important pediatric disorders in which TrkB is known to play an important role such as childhood psychiatric disorders [68–70], neuroblastomas [71], and Rett syndrome [72, 73].

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