Neuroinflammation and Neurosteroidogenesis: Reciprocal Modulation During Injury to the Adult Zebra Finch Brain

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Introduction

Steroids organize, reorganize, and activate the developing, juvenile, and adult CNS and are thus considered critical modulators of brain and behavior throughout the vertebrate lifespan (Gurney and Konishi, 1980; Breedlove and Arnold, 1981; MacLusky and Naftolin, 1981; Arnold and Gorski, 1984; McEwen, 2002; McEwen and Milner, 2017). Estrogens like 17β-estradiol (E2) are known organizers of masculine and feminine sexual behavior (Gurney and Konishi, 1980; MacLusky and Naftolin, 1981; Adkins-Regan and Ascenzi, 1990), although they also activate juvenile and adult behaviors in both sexes. Indeed, the range of physiological and behavioral endpoints affected by E2 has increased considerably and now includes, but is not limited to, the regulation of copulation, aggression, mood, balance, learning, and memory. Included in this list is a more recently discovered role for this steroid in the regulation of neuroplasticity and the preservation of neural circuits.

Influence of E2 on the Injured Brain

We have now learned that, in addition to E2-mediated plasticity of the normal brain, the injured brain is profoundly affected by this steroid. Indeed, premenopausal women have a lower risk of stroke compared with age-matched men (Barrett-Connor and Bush, 1991), and hormone replacement has been reported to decrease the risk of neurotrauma associated with cardiovascular disease (Grady et al., 1992). Interestingly, following traumatic brain injury (TBI), although there is no sex difference in the duration of unconsciousness following injury, the predicted outcome and recovery of females are better than for males (Groswasser et al., 1998). Taken together, these data suggest the possibility that endocrine factors may be responsible for some aspects of protection associated with neurotrauma.

In support, animal studies strongly suggest a role for E2 in neuroprotection and brain injury. Hall and colleagues (1991) reported lower levels of necrosis in females relative to males following experimental ischemia in gerbils, and embolic infarcts in rats (Nagpal et al., 1996). This bias also holds true for mice following medial carotid artery occlusion (Delpy et al., 2005; Brown et al., 2010; Fairbanks et al., 2012; Liu and McCullough, 2012). Further, infarcts from strokelike injuries are larger in metestrus rodents compared with those in estrus (high E2) and are inversely related to circulating E2 levels (Sohrabji and Williams, 2013). Additionally, ovariectomy increases subsequent infarct size relative to sham surgeries, and infarct sizes increase further the longer the animal is deprived of ovarian estrogens (Selvamani and Sohrabji, 2010).

Thus, the data reveal a neuroprotective effect of circulating E2 following brain trauma in multiple species that may involve several cellular mechanisms, including cell turnover. In many vertebrates, E2 is a well-established regulator of adult neurogenesis, neuronal survival, and neuronal death. E2 is an effective protectant across a broad range of neural insults. As many *in vitro* and *in vivo* studies have found, E2 protects neurons against cell damage and death such as that caused by serum deprivation (Green et al., 1996), glutamate (Mize et al., 2003), excitotoxicity (Garcia-Segura et al., 1999a), or mechanical injury (Peterson et al., 2001). Indeed, E2 is neuroprotective in several experimental models, including stroke and multiple sclerosis (Brown et al., 2010), and involves the action of estrogens on apoptotic and inflammatory pathways (Delpy et al., 2005). We are now beginning to learn that the source of this steroid is not limited to the periphery, but also involves an increase in neural synthesis of E2, particularly following damage to the brain.

Induction and consequences of injury-associated aromatase expression in reactive astrocytes

E2 is synthesized in many tissues, including the ovaries, adipose tissue, and placenta (Simpson et al., 2002). The brain also synthesizes E2 via the neuronal expression of aromatase (*E-synthase*) (MacLusky and Naftolin, 1981; Peterson et al., 2004). Neuronal aromatization has been intensely studied in many vertebrates because of its pivotal role in the organization and activation of reproductive behaviors (MacLusky and Naftolin, 1981; Balthazart et al., 1983; Balthazart and Schumacher, 1984; Adkins-Regan and Ascenzi, 1990). However, the previous decade has taught us much about aromatization in nonneuronal cells and the role that glial-derived E2 plays in neuroprotection (Garcia-Segura and McCarthy, 2004; Saldanha et al., 2010, 2013).

In mammals and birds, various forms of neural insult result in a dramatic upregulation of aromatase in reactive astrocytes at the site of damage (Azcoitia et al., 2002; Garcia-Segura and McCarthy, 2004; Saldanha et al., 2010). Specifically, excitotoxic damage to the hippocampus, a stab wound to the cerebral cortex, or a penetrating wound to the entopallium all induce astrocytic aromatase expression in rats and birds (Garcia-Segura et al., 1999b; Peterson et al., 2001, 2004; Azcoitia et al., 2002; Rau et al., 2003; Wynne and Saldanha, 2004; Saldanha et al., 2005; Wynne

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et al., 2008). The upregulation of aromatase (and resultant E2 provision) is neuroprotective, as site-specific aromatase inhibition increases (Wynne and Saldanha 2004) and E2 administration decreases (Saldanha et al., 2005) the extent of damage after mechanical injury and other neural insults (Saldanha et al., 2013). More specifically, injection of the aromatase inhibitor fadrozole results in larger injuries and more apoptosis relative to the vehicle alone (Azcoitia et al., 2002; Wynne and Saldanha 2004; Wynne et al., 2008). In the zebra finch, the inhibitory influence of local aromatization on apoptosis is potent enough to completely mask the wave of secondary degeneration consistently observed in the injured mammalian brain (Benkovic et al., 2006). This wave of secondary degeneration, however, is clearly observable upon aromatase inhibition in the injured songbird brain (Wynne et al., 2008). The influence of induced aromatization on indices of degeneration is similar but not identical in the rodent brain. Aromatase expression is induced in astrocytes following various forms of insult in the rodent brain (Azcoitia et al., 2003; Garcia-Segura and Melcangi 2006; Arevalo et al., 2015). Further, pharmacological or genetic inhibition of aromatase results in greater neuropathy following mechanical brain damage in rodents (Azcoitia et al., 2001). These data suggest that in multiple species, the induction of aromatase is key in controlling brain damage following neural insult.

In contrast, aromatase inhibition with concomitant replacement with E2 dramatically reverses the aforementioned effects in songbirds with corresponding decreases in the size of injury and lower levels of cell death, including apoptosis (Saldanha et al., 2005). In agreement, peripheral or central administration of E2 has been found neuroprotective in rats and mice (Garcia-Segura and McCarthy, 2004). The protective effects of E2 provision also involve mechanisms that may repair damaged tissue, as evidenced by the observation in the songbird that E2 replacement around sites of a penetrating central injury increases cytogenesis and neurogenesis (Walters et al., 2011).

This influence on multiple indices of cell turnover (most if not all of which may preserve and/or rebuild neural circuits) provides a promising target for therapies that seek to limit neurodegeneration and promote recovery following TBI. In fact, understanding the specific insult-dependent signal that is responsible for rapidly inducing aromatase expression and E2 provision may be key to developing such therapies.

The aforementioned studies have laid the foundation for a recent expansion in the literature about the role of sex, steroids, and their mechanism of function following TBI (Gibson et al., 2008; Berry et al., 2009; Herson et al., 2009; Chakrabarti et al., 2015; Brotfain et al., 2016). In general, the data all point to increased resilience following TBI in women compared with men (Ponsford et al., 2008; Yeung et al., 2011), a pattern also reflected in several studies in rodent models (Sarkari et al., 2010; Shahrokhi et al., 2010; Day et al., 2013). Indeed, neuroprotective estrogens during TBI appear to work via the more recently discovered membrane form of the receptor, G-protein coupled estrogen receptor-1 (GPER1), which provides a mechanism for rapid effects of these steroids on various aspects of neuroplasticity (Day et al., 2013; Wang et al., 2017). The neuroprotective effects of E2 are echoed by similar effects of progesterone in rodents (Feeser and Loria, 2011; Stein, 2013), although these patterns are not well supported by more recent clinical trials in humans (Lin et al., 2015; Stein, 2015; Zeng et al., 2015).

Brain injury may induce aromatase expression in astrocytes via alternative transcripts

What is responsible for aromatase expression in reactive astrocytes following neural insult? Our laboratory first decided to take a molecular approach to answering this question by entertaining the hypothesis that the aromatase transcript expressed in astrocytes following brain damage could be a novel transcript variant induced only by factors associated with neurotrauma. The number of genes for aromatase varies across vertebrates. Humans, mice, and zebra finches have one gene that, owing to variance in promotors and/or splice events, is expressed differentially across tissues (Ramachandran et al., 1999). For example, Yague and colleagues (2006) reported at least four different isoforms of exon 1 in humans. Zebrafish, goldfish, and pigs have multiple copies of the *cyp19* (aromatase) gene, and these are also differentially expressed in tissues, including ovary and brain (Robic et al., 2014). Importantly, all these genes, splice variants, and tissue-specific promotors make a single, well-conserved protein product that varies between 50 kD and 55 kD in size.

Given that multiple aromatase isoforms could produce the same protein product, Wynne and colleagues (2008) tested the hypothesis that the single zebra finch gene was alternatively spliced in ovarian follicular cells, neurons, and astrocytes. In the zebra finch, a single aromatase gene at Exon 1 is alternatively spliced and is expressed differentially

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in the brain (exon 1a) and ovary (exon 1b) (Ramachandran et al., 1999). After successfully discriminating between the two known transcripts using PCR, we then used overlapping primers along with 5' and 3' RACE (rapid amplification of cDNA ends) to isolate the entire product of the aromatase transcript specifically upregulated by injury (Wynne et al., 2008). Upon sequencing, this product was found to be exactly the same as the known brain transcript (containing exon 1a). These data suggest that the neural expression of aromatase occurred via the expression of identical transcripts in both neurons and astrocytes.

Brain injury is accompanied by a host of neural responses including, but not limited to, cell death and neuroinflammation. Either (or both) these processes could involve signaling molecules that may also serve as inducers of aromatase in astrocytes. Importantly, the almost invariable coincidence of these processes makes it very difficult to separate them. However, inducing inflammation in the absence of substantial cell death proved to be a more rewarding avenue of pursuit in our search for factors that induce astrocytic aromatase expression.

Inflammation induces aromatase expression

In very general terms, brain damage is characterized by two phases, the first of which results in tissue damage and cell death from the force of injury. The second phase involves inflammatory signals, including increases in cytokines, chemokines, and prostaglandins, that can occur within minutes of injury and last for months (Rothwell and Strijbos, 1995; Ghirnikar et al., 1998; Marciano et al., 2002). Although the initial activation of inflammation is neuroprotective, the chronic activation can lead to increased brain damage via breakage of the blood-brain barrier, production of reactive oxygen species, or the amplification of proinflammatory signaling.

Inflammatory processes themselves may play an inductive role in the expression of aromatase following penetrating brain injury. Major proinflammatory signals, which include cytokines, prostaglandin E2 (PGE2), and NF-κB, have been shown to regulate aromatase expression in the periphery. More specifically, inflammatory signals regulate aromatase in normal and malignant breast tissue (Purohit et al., 1995; Singh et al., 1997; Purohit et al., 2005; Morris et al., 2011). It is hypothesized that cyclooxygenase-2 (COX-2)–derived PGE2 stimulates PKA (protein kinase A) production, which results in *cyp19* transcription and thereby increases in aromatase expression. The proinflammatory cytokine interleukin-6 (IL-6) has been shown to regulate aromatase expression and E2 synthesis within tumors in endometrial cancer cells (Che et al., 2014). IL-6 has also been shown to increase aromatase expression in other forms of cancer, including cervical and non-small-cell lung carcinoma (Irahara et al., 2006; Veerapaneni et al., 2009; Miki et al., 2010).

Although much evidence focuses on the regulation of aromatase by inflammatory signals in the periphery, further evidence suggests that central inflammation is capable of regulating central aromatase expression. In the neonatal rat, administration of PGE2 increases aromatase and E2 content in the developing rat cerebellum, and treatment with the COX inhibitor indomethacin prevents this effect, with dramatic effects on dendritic morphology and neurophysiology (Dean et al., 2012a,b). Thus, local COX activity and consequent PGE2 synthesis can regulate aromatase activity in the developing mammalian brain.

Inflammation also induces glial aromatase expression in brain injury models. An experiment done in our lab found that application of the toxin phytohemagglutinin (PHA) induces glial aromatase expression in the absence of detectable cell death (Duncan and Saldanha, 2011). However, because PHA stimulates multiple components of the inflammatory pathway, including the stimulation of macrophages, T-cells, cytokines, and prostaglandins, the specific signal that induces glial aromatase remained unclear (Phillips et al., 1978; Duncan and Saldanha, 2011). Given the previous data in neonatal rats, we hypothesized that in zebra finches, COX activity may be necessary for the induction of glial aromatase and consequent E2 synthesis following a penetrating brain injury.

The induction of aromatase following brain injury: the influence of sex

To test this hypothesis, we administered indomethacin, a nonspecific COX-1/2 inhibitor, during a penetrating brain injury in adult male and female zebra finches (Pedersen et al., 2017). We found that COX activity is necessary for injury-induced E2 and is detectable in temporally distinct patterns between sexes. First, we measured central PGE2 content at 6 h or 24 h after injury. At both time points, PGE2 was decreased in the hemisphere treated with indomethacin, suggesting that our treatment was effective at both time points. However, the temporal pattern of aromatase induction following brain injury appears to differ between the sexes. More specifically,

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6 h after injury, there is no evidence of injury-induced aromatase expression or a change in local E2 levels in males. However, females at the same time point displayed robust increases in E2. This induction of local E2 is severely curtailed by the administration of indomethacin, suggesting that COX activity is necessary for injury-induced aromatization (Mehos et al., 2015; Pedersen et al., 2017). The effect of indomethacin on aromatase expression and central E2 content is evident in males, however, at 24 h postinjury. Indeed, in males, COX inhibition prevents the increase of aromatase and E2 content following brain injury at this time point. Interestingly, also at this time (and despite lower PGE2 levels), in females the E2 content around injuries injected with indomethacin did not differ from E2 levels around injuries treated with vehicle. These data strongly suggest that aspects of injury-induced inflammatory signaling are in part responsible for the induction of E2 following brain damage. The factors that sustain injury-induced aromatase expression in either sex is unknown.

The temporal difference in the COX-dependent induction of aromatase expression may reflect a basic sex difference in the induction patterns of glial aromatase. Previous reports from our lab found that females induce glial aromatase faster than males following a penetrating brain injury to the entopallium (Saldanha et al., 2013). Females have inductions of glial aromatase as soon as 2 h postinjury, whereas they are not evident in males until 24 h. Interestingly, by 24 h, the sex difference disappears, and both males and females have similar numbers of aromatase-expressing cells around the site of damage. A similar female-biased sex effect occurred following penetrating injury to the zebra finch cerebellum (Mirzatoni et al., 2010). The result of indomethacin preventing the induction of E2 in a temporally distinct, sex-specific manner may be a reflection of a sex difference in the time course of aromatase induction. Current work in our lab is exploring mechanisms underlying this sex difference.

Multiple reports from our lab have found basal and injury-induced sex differences in cytokine expression (Saldanha et al., 2013; Pedersen et al., 2016). For example, following injury to the entopallium, females induced glial aromatase faster than males while having larger increases in IL-1β (Saldanha et al., 2013). It is difficult to dissect the time course of proinflammatory signals, such as cytokines and PGE2, after brain injury. However, these sex differences in the time course of cytokine and PGE2 induction following injury may be important to investigate. This is of special significance given the sex difference between basal and induced inflammatory signals following injury, and given that inflammation seems to regulate aromatase and E2 expression. We have now begun to understand that the inductive role of inflammatory signaling on aromatization appears to be part of a reciprocal relationship as local increases in estrogens are responsible for decreases in chronic neuroinflammation.

Astrocytic aromatization decreases indices of neuroinflammation

Sex steroids can dramatically influence inflammation, and the evidence strongly suggests that E2 can exacerbate or inhibit several indices of inflammation in a diverse set of tissues. Indeed, the chronic inflammatory conditions that accompany several human diseases, including rheumatoid arthritis, osteoporosis, asthma, endometriosis, and obesity, are strongly influenced by E2. However, although E2 exacerbates inflammation in endometriosis and asthma (Bulun et al., 2012; Keselman and Heller, 2015), the data suggest a strong anti-inflammatory role for this steroid in rheumatoid arthritis and osteoporosis (Sapir-Koren and Livshits, 2017). Hypotheses explaining this differential influence across tissues abound and are beyond the scope of this review; however, across many species, there appear to be consistent reports of an anti-inflammatory role for E2 in the acute regulation of several components of the immune cascade.

We have found that the anti-inflammatory effect of E2 extends into the traumatized brain. As mentioned earlier, mechanical damage to the finch brain increases local E2 by about fourfold (Mehos et al., 2015). We queried the role of induced aromatization following brain damage by performing bilateral injuries in adult birds. One hemisphere received the aromatase inhibitor fadrozole, whereas the contralateral hemisphere received vehicle (Pedersen et al., 2016). Twenty-four hours later, we found exaggerated levels of IL1-β and COX-2 transcription in the hemisphere injected with fadrozole relative to vehicle. These data suggest that the inhibition of induced aromatization during brain damage results in a sustained level of inflammation. In agreement, levels of prostaglandin E2 were elevated in the hemisphere injected with fadrozole relative to the vehicle-treated contralateral hemisphere, suggesting that local aromatization may be responsible for the anti-inflammatory effects observed.

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To test the E2 dependency of the effect above, we inflicted bilateral penetrating injuries and injected the aromatase inhibitor fadrozole into adult zebra finches of both sexes. In one hemisphere, however, we concurrently injected E2 to assess the potential local influence of this steroid on multiple indices of inflammation. In the hemisphere injured in the presence of E2, the expression of COX-2 was lower relative to the contralateral side (Pedersen et al., 2016). This expression apparently influenced prostaglandin levels, as hemispheres with E2 also had lower levels of PGE2 (Pedersen et al., 2016). These data strongly support an anti-inflammatory role for E2 during brain injury.

Sex differences in E2 modulation of neuroinflammation following brain injury

Previous studies have revealed a strong interaction between estrogens and the innate immune system. Resident macrophages isolated from female mice are more plentiful and express higher levels of toll-like receptors compared with those in males (Scotland et al., 2011), perhaps suggesting a higher sensitivity of the female immune system. Indeed, tumor necrosis factor alpha (TNF-α) and IL-1β increase in women with low circulating E2 as a result of either natural (Pfeilschifter et al., 2002) or surgical menopause (Pacifici et al., 1991). These sex differences appear to be caused by differences in circulating E2; for example, ovariectomized mice have higher neural cytokine expression following peripheral endotoxin treatment relative to sham controls (Brown et al., 2010), suggesting an anti-inflammatory role for circulating E2. In agreement, indices of inflammation are higher in postmenopausal women and ovariectomized mice compared with premenopausal, age-matched controls and intact animals, respectively. Specifically, the expression and secretion of TNF-α, IL-1β, and IL-6 are higher at times of low circulating E2 relative to controls, as is the expression of their cognate receptors (Pfeilschifter et al., 2002). The present data extend these findings to the brain by demonstrating a role for injury-induced aromatization within the CNS—one that involves a potent inhibition of multiple components of the inflammatory cascade within neural tissue.

There appear to be differences in the anti-inflammatory effects of E2 between the sexes. Upon aromatase inhibition, injury-associated levels of TNF-α and IL-1β are higher in females than in controls, but only TNF-α remains high in males. In partial agreement, replacement with E2 lowers TNF-α in males and IL-1β in females, but not vice versa. These data suggest that the initial portions of the inflammatory cascade may be influenced by aromatization differently between sexes. It is important to point out, however, that regardless of these differences in cytokine expression, downstream inflammatory signaling does not appear to be sex-specific. Indeed, the inhibition of aromatase and E2 replacement exaggerate and mitigate injury-associated COX expression in both sexes (Pedersen et al., 2016).

Previous studies have hypothesized that cytokines may serve different biological functions in men and women (Lynch et al., 1994). Thus, it is likely that E2 manipulation may affect inflammation in a sexually differentiated manner. Experiments that vary the severities of injury and time points of injury need to be explored in order to increase confidence in this interpretation. However, therapies that seek to control injury-associated inflammation may need to be tailored to these important sex differences in the temporal and cytokine-specific pattern of neural changes following various types of insult.

Conclusion

Although much work had focused on the neuroprotective role of glial aromatase and consequent E2 synthesis, the mechanisms regulating this influence were unknown. We have presented a novel relationship between immune and endocrine systems in the brain, which appears to be sexually differentiated. These latent sex differences, however, ultimately achieve the same result: the induction of astrocytic aromatization of E2 and consequent anti-inflammatory effect of E2, via the decrease in PGE2.

This feedback between neuroimmune and neuroendocrine signaling may serve as a unique model of neuroprotection. The release of inflammatory factors following brain injury can exacerbate neurodegeneration (Rothwell and Strijbos, 1995; Ghirnikar et al., 1998; Denes et al., 2010). However, these inflammatory factors have the ability to shift from neurodegenerative to neuroprotective via PGE2-dependant increases in aromatase and E2, which decrease inflammatory signaling. This ability may be important to exploit in a therapeutic context, given that chronic elevation of inflammatory signaling is notable in many disorders, including depression, Alzheimer's disease, Parkinson's disease, stroke, and TBI (Perry, 2004; Turgeon et al., 2006; Perry et al., 2007).

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