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Review article

Brain-derived estrogen and neural function

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ABSTRACT

Although classically known as an endocrine signal produced by the ovary, 17 β -estradiol (E₂) is also a neurosteroid produced in neurons and astrocytes in the brain of many different species. In this review, we provide a comprehensive overview of the localization, regulation, sex differences, and physiological/pathological roles of brain-derived E₂ (BDE₂). Much of what we know regarding the functional roles of BDE₂ has come from studies using specific inhibitors of the E₂ synthesis enzyme, aromatase, as well as the recent development of conditional forebrain neuron-specific and astrocyte-specific aromatase knockout mouse models. The evidence from these studies support a critical role for neuron-derived E₂ (NDE₂) in the regulation of synaptic plasticity, memory, socio-sexual behavior, sexual differentiation, reproduction, injury-induced reactive gliosis, and neuroprotection. Furthermore, we review evidence that astrocyte-derived E₂ (ADE₂) is induced following brain injury/ischemia, and plays a key role in reactive gliosis, neuroprotection, and cognitive preservation. Finally, we conclude by discussing the key controversies and challenges in this area, as well as potential future directions for the field.

1. Introduction

Aromatase is a cytochrome P450 enzyme that drives conversion of androgen precursors into estrogens (Fig. 1) (Blakemore and Naftolin, 2016; Simpson et al., 2002). The aromatase-driven catalysis process involves hydroxylation of androgen precursors using three molecules each of NADPH and oxygen to produce one molecule of estrogen (Ryan, 1959). Aromatase is encoded by a single gene, CYP19, which is located

on the 21.2 region of chromosome 15 in humans (Simpson et al., 2002). This gene is 123 kb in length, and is expressed in many tissues, including the gonads, bone, breast, adipose, vascular tissue, skin, placenta and brain (Stocco, 2012). Tissue-specific transcripts of aromatase are produced from the alternative use of several first exons that are promoter-specific (Fig. 2) (Bulun et al., 2004; Simpson et al., 1993). Splicing of the untranslated first exons into the coding exons 2 through 10 produces multiple different aromatase transcripts; however, all of the

Abbreviations: 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; AD, Alzheimer's disease; ADE₂, astrocyte-derived estradiol/estrogen; AMG, amygdala; APP, amyloid precursor protein; ATD, 1,4,6-androstatriene-3,17-dione; BDE₂, brain-derived estradiol/estrogen; BDNF, brain-derived neurotrophic factor; CaMKII, calcium/calmodulin-dependent protein kinase II; c/EBP, enhanced CCAAT-enhancer binding proteins; CREB, cAMP response element binding protein; DHT, 5 α -dihydrotestosterone; E₂, 17 β -estradiol; EGFP, enhanced green fluorescent protein; EP3/4, E-prostanoid receptor 3/4; ER α , estrogen receptor alpha; ER β , estrogen receptor beta; ERK, extracellular signal regulated kinase; FBN-ARO-KO, forebrain neuronal aromatase knockout; GCI, global cerebral ischemia; GFAP, glial fibrillary acidic protein; GFAP-ARO-KO, astrocyte aromatase knockout; GnIH, gonadotropin inhibitory hormone; GnRH, gonadotropin releasing hormone; HFS, high frequency tetanic stimulation; HPC, hippocampus; HPLC, high performance liquid chromatography; HYP, hypothalamus; IHC, immunohistochemistry; OVX, ovariectomized; LIF, leukemia inhibitory factor; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; LH, luteinizing hormone; LPS, lipopolysaccharide; LTD, long term depression; LTP, long term potentiation; NCM, caudomedial nidopallium; NDE₂, neuron-derived estradiol/estrogen; P45017 α , P450 17 α -hydroxylase; P450scc, P450 side chain cleavage; PEPCK, phosphoenolpyruvate carboxykinase; PET, positron emission tomography; PGE₂, prostaglandin E₂; POA, preoptic area; PSD, post-synaptic density; PSD95, postsynaptic density 95; RORA, retinoic acid-related orphan receptor-alpha; RT-PCR, reverse transcriptase-polymerase chain reaction; STAr, steroidogenic acute regulatory protein; VMN, ventromedial nucleus.

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transcripts code for the same protein. Exon 1.f has classically been considered to be the brain-specific variant. However, ovarian-specific exon PII and adipose-specific exons 1.3 and 1.4 are also expressed in the brain of rodents and humans (Prange-Kiel et al., 2016; Yague et al., 2006). It should be mentioned that teleost fish are unique in that they have two aromatase isoforms, CYP19a which encodes aromatase A, and CYP19b, which encodes aromatase B (Tchoudakova and Callard, 1998). CYP19a is expressed in the gonads, while CYP19b is expressed in both the brain and gonads. Although these two genes are structurally different, they have similar catalytic activities and over 20 different regulatory sites in the promoter, including response elements for sex steroid receptors, and several transcription factors that regulate neurogenesis (Piferrer and Blazquez, 2005).

Estrogens, the product of aromatase activation, are steroid hormones that can act upon estrogen receptors in tissues throughout the body and brain. The most potent and most studied estrogen is 17 β -estradiol (E₂), while the other estrogens, estrone (E₁) and estriol (E₃), are considered weak estrogens. E₂ has been implicated in the regulation of many diverse physiological and pathological processes, including reproduction, sexual differentiation and behavior, cancer biology, bone physiology, synaptic plasticity, cognitive function, anti-inflammatory actions and neuroprotection (Azcoitia et al., 2018; Boon et al., 2010; Brann et al., 2007; Brocca and Garcia-Segura, 2019; Cortez et al., 2010; Dhandapani and Brann, 2003; Emmanuelle et al., 2021; Khan et al., 2013; Kramar et al., 2013; Saldanha, 2020; Vegeto et al., 2008). While the role of gonadal-derived E₂ has been studied extensively, the roles and functions of brain-derived E₂ (BDE₂) has received less attention and has only recently begun to be fully appreciated. Hence, this review will focus on the localization, regulation and functions of BDE₂ in the brain. Much of the work in this area has been conducted in rodents and the songbird. However, where available, we will present and discuss findings in other species including humans and non-human primates. Much of what we know about the roles and functions of BDE₂ in the brain has come from studies using pharmacological aromatase inhibitors (see Fig. 3). However, since both neurons and astrocytes can produce E₂, using such a cell non-specific pharmacological approach provides challenges in determining the specific role of neuron-derived E₂ (NDE₂) versus astrocyte-derived E₂ (ADE₂) in the brain. Whole body global aromatase knockout mice support a role for E₂ in anti-inflammation, synaptic plasticity and cognition, and neuroprotection from neurodegenerative disorders (Simpson et al., 2002). However, these studies are

poorly suited to distinguish the role and specific contributions of brain-derived versus gonadal-derived aromatase/E₂ to these effects. Recent work by our group (Lu et al., 2019, 2020; Wang et al., 2020) using brain cell-specific aromatase knockout animal models has helped address this issue and given important insights on the respective roles and functions of NDE₂ versus ADE₂ in the brain in both physiological and pathological states. We will review this emerging work, as well as discuss existing controversies, and potential future directions for advancement of knowledge in this important area.

2. Aromatase localization in the brain

2.1. Human

Table 1 shows a summary of brain localization of aromatase in various species. A full description of the localization of aromatase for each species is provided below. The first report of aromatase activity in the brain was made by Naftolin and coworkers when they measured conversion of radiolabeled androgen precursors to estrogens using human fetal brain homogenates and demonstrated the human fetal diencephalon and limbic system possess significant aromatase activity (Naftolin et al., 1971). Subsequent studies using RT-PCR of adult human brain samples revealed highest aromatase mRNA expression in the hypothalamus, amygdala, pons, thalamus, hippocampus, temporal cortex, and frontal cortex (Sasano et al., 1998; Steckelbroeck et al., 1999; Stoffel-Wagner et al., 1999, 1998). Additional work confirmed high expression of aromatase mRNA in the human hippocampus, temporal cortex, and frontal cortex, and found no sex differences in aromatase expression in these brain regions (Stoffel-Wagner et al., 1999). Furthermore, positron emission tomography (PET) imaging using radiolabeled aromatase inhibitors also confirmed widespread aromatase localization in the human brain, with highest concentrations in the thalamus and amygdala, followed by preoptic area (POA), hippocampus, cortex, putamen, cerebellum and white matter (Biegon, 2016). PET imaging further confirmed that there are no significant sex differences in aromatase levels in the human brain except for greater levels in the male left hypothalamus (Takahashi et al., 2018). PET imaging also revealed that regional brain uptake of the radiolabeled aromatase inhibitor, ¹¹C-vorozole did not vary across the menstrual cycle in premenopausal women (Biegon et al., 2015). Immunohistochemical studies to examine the cellular and subcellular localization of aromatase showed

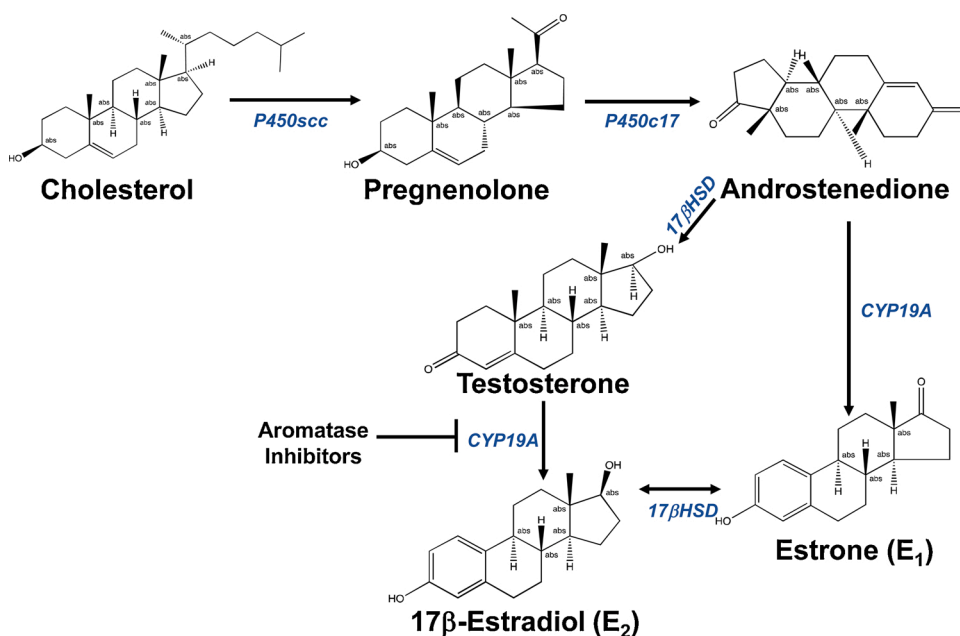


Fig. 1. Simplified Biosynthetic Pathway for Estrogens. Estrogen synthesis begins with conversion of cholesterol to pregnenolone in mitochondria. Through a series of steps, pregnenolone is converted into androstenedione, which is converted into testosterone and estrone (E₁). Testosterone is then converted into 17 β -estradiol (E₂) through the action of aromatase (CYP19A). As also shown, CYP19A can be inhibited by various aromatase inhibitors for research purposes and for therapies. Chemical structures were generated from the ChemSpider webpage (<http://www.chemspider.com>).

widespread aromatase expression in pyramidal neurons in the human temporal cortex and CA1-CA3 regions of the hippocampus, granule cells of dentate gyrus, in a small number of astrocytes, and in some interneurons in the normal and epileptic human brain (Azcoitia et al., 2011; Yague et al., 2010, 2006). Light and electron microscopy ultrastructural studies in the human brain further demonstrated aromatase immunoreactivity throughout the neuronal cell body, including dendrites and axonal processes, and in numerous boutons with synaptic vesicles (Naftolin, 1994; Naftolin et al., 1996). In addition, axon terminals were found to form synapses with immuno-negative and immuno-positive dendrites and neuronal cell bodies (Naftolin, 1994). The synaptic localization of aromatase suggests a possible neuro-modulator or neurotransmitter role for neuron-derived estrogen, which will be discussed in a subsequent section.

2.2. Non-human primate

Similar to the human, early studies revealed high aromatase activity in the monkey hypothalamus, amygdala, hippocampus and cortex (Flores et al., 1973; MacLusky et al., 1986). Later studies using *in situ* hybridization confirmed high aromatase mRNA-containing neurons in hypothalamic areas, with highest expression observed in the medial preoptic nucleus, bed nucleus of the stria terminalis and anterior hypothalamus, as well as cortical and medial amygdaloid nucleus, and basal amygdala nucleus – areas important in expression of emotional behaviors and memory processing (Roselli et al., 2001). Studies using RT-PCR also demonstrated significant aromatase expression in the amygdala, mediobasal hypothalamus, hippocampus and prefrontal cortex of the ovariectomized female monkey (Sorwell et al., 2012). At the protein level, immunohistochemical studies confirmed significant aromatase immunoreactive protein localization in the monkey temporal cortex, hippocampal CA1-3 pyramidal neurons, granule neurons of the dentate gyrus, and some interneurons (Yague et al., 2008). Additional studies demonstrated that aromatase localization occurred throughout the neuronal cell body, including dendrites and axons, and in boutons that contained synaptic vesicles (Naftolin et al., 1996).

2.3. Rat

The first studies to localize aromatase in the rat brain used activity assays and focused on the hypothalamus. These studies demonstrated high activity in the male and female hypothalamus (Naftolin et al., 1972), POA and mediobasal hypothalamus (Selmanoff et al., 1977). Subsequent studies using a microsomal based aromatase activity assay and high-performance liquid chromatography (HPLC) measurement of E₂ performed a more widespread analysis of the brain and showed the highest aromatase activity and E₂ levels in the amygdala, POA and hippocampus of the female rat brain (Li et al., 2016). RT-PCR studies similarly found high expression of aromatase mRNA in the amygdala,

bed nucleus of the stria terminalis and POA, followed by the hippocampus and cingulate cortex, with low levels in the brainstem and cerebellum of the adult male and female rat brain (Tabatadze et al., 2014). In keeping with the hippocampus being a site of E₂ production, significant mRNA expression for the steroidogenic enzymes necessary for E₂ synthesis including aromatase, P450 side chain cleavage (P450scc), P450 17 α -hydroxylase (P45017 α), 17 β -hydroxy steroid dehydrogenase (17 β -HSD), 3 β -hydroxysteroid dehydrogenase (3 β -HSD) was demonstrated in the rat hippocampus (Hojo et al., 2004; Mukai et al., 2006). The basal concentration of E₂ was reported to range from 1-8 nM in the male rat hippocampus, and from 0.5 to 2 nM in the rat female hippocampus (Hojo et al., 2004; Mukai et al., 2010, 2006), which is significantly greater than the concentration in the blood. E₂ in the hippocampus was reported to be very stable and not significantly converted to other metabolites (Hojo et al., 2004). Further studies of the rat hippocampus revealed that aromatase is localized in neurons basally. For instance, immunohistochemical studies demonstrated significant aromatase localization in pyramidal neurons of the adult male and female rat hippocampal CA1-CA3 regions, and in granule neurons in the dentate gyrus (Hojo et al., 2004; Mukai et al., 2006; Zhang et al., 2014). *in vitro* studies further confirmed aromatase enzymatic activity in neurons, with no activity observed in astrocytes or oligodendrocytes (Negri Cesi et al., 1992, 1993). Additional studies revealed aromatase was localized in pre- and post-synaptic compartments and the endoplasmic reticulum in the rat hippocampus (Hojo et al., 2004; Mukai et al., 2006; Zhang et al., 2014), and was located at the synapse and in presynaptic terminals in cultured rat cortical neurons (Srivastava et al., 2010). This neuronal and synaptic localization of aromatase is similar to the results observed in the human and non-human primate, as well as in the mouse and bird, as will be discussed below.

2.4. Mouse

Studies in mice are less numerous, but generally consistent with aromatase localization observed in the rat brain. For instance, significant aromatase localization has been reported in the mouse hypothalamus, amygdala, hippocampus and cerebral cortex by immunohistochemistry and RT-PCR (Balthazart et al., 1991a, b; Bender et al., 2017; Beyer et al., 1994a; Ivanova and Beyer, 2000; Lu et al., 2019; Wang et al., 2020). Similar to studies in the rat, aromatase was demonstrated only in neurons of the mouse hippocampus, with no localization observed in astrocytes basally (Lu et al., 2019, 2020; Wang et al., 2020). *In vitro* studies of cultured mouse neurons and astrocytes confirmed that aromatase was only expressed in neurons and not astrocytes (Beyer et al., 1994a). Mice engineered to express enhanced green fluorescent protein (EGFP) upon aromatase activation showed widespread aromatase expression in the brain, with highest EGFP-positive cell bodies and fibers noted in the amygdala, hypothalamus and bed nucleus of the stria terminalis. In many mouse brain areas,

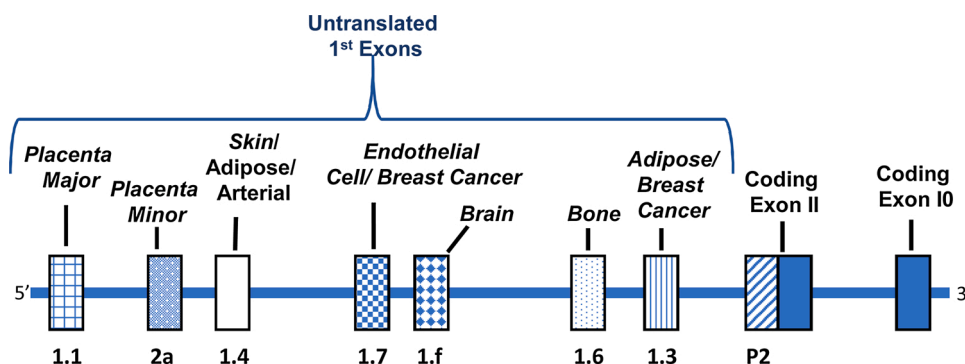


Fig. 2. Partial Aromatase Gene Structure. Tissue-specific promoters in untranslated first exons are responsible for tissue-specific transcripts of aromatase. Promoter 1.f is typically considered the brain-specific promoter; however, promoters 1.3 and 1.4 have also been reported to be expressed in the brain.

AROMATASE INHIBITORS

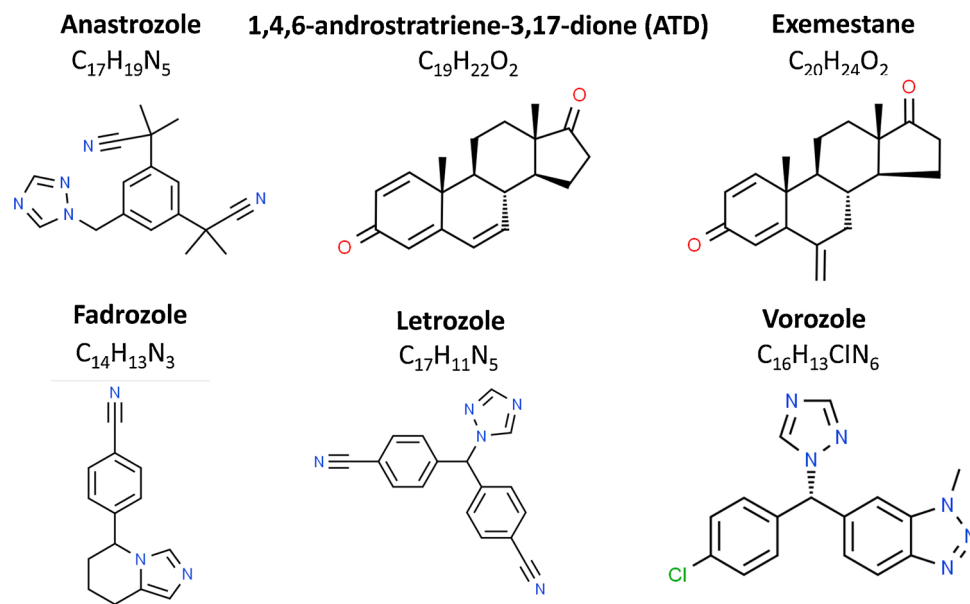


Fig. 3. Primary aromatase inhibitors used to inhibit aromatase activity in the brain in animals and humans. Chemical structures were generated from the ChemSpider webpage (<http://www.chemspider.com>).

EGFP-positive cells co-expressed estrogen receptor- α (ER α) and estrogen receptor- β (ER β) or the androgen receptor (Stanic et al., 2014). Collectively, these studies show that similar to observations in monkeys and humans, aromatase is widely distributed in neurons at synaptic locations in both the rat and mouse brain. This suggests that aromatase and BDE₂ may regulate synaptic function in the brain. Indeed, there is growing evidence supporting a synaptic role for BDE₂, as will be discussed in a subsequent section.

2.5. Bird

One of the earliest reports of brain localization of aromatase in birds was a study that demonstrated significant aromatase activity in the bird forebrain (Callard et al., 1978). Interestingly, this study also confirmed a similar significant aromatase activity level in the forebrain of the snake, sea turtle, opossum, urodele amphibian, teleost and skate (Callard et al., 1978). Immunohistochemical studies in the Japanese quail revealed significant aromatase protein localization in the medial preoptic nucleus, septal region, ventromedial and tuberal hypothalamus, with aromatase and estrogen receptor colocalized in many of the regions (Balthazart et al., 1991b). Aromatase activity was found in synaptosomes, and electron microscopy studies revealed aromatase was present in synaptic boutons in the Japanese quail brain (Balthazart et al., 1991b). Further work confirmed that aromatase is expressed in the POA in many bird species including canaries, house sparrows, zebra finches, ring doves, swifts, grey partridges, barn owls and budgerigars (Metzdorf et al., 1999).

It is important to note that the songbird telencephalon expresses aromatase much more widely and with much higher activity than observed in the quail, which has high aromatase expression localized primarily to the hypothalamus. Thus, the songbird has been particularly useful in determining the role of aromatase and BDE₂ in neuroplasticity, memory, and behavior. In situ hybridization documented widespread aromatase localization in the adult zebra finch brain, with highest localization observed in the POA, hypothalamus, hippocampus and neostriatum (Shen et al., 1994). Subsequent work demonstrated aromatase was localized in pre-synaptic boutons in the zebra finch

hippocampus and high vocal centre brain areas and that males had more synaptic profiles with aromatase than females (Peterson et al., 2005). In addition, aromatase protein and activity were demonstrated in synaptosomes of the male and female zebra finch anterior and posterior telencephalon, with males exhibiting the highest levels (Rohmann et al., 2007). The aromatase-positive pre-synaptic boutons in the zebra finch brain were found to always innervate aromatase-negative post-synaptic elements (Peterson et al., 2005). Further work demonstrated that aromatase activity was elevated in forebrain synaptic terminals in male zebra finches that were singing for 30 min (Remage-Healey et al., 2009).

2.6. Fish, amphibians and reptiles

As mentioned previously, teleost fish have two aromatase isoforms, CYP19a which encodes aromatase A and is expressed in the gonads, and CYP19b, which encodes aromatase B and is expressed in the brain and gonads (Tchoudakova and Callard, 1998). Another unique characteristic of teleost fish is that aromatase is expressed exclusively in radial glial cells in the brain, and not in neurons (Forlano et al., 2001). While radial glial cells generally disappear in mammals after development (Mori et al., 2005), they remain in the brain of adult fish. In zebrafish, aromatase expression is highest in the telencephalon, POA, thalamus, hypothalamus, optic tectum, and torus semicircularis (Menuet et al., 2005; Pellegrini et al., 2007). Further work demonstrated that aromatase B is upregulated in radial glial cells in the POA and mediobasal hypothalamus by E₂ (Menuet et al., 2005). Interestingly, bromodeoxyuridine treatment coupled with IHC revealed that aromatase-positive radial glial cells divide in the zebrafish brain, and over time move away from the ventricles with some differentiating into neurons (Pellegrini et al., 2007). IHC and *in situ* hybridization showed a similar aromatase localization pattern in the brain of rainbow trout with highest levels in the POA and hypothalamus, ventricles of telencephalon and ventral dien-cephalon (Menuet et al., 2003). In contrast to teleost fish, aromatase is encoded by a single gene CYP19a1 in amphibians (Iwabuchi et al., 2007). In the amphibian xenopus, aromatase gene expression in the brain occurred from early developmental stages to metamorphosis, with highest expression in the POA and caudal hypothalamus (Urbatzka

Table 1
Summary of Brain Localization of Aromatase.

Species	Year	Detection Technique	Brain Areas Localized
Human	1971	Aromatase Activity Assay (AAA)	<ul style="list-style-type: none"> Fetal diencephalon (Naftolin et al., 1971)
Rat	1972	AAA	<ul style="list-style-type: none"> Male and female anterior hypothalamus (HYP) (Naftolin et al., 1972)
Turtle	1977	AAA	<ul style="list-style-type: none"> Strio-amygdaloid complex, HYP (Callard et al., 1977)
Rat	1977	AAA	<ul style="list-style-type: none"> Preoptic area (POA), mediobasal HYP (Selmanoff et al., 1977)
Bird, Snake, Shark, Teleost	1978	AAA	<ul style="list-style-type: none"> Forebrain (Callard et al., 1978)
Rat	1984	AAA	<ul style="list-style-type: none"> Amygdala (AMG), POA, HPC, cerebral cortex, cerebellum, anterior pituitary. In female, no change in estrous cycle or after ovariectomy (Roselli et al., 1984)
Rat	1984	AAA	<ul style="list-style-type: none"> Castration decreased aromatase activity in POA by 60 % (Roselli and Resko, 1984)
Monkey	1986	AAA	<ul style="list-style-type: none"> Highest in HYP, also detected in AMG, hippocampus (HPC), cortex (MacLusky et al., 1986)
Songbird	1990	AAA	<ul style="list-style-type: none"> Nuclei of the song system, Female greater E₂ production than male (Vockel et al., 1990)
Mouse	1991	Immunohistochemistry (IHC)	<ul style="list-style-type: none"> High in medial and tuberal HYP (Balthazart et al., 1991a, b)
Quail	1991	IHC	<ul style="list-style-type: none"> Majority of aromatase-positive neurons in HYP colocalize ERα (Balthazart et al., 1991b)
Rat	1993	AAA	<ul style="list-style-type: none"> Aromatase activity in neurons, low in astrocytes, absent in oligodendrocytes (Negri Cesi et al., 1993)
Chicken	1994	IHC	<ul style="list-style-type: none"> Aromatase high in HYP, HPC (Beyer et al., 1994b)
Mouse	1994	IHC	<ul style="list-style-type: none"> Aromatase immunoreactivity in mouse cultures of hypothalamic and cortical cells, only in neurons (Beyer et al., 1994a)
Songbird	1994	In situ hybridization (ISH)	<ul style="list-style-type: none"> Aromatase mRNA detected in POA, HYP, HPC, neostriatum (Shen et al., 1994)
Rat	1994, 1998	ISH	<ul style="list-style-type: none"> Aromatase mRNA highest in POA, medial preoptic nucleus, bed nucleus of stria terminalis, AMG (Lauber and Lichtensteiger, 1994; Roselli et al., 1998)
Human	1996	Reverse transcriptase- polymerase chain reaction (RT-PCR)	<ul style="list-style-type: none"> Aromatase mRNA highest in pons, thalamus, HYP and HPC (Sasano et al., 1998)
Human	1998	RT-PCR, AAA	<ul style="list-style-type: none"> Aromatase mRNA and activity detected in temporal lobe cortex (Steckelbroeck et al., 1999; Stoffel-Wagner et al., 1998)
Quail, Rat, Monkey, Human	1996	AAA	<ul style="list-style-type: none"> Aromatase activity high in human cerebral cortex vs. subcortical regions (10443682) POA, HYP, limbic areas, dendrites, perikaryal, axons, synaptic vesicles (9053779)
Rat, mouse	1999	IHC and AAA	<ul style="list-style-type: none"> Aromatase immunoreactivity/activity induced in HPC astrocytes after kainic acid or penetrating injury (Garcia-Segura et al., 1999)
Human	1999	RT-PCR	<ul style="list-style-type: none"> Aromatase mRNA expressed in temporal and frontal cortex and HPC, No sex difference (Stoffel-Wagner et al., 1999)
Rat	2003, 2004	RT-PCR, IHC, electron microscopy (EM), high performance liquid chromatography (HPLC)	<ul style="list-style-type: none"> In male rat HPC pyramidal neurons (Shibuya et al., 2003)
Zebra Finch	2004, 2005	IHC, EM	<ul style="list-style-type: none"> Aromatase localized in pre- and post-synaptic sites in male rat HPC, E₂ synthesized in HPC and increased by NMDA, E₂ very stable in and not significantly metabolized (Hojo et al., 2004)
Human	2006	RT-PCR, IHC	<ul style="list-style-type: none"> Aromatase and NMDAR co-expressed in HPC neurons (Saldanha et al., 2004), aromatase is presynaptic (Peterson et al., 2005)
Rat	2006	RT-PCR, Western blot (WB), IHC, EM, HPLC	<ul style="list-style-type: none"> Aromatase expressed in pyramidal neurons of temporal cortex, small number astrocytes positive, no expression GABAergic interneurons (Yague et al., 2006)
Monkey	2008	IHC	<ul style="list-style-type: none"> E₂ synthesized locally in rat HPC neurons, Basal conc was 1 nM in HPC. Aromatase, P450scc, 3β-HSD, 17β-HSD, STAR all expressed in HPC neurons (Mukai et al., 2006)
Human	2010	IHC	<ul style="list-style-type: none"> Aromatase expressed in temporal cortex, HPC pyramidal neurons, dentate gyrus (DG) granule cells, and some interneurons (Yague et al., 2008)
Rat	2010	IHC	<ul style="list-style-type: none"> Widespread aromatase expression in HPC pyramidal neurons, DG granule cells, and interneurons in normal and epileptic HPC tissue (Yague et al., 2010)
Zebra Finch	2013	IHC	<ul style="list-style-type: none"> Aromatase staining present in synapses and presynaptic terminals of cultured rat cortical neurons (Srivastava et al., 2010)
Rat	2014	IHC	<ul style="list-style-type: none"> Aromatase expression constitutive in neurons and induced in astrocytes (Saldanha et al., 2013)
Rat	2014	RT-PCR	<ul style="list-style-type: none"> Aromatase expression constitutive in HPC and cortical neurons, induced in astrocytes after cerebral ischemia (Zhang et al., 2014)
Rat	2016	Microsome-based AAA using liquid chromatography-mass spectrometry (LC-MS)	<ul style="list-style-type: none"> Long-form of aromatase expressed in many brain regions, no sex difference in dorsal HPC expression and no regulation by gonadal/hormone (Tabatadze et al., 2014)
Mouse	2019	IHC, WB, High Sensitivity Enzyme-linked immunosorbent assay (ELISA)	<ul style="list-style-type: none"> Distribution of aromatase long-form mRNA correlated with AA and was highest in AMG, POA and HPC. (Li et al., 2016)
Mouse	2020	IHC, WB, High Sensitivity ELISA	<ul style="list-style-type: none"> Aromatase expression in HPC and cortex of male and female mouse, E₂ levels in HPC and cortex ~1 nM. Genetic deletion of forebrain neuronal aromatase leads to defects in synaptic plasticity, LTP and memory (Lu et al., 2019)
Mouse	2020	IHC, WB, High Sensitivity ELISA	<ul style="list-style-type: none"> Astrocyte aromatase and E₂ increased in HPC after cerebral ischemia. Genetic deletion of forebrain neuronal or astrocyte aromatase leads to reduced astrocyte activation and attenuated neuroprotection after cerebral ischemia (Lu et al., 2020; Wang et al., 2020)

et al., 2007). It was further found that the aromatase gene is strictly expressed in neurons and not in radial glial cells in the xenopus brain and was not sexually dimorphic (Coumilleau and Kah, 2015). In reptiles, aromatase activity was demonstrated to be high in the forebrain (Callard et al., 1977; Callard et al., 1978). IHC in the red-sided garter

snake demonstrated that aromatase is localized in the POA, anterior hypothalamus, nucleus sphericus and septum (Krohmer et al., 2002), and inhibition of aromatase activity by administration of an aromatase inhibitor revealed a role for brain aromatase in courtship behavior in the red-sided garter snake (Krohmer, 2020).

3. Aromatase regulation in the brain

Aromatase and BDE₂ levels in the brain can be regulated by both transcriptional and post-transcriptional mechanisms, as well as a diverse array of intrinsic and extrinsic factors (Fig. 4). In this section, we will review these key mechanisms and factors that control aromatase expression/activity and E₂ production in the brain.

3.1. Phosphorylation

There is considerable evidence that phosphorylation is a key mechanism to rapidly regulate aromatase activity and BDE₂ production. For instance, early work in quail showed that Ca²⁺-dependent phosphorylation of aromatase caused a rapid decrease of brain aromatase activity in hypothalamic homogenates and explants (Balthazart et al., 2005, 2001a; Balthazart et al., 2001b). Further work revealed that quail aromatase has 15 predicted consensus phosphorylation sites, and that protein kinase C and protein kinase A are involved in phosphorylation control of aromatase activity (Balthazart et al., 2005). Hayasi and Harada (Hayashi and Harada, 2014) reported that in human JEG-3 cells aromatase is phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) and dephosphorylated by calcineurin, with catalytic activity being reversibly regulated. Additional work revealed that acid phosphatase blocks the inhibiting effects of Ca²⁺-dependent phosphorylation on aromatase activity in quail (Balthazart et al., 2005). It was also shown that sexual interaction decreases aromatase activity within minutes in male quail medial preoptic nucleus (Cornil, 2018; de Bournonville et al., 2017a, b). The authors proposed that the rapidity of the effect suggests a nongenomic mechanism is involved that may involve glutamate signaling and Ca²⁺-dependent phosphorylation of aromatase (de Bournonville et al., 2017a). Indeed, glutamate is increased in the medial POA of the male quail during sexual interaction and injection of the glutamate agonist, kainate into the medial preoptic nucleus led to a significant decrease of aromatase activity in male quail (de Bournonville et al., 2017b). Similar to the observation in quail, a rapid decrease of

aromatase activity was also observed in the male and female zebra finch hypothalamus, hippocampus and caudomedial nidopallium (NCM) by Ca²⁺-dependent phosphorylation (Comito et al., 2016). In further support of a role for Ca²⁺ in regulating brain aromatase, depletion of Ca²⁺ stores in zebra finch forebrain and rat hippocampal neurons in culture resulted in increased release of E₂ (Fester et al., 2016; Remage-Healey et al., 2011) and dephosphorylation of aromatase (Remage-Healey et al., 2011). Intriguingly, E₂ treatment increased phosphorylation of aromatase and increased aromatase protein levels in rat hippocampal neurons, suggesting that E₂ can regulate aromatase in the rat hippocampus (Remage-Healey et al., 2011).

Subsequent work used liquid chromatography with tandem mass spectrometry analysis to identify phosphorylation sites in human aromatase (Ghosh et al., 2019). The study revealed that human aromatase has as many as 19 phosphorylation sites, of which T⁴⁶², T162, H⁴⁷⁵/S⁴⁷⁸, and Y³⁶¹ are major and reproducibly detectable (Ghosh et al., 2019). Phosphorylation sites T⁴⁶², S118 and S⁴⁷⁸ are highly homologous between species, being present in human, monkey, rat, zebra finch, mouse and chicken. Phosphorylation site Y³⁶¹ is present in human, monkey, zebra finch, chicken and quail, but not in rat or mouse. Interestingly, phosphorylation of Y³⁶¹ was shown to enhance aromatase activity, and phosphorylation of S⁴⁷⁸ in the active site access channel was proposed to also be significant as previous work implicated S⁴⁷⁸ in catalysis (Ghosh et al., 2009; Kao et al., 2001). An important point to consider is that phosphorylating and dephosphorylating conditions likely work together to regulate aromatase activity. In support of this possibility, work using zebra finch brain homogenates found that acid phosphatase increases aromatase activity in low to moderate phosphorylating conditions, while the opposite effect is observed under high phosphorylating conditions (Hovsepian-Ruby, 2017). In addition, low to moderate levels of acid phosphatase increased aromatase activity and Y³⁶¹ phosphorylation, while high concentrations strongly inhibited aromatase activity and Y³⁶¹ phosphorylation (Hovsepian-Ruby, 2017). Another investigative group performed microsecond-long all-atom molecular dynamics simulations to determine how phosphorylation regulates human

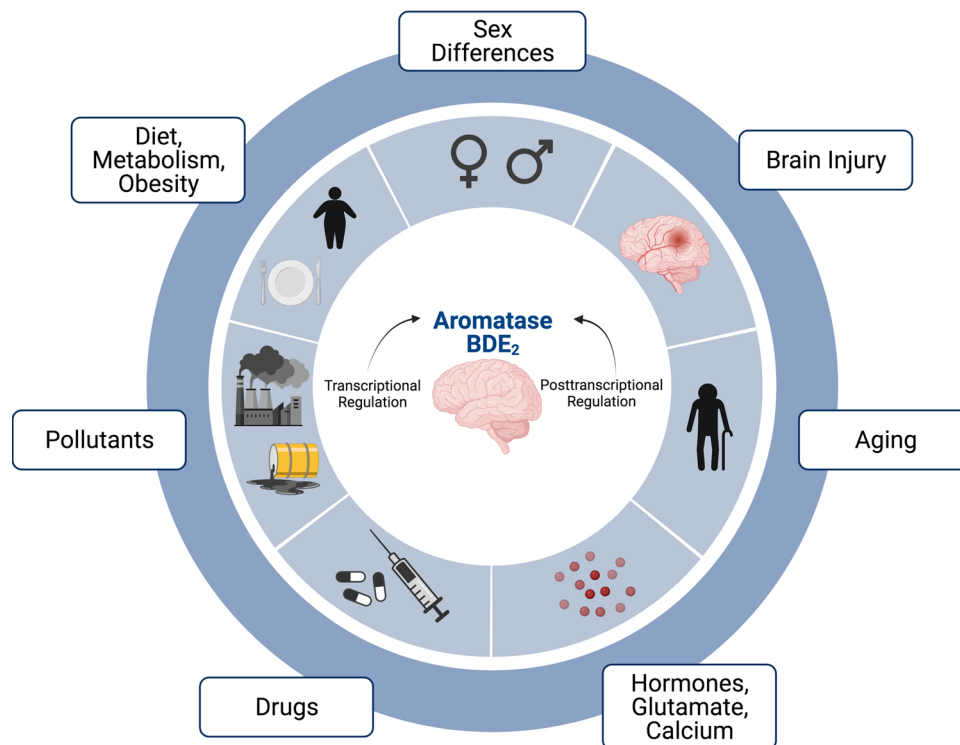


Fig. 4. Summary diagram illustrating multiple processes and factors that have been implicated to regulate brain aromatase. See text for full description and discussion. BDE₂ = brain-derived 17 β -estradiol. Created with BioRender.com.

aromatase catalyzed estrogen synthesis (Ritacco et al., 2019). They proposed that phosphorylation of residue Y³⁶¹ in aromatase leads to stabilization of its adduct with the CYP450 reductase flavin mononucleotide domain (Ritacco et al., 2019). This may facilitate estrogen biosynthesis by favoring aromatase as it competes with other proteins that require CYP450 reductase's electron supply. Finally, a myc-tagged mouse aromatase construct transfected into COS cells or HEK-293T cells was used to further study phosphorylation of mouse aromatase (Miller et al., 2008). The study demonstrated that mutation of S118 to Ala blocked phosphorylation and led to destabilization of aromatase, suggesting that S118 may have an important structural role (Miller et al., 2008). Using a phosphomimetic S¹¹⁸ D mutant, their work provided evidence that S118 phosphorylation decreases aromatase activity (Miller et al., 2008). Taken as a whole, the above studies demonstrate that phosphorylation can either increase or decrease aromatase activity depending on the site(s) phosphorylated. Unfortunately, there are few studies that have examined phosphorylation of brain aromatase in physiological or pathological processes, and thus work to address this deficit is very much needed.

3.2. Glutamate

Glutamate, the major excitatory amino acid transmitter in the brain, has been shown to rapidly regulate neural aromatase activity and BDE₂ levels, although species and brain region-specific differences exist. For example, glutamate agonists rapidly decreased aromatase activity in quail hypothalamic explants (Balthazart et al., 2006). The rank order of effectiveness was kainate > AMPA > NMDA. Likewise, glutamate retrodialysis was shown to induce a significant decrease of local E₂ levels in the NCM of the zebra finch (Remage-Healey et al., 2008). The glutamate receptor responsible for mediating the effect was not studied, but aromatase and NMDA receptors have been shown to be colocalized in hippocampal neurons of zebra finch (Saldanha et al., 2004), which raises the possibility that NMDAR may mediate the effect. In contrast to the reports in quail and zebra finch, glutamate administration was shown to be stimulatory to BDE₂ release in the rat as *in vivo* kainate administration more than doubled E₂ release in the rat hippocampus within two hours (Sato and Woolley, 2016). Similarly, NMDA treatment nearly doubled E₂ release in male rat hippocampal slices incubated *in vitro* (Hojo et al., 2004). The stimulatory effect of NMDA was fully suppressed by treatment with an NMDAR antagonist, MK-801 (Hojo et al., 2004). NMDA also induced production of pregnenolone, an upstream precursor to E₂, and this effect was found to be dependent upon NMDAR and Ca²⁺ influx (Shibuya et al., 2003). Rune's group found a biphasic effect of NMDA on E₂ release from rat hippocampal neurons in culture - a decrease noted at 30 min after NMDA treatment followed by a significant increase at 60 min post-treatment (Fester et al., 2016). Finally, the NMDA receptor agonist, D-aspartate (Errico et al., 2015) increased aromatase mRNA and protein as well as brain E₂ levels in the frog, an effect that involved CREB signaling (Burrone et al., 2012; Santillo et al., 2013). Since glutamatergic signaling is a major component of excitatory neurotransmission in the brain, its ability to rapidly regulate BDE₂ release could suggest a role for BDE₂ in neurotransmission. Indeed, numerous studies have now provided evidence that BDE₂ regulates synaptic function, cognition and memory (Brandt et al., 2020; Lu et al., 2019; Mukai et al., 2010, 2006), as will be discussed in a subsequent section.

3.3. Transcriptional regulation

In addition to phosphorylation, there is also abundant evidence that aromatase is regulated at the transcriptional level. For instance, treatment with a hypomethylating agent, 5-aza-2'-deoxycytidine caused a 10-fold increase of brain-specific 1.f aromatase promoter transcription and an ~80% increase in aromatase activity in human glioblastoma cells (Tan et al., 2017). There was no effect on the PII aromatase promoter. This finding indicates that methylation can inhibit or restrain aromatase

expression and activity in glioblastoma cells. It would be interesting to determine whether methylation can similarly regulate aromatase expression/activity in normal non-transformed neuronal and glia cells in the brain. Regarding the mechanisms underlying transcriptional control of the brain-specific 1.f aromatase promoter, the orphan transcription factor ARP-1 has been shown to bind to the brain-specific exon 1 cis-acting element aro-A1 and induce the 1.f promoter (Honda and Harada, 2020). A functional regulatory role for the interaction was demonstrated via ARP-1 knockdown in mouse neurons, which significantly decreased aromatase induction. Further work by this group revealed that the Lim-homeodomain protein, Lhx2, also appears to be involved in brain-specific 1.f aromatase transcriptional control (Honda et al., 2012). Lhx2 was identified as binding to the mouse brain-specific exon 1 aro-B cis-acting element, and a reporter assay demonstrated Lhx2-dependent aromatase promoter activity was suppressed by siRNA knockdown of Lhx2 expression (Honda et al., 2012). Finally, the human aromatase gene was shown to contain two binding sites for the hormone-dependent transcription factor, retinoic acid-related orphan receptor-alpha (RORA) (Sarachana et al., 2011). Further work indicated a transcriptional regulatory role for RORA as its overexpression in human neuroblastoma cells led to a ~10-fold increase of aromatase expression (Sarachana et al., 2011). The authors further found that estrogen increases RORA expression in the brain, and that expression of RORA and aromatase were both significantly decreased in the frontal cortex of autistic subjects, which the authors postulate may contribute to the disorder (Sarachana et al., 2011).

3.4. Gonadectomy and sex differences

Castration in male rats was found to significantly decrease aromatase mRNA levels in the male rat POA but not in the hippocampus or cingulate cortex (Roselli et al., 1998; Tabatadze et al., 2014). At the enzyme activity level, castration was shown to decrease aromatase activity in the POA (Roselli et al., 1984; Roselli and Resko, 1984), but not in the amygdala or cortex (Roselli et al., 1984). These findings suggest that the testis can regulate brain aromatase and E₂ levels, especially in the hypothalamus. The regulatory effect of the testis may be mediated by the gonadal hormone, testosterone, as several studies have shown that testosterone can regulate brain aromatase and E₂ levels (Roselli et al., 1984; Roselli and Resko, 1984). Interestingly, additional studies revealed aromatase activity did not change in the female rat POA after ovariectomy or during the estrus cycle (Roselli et al., 1984). Several studies have demonstrated that sex differences exist for aromatase expression in the amygdala and POA of the rat brain. For instance, aromatase mRNA levels in the amygdala were reported to be significantly higher in males as compared to females (Tabatadze et al., 2014). Further work found that E₂ levels are significantly higher in the amygdala and POA of male rats as compared to levels in ovariectomized females (Li and Gibbs, 2019). Sex differences were demonstrated in the zebra finch brain with greater aromatase activity observed in the male amygdala and hypothalamus/POA as compared to female (Roselli et al., 1984). The reasons for the sex differences in the zebra finch brain are unclear but they may relate to regulation of key functions controlled by these brain regions, including reproductive behavior, learning and memory, song-recognition, and singing. Sex differences in aromatase expression in the brain could be due to regulation by gonadal hormones and/or sex chromosome complement. Indeed, there is significant evidence of gonadal hormone regulation of aromatase in the brain of various species as described in the next section below. Only a few studies have examined the possibility of sex chromosome complement regulation of aromatase expression and this work was performed in the developing mouse brain (Cisternas et al., 2017, 2015). The investigators used the four core genotypes mouse model in which the sex chromosome complement and gonadal sex are dissociated and found that 16 day old XY mouse had higher aromatase expression in the anterior amygdala and stria terminalis than XX embryos, independent of gonadal sex

(Cisternas et al., 2015). Interestingly, E₂ or dihydrotestosterone enhanced aromatase expression in cultured amygdaloid neurons from XX embryos but not XY embryos, and this effect was also independent of gonadal sex and appeared to be mediated by ER β (Cisternas et al., 2017, 2015). It would be interesting to conduct additional studies to determine whether aromatase regulation by sex chromosome complement extends to other brain regions, other ages, and to other species.

In contrast to the sex differences observed in the amygdala, POA and stria terminalis, examination of aromatase mRNA levels and immunoreactive aromatase expression in the hippocampus of the rat did not reveal any sex differences (Fester et al., 2012; Tabatadze et al., 2014), and E₂ levels were not significantly different in the intact male hippocampus versus the ovariectomized female rat hippocampus (Li and Gibbs, 2019). In addition, measurement of E₂ in the supernatant of cultured female and male hippocampal neurons also revealed no significant sex differences (Fester et al., 2012). However, a sex difference in E₂ levels in the hippocampus of the intact male rat versus the intact female proestrus rat has been reported, with hippocampal E₂ levels as high as 8 nM reported in the intact male rat versus 1.7 nM in the intact proestrus female rat (Mukai et al., 2010). These contradictory reports on sex differences in hippocampal E₂ levels may be due to differences in the study design, including 1) differences due to measuring mRNA and protein as compared to the actual product E₂, 2) differences in comparing intact males to ovariectomized females versus comparing intact males to intact females, and 3) differences due to *in vitro* versus *in vivo* approaches.

3.5. Hormones

There is growing evidence that brain aromatase is regulated by both steroid and peptide hormones. Testosterone, a substrate for estrogen production, was shown to increase aromatase activity in the POA of castrate male Japanese quail (Balthazart et al., 1990) as well as castrate male and female rat POA, periventricular magnocellular nucleus and posterior medial hypothalamic nucleus (Abdelgadir et al., 1994; Roselli et al., 1984), and in the nuclei of the song system in the castrate male zebra finch (Vockel et al., 1990), and suprachiasmatic nucleus, paraventricular nucleus, ventromedial nucleus and lateral hypothalamus of the castrate male monkey (Roselli and Resko, 1989). The regulation of aromatase activity in the brain by testosterone appears to be exerted at the transcriptional level as testosterone was found to cause an associated increase of aromatase mRNA levels in the brain of several species (Abdelgadir et al., 1994; Harada et al., 1992).

In addition to testosterone, E₂ has also been demonstrated to regulate expression of brain aromatase. Using a mouse hypothalamic neuronal cell line, it was shown that ER α interacts with the 1.f promoter and that E₂ treatment increased aromatase mRNA, an effect reversed by an ER antagonist or ER α knockdown (Yilmaz et al., 2009). Likewise, E₂ increased aromatase mRNA levels in the ovariectomized female rat amygdala (Tabatadze et al., 2014). Similarly, tonic E₂ treatment was found to increase aromatase expression in the female mouse hippocampus; however, phasic E₂ treatment was shown to actually be inhibitory (Iivonen et al., 2006). Finally, short-term E₂ replacement decreased aromatase expression in the monkey hippocampus (Sorwell et al., 2012). Unfortunately, none of these studies examined aromatase activity or E₂ levels, so it is unclear whether the aromatase expression results actually contributed to changes in BDE₂ levels. Furthermore, it is unclear why some studies found stimulatory effects of E₂ while others reported inhibitory effects. It is possible the divergent results could be due to study differences, including species differences, examining different brain locations, utilizing *in vitro* versus *in vivo* studies, and/or employing different E₂ treatment regimens.

In addition to testosterone and E₂, progestins and glucocorticoids have also been implicated to transcriptionally regulate the expression of brain aromatase. For instance, R5020, a synthetic progestin, was shown to increase aromatase promoter 1.f transcription in mouse hypothalamic

neuronal cell lines, an effect that was reversed by the progesterone receptor antagonist RU486 and siRNA knockdown of the progesterone receptor (Yilmaz et al., 2011). The physiological importance of this regulatory effect remains unclear. However, progesterone has been reported to act in the hypothalamus to help amplify induction of the gonadotropin releasing hormone (GnRH) surge and luteinizing hormone (LH) surge (Lee et al., 1990; Mahesh and Brann, 1998; Stephens et al., 2015). Interestingly, Terasawa and coworkers (Kenealy et al., 2017; Terasawa, 2018) recently demonstrated a role for BDE₂ in GnRH and LH surge induction in ovariectomized monkeys. It would therefore be interesting to perform studies to examine whether progesterone enhances hypothalamic BDE₂ *in vivo* and whether this effect contributes to progesterone's ability to amplify the GnRH and LH surge. One of the most profound regulations of 1.f promoter transcription reported to date was the finding that the glucocorticoid, dexamethasone can induce up to a 98-fold increase of aromatase mRNA and protein in mouse hypothalamic neuronal cell lines (Brooks et al., 2012). This effect required mediation by the glucocorticoid receptor as it was blocked by a glucocorticoid receptor antagonist and by siRNA knockdown of the glucocorticoid receptor (Brooks et al., 2012). Furthermore, acute stress, which increases the release of corticosterone from the adrenal, was shown to significantly enhance aromatase activity in the POA of quail (Dickens et al., 2013), and to rapidly increase aromatase expression and E₂ levels in the paraventricular nucleus of proestrus and ovariectomized female rats (Liu et al., 2011). All of these studies focused on regulatory effects in the hypothalamus. It would also be interesting to examine the hippocampus and amygdala, as well as other brain regions for stress and glucocorticoid regulation of aromatase and BDE₂ levels. The POA is known to control gonadotropin secretion, reproduction and sexual behavior, while the paraventricular nucleus is involved in osmoregulation, appetite control, and the body's response to stress. Thus, the regulation of hypothalamic BDE₂ by acute stress and glucocorticoids could be related to one or more of these functions.

In addition to steroid hormones, peptide hormones have also been shown to regulate aromatase activity and BDE₂ levels. Of significant interest, the peptide hormone, GnRH was shown to have a biphasic effect upon E₂ synthesis in postnatal rat hippocampal slices and hippocampal neurons in culture (Fester et al., 2012; Prange-Kiel et al., 2008). Low doses (10 nM) of GnRH stimulated E₂ synthesis while higher doses (500 nM) were inhibitory. The effects of GnRH appeared to be specific as they were blocked by a GnRH receptor antagonist. Another study showed that intracerebroventricular injection of GnRH also increased E₂ levels in the hippocampus (Marbouti et al., 2020b). The physiological significance of the GnRH effects on BDE₂ are unclear, but they could be related to regulation of synaptic function and memory. In support of this possibility, spine synapse density was shown to be upregulated by GnRH in rat hippocampal neuron cultures, and this effect was blocked by the aromatase inhibitor letrozole (Prange-Kiel et al., 2008). Furthermore, hippocampal administration of GnRH was shown to enhance memory in ovariectomized rats, an effect that was also blocked by central administration of letrozole (Nelson et al., 2016). Finally, intriguingly, another peptide hormone, gonadotropin-inhibitory hormone (GnIH), was recently shown to increase aromatase activity and E₂ synthesis in the POA in male quail (Ubuka et al., 2014). The investigators also demonstrated that aromatase cells express GnIH receptor mRNA, and that GnIH fibers form close appositions to aromatase immunoreactive cells in the POA, as well as the bed nucleus of the stria terminalis, mediobasal hypothalamus and periaqueductal grey of the male quail (Ubuka et al., 2014). The authors suggest that GnIH increases BDE₂ above its optimal level which may play a role in the ability of GnIH to inhibit aggressive and sexual behavior in males (Ubuka et al., 2014). However, further studies are needed to explore this hypothesis.

3.6. Aging, diet, metabolism and obesity

There is growing evidence that brain aromatase and BDE₂ levels are

significantly regulated by aging and diet. In the human, PET imaging revealed that normal aging and postmenopausal status were associated with decreased uptake of ^{11}C -vorozole throughout the brain in both men and women, indicating that aging decreases aromatase levels in the human brain (Biegon et al., 2015). In the rat, two studies reported that aromatase expression and activity in the hypothalamus of the rat is unaffected by age (Munetomo et al., 2015; Roselli et al., 1986), while a third study found that the number of immunoreactive aromatase neurons in the POA were decreased in 36-month-old as compared to 6-month-old male quail (Dellovade et al., 1995). Likewise, aromatase expression and E_2 levels were found to be decreased in the hippocampus of 19-month-old versus 10-month-old female rats (Chamniansawat and Sawatdiyaphanon, 2018). Aromatase protein expression was also demonstrated to be decreased in the hippocampus of 20-month-old versus 3-month-old female mice (Zhao et al., 2017). While aromatase expression in the monkey hippocampus was unchanged with aging, expression of 17α -hydroxylase and 3β -HSD, key steroidogenic enzymes necessary for E_2 synthesis, were significantly decreased in the hippocampus of aged monkeys (Sorwell et al., 2012). As a whole, these studies suggest that aromatase and brain E_2 levels may be decreased by aging in certain brain areas, with the hippocampus being most consistently reported to exhibit an age-related decrease of aromatase and E_2 levels. Since BDE_2 has been implicated in the regulation of many key brain processes, its decrease with aging may contribute, in part, to the age-related decline in key brain processes/functions such as synaptic plasticity, memory and cognition, anti-inflammatory effects, reproductive function, and neuroprotection (Brandt et al., 2020; Duncan and Saldanha, 2020; Garcia-Segura et al., 2003; Lu et al., 2019, 2020; Saldanha, 2020; Terasawa, 2018; Wang et al., 2020).

The effect of diet upon brain aromatase has been little studied, although there has been some work examining the effect of flavonoids and phytoestrogens, as well as hypoglycemia and obesity/body mass index (BMI) on brain aromatase in rodents and humans. Flavonoids are found in many plants and represent the most common polyphenolic compounds in human diet. Red wine is a well-known flavonoid, and *in vivo* studies revealed a significant stimulatory effect of chronic (8-week) treatment of red wine on aromatase expression and activity in the rat hippocampus (Monteiro et al., 2008). The effect in the hippocampus is unlikely to be due to procyanidins in red wine as a similar 8-week treatment with procyanidins had no effect on hippocampal aromatase expression or activity (Monteiro et al., 2008). Since red wine has been shown in many studies to have anti-oxidant properties and to be neuroprotective (Amodio et al., 2006; Basli et al., 2012), the authors suggested that the neuroprotective effect of red wine may involve mediation by aromatase and BDE_2 (Monteiro et al., 2008). However, this intriguing suggestion remains to be tested. In contrast to the effect of flavonoids, phytoestrogens, which are estrogen-like compounds derived from plants, were found to have no effect upon brain aromatase levels in male rats (Lephart et al., 2001; Weber et al., 2001). However, another study found that administration of the phytoestrogen, genistein to ovariectomized rats increased protein synthesis in the brain and this effect was blocked by the aromatase inhibitor, fadrozole (Lyou et al., 2008). The authors suggest that aromatase in the peripheral and in the brain helps mediate the effects of genistein on protein synthesis in the brain (Lyou et al., 2008). PET imaging of the human brain using ^{11}C -vorozole revealed that obesity (BMI) was associated with lower aromatase availability in the amygdala in both male and females, although the mechanism underlying this effect is unclear (Biegon et al., 2020). Interestingly, the investigators found that aromatase availability in the amygdala was positively correlated with personality trait constraint, and they suggested that the brain's capacity to make E_2 may influence the risk of obesity and self-control in men and women (Biegon et al., 2020).

Finally, recent evidence suggests that BDE_2 may regulate glucose homeostasis through actions in the ventromedial nucleus (VMN), an important site for control of glucose homeostasis in the body.

Quantification of E_2 levels in the male rats revealed that acute and chronic hypoglycemia, respectively enhanced or decreased E_2 levels in both the rostral and caudal VMN (Bheemanapally et al., 2020). In female rats, acute hypoglycemia increased E_2 levels in the rostral VMN but not in the caudal VMN, while chronic hypoglycemia increased E_2 in the caudal VMN with no change observed in the rostral VMN. The authors suggest that BDE_2 in the VMN may contribute to regulation of glucose homeostasis (Bheemanapally et al., 2020). In possible support of this suggestion, inhibition of BDE_2 synthesis in the VMN by letrozole administration attenuated hypoglycemic upregulation of the energy regulator proteins, 5-AMP-activated protein kinase (AMPK) and glucagon in male and female rats (Uddin and Briski, 2021). Furthermore, letrozole treatment was shown to inhibit hypoglycemic-induced glycogen elevation in the VMN, while exerting a stimulatory effect in females (Ibrahim et al., 2020). The authors suggest that BDE_2 facilitates hypoglycemic induction of VMN glycogen levels in males, but acts to inhibit glycogen levels in hypoglycemic females (Ibrahim et al., 2020).

3.7. Environmental pollutants

Of significant note, several groups have demonstrated that environmental pollutants can significantly increase expression of brain aromatase and BDE_2 release. For example, tributyltin, an environmental pollutant and retinoid X receptor binder, is an organic compound of tin and is a widely used pesticide in marine environments. It can penetrate the blood brain barrier and accumulates in the brain. Interestingly, 48-h treatment with tributyltin (0.1 μM) was shown to increase aromatase expression and led to a 2-fold increase of E_2 release from rat hippocampal slices (Munetsuna et al., 2014). Further evidence of a role for retinoid X receptors in regulation of aromatase and BDE_2 was demonstrated by studies showing that treatment of rat hippocampal slices with bexarotene, a retinoid X receptor agonist, also increased E_2 levels as well as aromatase expression (Ishihara et al., 2019). Bexarotene also attenuated oxygen-glucose deprivation-induced neuronal cell death, and this was suggested to be mediated by BDE_2 , as it was blocked by letrozole (Ishihara et al., 2019). Intriguingly, the human brain-specific aromatase promoter has six retinoid X receptor half sites, two of which were responsible for the increased aromatase expression by bexarotene (Ishihara et al., 2019). Furthermore, 9-cis-retinoic acid, which can also activate retinoid X receptors, was also shown to induce a 1.7 fold increase of aromatase protein and a 2-fold increase of *de novo* synthesis of E_2 from rat hippocampal slices (Munetsuna et al., 2009). Another well-known environmental toxin, dioxin, has also been shown at doses as low as 1 pM to significantly increase aromatase expression and activity in human glioma cells via an effect that involved extracellular signal regulated kinases (ERK) activation and enhanced CCATT-enhancer binding proteins (C/EBP) binding activity within exon 1.f promoter (Tan et al., 2013). Furthermore, bisphenol A, a plastics monomer and hormonally active pollutant, induced robust brain-specific expression of aromatase in the zebrafish embryo, an effect that involved nuclear estrogen receptors (Chung et al., 2011). The reason why BDE_2 is induced by environmental pollutants is unclear. However, environmental pollutants have been implicated to have a detrimental effect upon the brain as they induce oxidative stress, inflammation and apoptosis in the brain (Hassoun et al., 1998; Mitra et al., 2013). Thus, induction of aromatase and BDE_2 by environmental pollutants could be a defense mechanism to protect the brain from the oxidative stress and inflammatory damage caused by the environmental pollutants. Further studies are needed to explore this possibility.

3.8. Drugs

Both recreational and medicinal drugs have been implicated to regulate brain aromatase. For instance, studies from multiple species, including humans, suggest that nicotine can inhibit brain aromatase levels. Studies in fetal and neonatal rats and mice found that nicotine

administration significantly inhibited forebrain aromatase activity (Bertilsson et al., 1976; von Ziegler et al., 1991). Likewise, PET imaging in female baboons revealed that nicotine administration dose-dependently decreased ^{11}C -vorozole uptake in the brain, with the amygdala and POA showing the largest reductions (Biegón et al., 2012, 2010), indicating that nicotine decreases aromatase levels in the non-human primate brain. Since the doses of nicotine used in the study produced plasma levels similar to those found in smokers, the findings raise the possibility that smoking may inhibit brain aromatase and BDE₂ levels, although this remains to be verified. Similar to the effects of nicotine, administration of the barbiturate, phenobarbital (3.5 g/kg for 5 days) in adult male mice was shown to result in a 50 % reduction of brain aromatase activity (Weidenfeld et al., 1983). Brain aromatase activity had returned to control group levels when examined five days after termination of phenobarbital treatment. Chronic (28 day) treatment with the antipsychotic drugs, clozapine and haloperidol, induced an increase in aromatase mRNA levels, but not protein, in male rat brain (Bogus et al., 2019). In contrast, the antipsychotic drug, olanzapine had no effect on aromatase mRNA or protein expression in the brain (Bogus et al., 2019). Since this study did not measure aromatase activity or BDE₂ levels, and clozapine and haloperidol regulation did not extend to aromatase protein levels, the significance of these findings are unclear and will require further investigation. Finally, morphine treatment was shown to increase aromatase expression in rat hippocampal neurons and in the brain of male mice and rats (Aloisi et al., 2010; Cui et al., 2011; Khazali and Mahmoudi, 2019), as well as E₂ release in rat hippocampal neurons *in vitro* (Cui et al., 2011). Interestingly, morphine regulation of BDE₂ appears to serve a neuroprotective effect as the protective effects of morphine against amyloid toxicity in rat and human neuronal cell cultures was blocked by siRNA knockdown of aromatase (Beyer et al., 1994a).

3.9. Brain injury and inflammation

As mentioned previously, aromatase is generally expressed only in neurons basally, with astrocytes showing little to no expression. However, in 1999 Garcia-Segura's group was the first to demonstrate that aromatase can be strongly induced in astrocytes in various regions of the male and female rat and mouse brain following a penetrating brain injury or injection of the excitotoxin kainic acid (Garcia-Segura et al., 1999). In kainic acid-injected animals, aromatase-positive astrocytes were observed in all brain regions that had significant neuronal damage, including the hippocampus, pyriform and entorhinal cortex, amygdala, and bed nucleus of the stria terminalis (Garcia-Segura et al., 1999). Interestingly, neuronal aromatase expression was not affected by kainic acid injection. Furthermore, in the penetrating injury model, aromatase-positive astrocytes were observed in all injured brain regions, including the striatum, corpus callosum, cortex, hippocampus, hypothalamus, and thalamus (Garcia-Segura et al., 1999). These findings indicate that aromatase is induced in astrocytes in most areas of the brain following excitotoxic or penetrating brain injury. Furthermore, penetrating brain injury also induced a significant increase of aromatase activity, indicating increased local E₂ production (Garcia-Segura et al., 1999). Subsequent studies in the zebra finch confirmed penetrating brain injury upregulated aromatase in astrocytes in the lesion site within 24–48 h after injury and demonstrated a parallel upregulation of local E₂ (Mehos et al., 2016; Peterson et al., 2001). Robust aromatase induction in astrocytes and local E₂ elevation has also been observed in the hippocampus at 2–7 days following global cerebral ischemia (GCI) in male and female rats and mice (Cincioglu et al., 2012; Kelicen Ugur et al., 2011; Lu et al., 2020; Wang et al., 2020; Zhang et al., 2014). Focal cerebral ischemia has also been shown to increase aromatase expression in the penumbra/peri-infarct area of the cerebral cortex in rats (Carswell et al., 2005; Zhong et al., 2017).

The mechanisms underlying aromatase induction after brain injury are not entirely clear. However, brain injury is known to induce several

pathological mechanisms that have been implicated in the regulation of brain aromatase, such as inflammation, cytokine and prostaglandin production, as well as induction of reactive oxygen species and oxidative stress (Lozano et al., 2015). In support of a potential regulatory role of inflammation and cytokines, it was found that expression of the cytokines, interleukin-1beta (IL-1 β) and interleukin-6 (IL-6) are increased several hours prior to the increase of aromatase in astrocytes following a penetrating injury to the brain of male zebra finch (Duncan and Saldanha, 2011). Furthermore, administration of lipopolysaccharide (LPS) or phytohemagglutinin to induce inflammation in the brain strongly induced expression of brain aromatase (Duncan and Saldanha, 2011; Sadasivam et al., 2014). Interestingly, LPS-induced acute inflammation was associated with increased brain expression of phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme (Sadasivam et al., 2014). Further work showed that inhibition of PEPCK by administration of the inhibitor glipizide significantly attenuated the inflammation-induced upregulation of aromatase expression in the brain, as well as the steroidogenic enzymes 3 β -HSD, 17 β -HSD and steroidogenic acute regulatory protein (StAR) (Sadasivam et al., 2014). The authors suggest that PEPCK may have an important role in regulating brain neurosteroidogenesis mediated by inflammation (Sadasivam et al., 2014). Interestingly, expression of proinflammatory cytokines was increased by inhibition of PEPCK, which the authors suggested could be due to the decreased production of neurosteroids (Sadasivam et al., 2014).

Additional work has implicated prostaglandin E₂ (PGE₂) as a key factor regulating aromatase and BDE₂. PGE₂ is increased in the male and female zebra finch brain after a penetrating injury (Pedersen et al., 2017, 2018), and central administration of the prostaglandin inhibitor, indomethacin was shown to reduce brain injury-induced elevation of aromatase and BDE₂ in the zebra finch brain (Pedersen et al., 2018). Use of more specific E-prostanoid (EP) receptor antagonists for EP3 and EP4 receptors implicated EP3 receptors in mediating BDE₂ release in the injured male zebra finch brain, while EP4 receptors were implicated in injury-induced BDE₂ release in females (Pedersen and Saldanha, 2017). Additionally, inflammation and PGE₂ have also been demonstrated to increase aromatase activity and BDE₂ in the immature rat and human cerebellum, with the PGE₂ effect similarly involving EP3 and EP4 mediation (Dean et al., 2012; Wright et al., 2019), which indicates that inflammation and PGE₂ regulation of aromatase and BDE₂ occurs in other brain areas and in multiple species. Finally, nitrosative stress/oxidative damage to sheep astrocytes or sheep neurons in culture was shown to increase aromatase gene expression and immunoreactive protein levels (Lepore et al., 2009, 2011). This finding raises the possibility that oxidative stress following brain injury may also contribute to enhanced E₂ production in the brain. As a whole, the studies indicate that trauma and ischemic injury to the brain causes a robust elevation of aromatase in astrocytes and a corresponding increase in BDE₂, possibly through induction of inflammation, cytokines, and prostaglandins. Many of the above studies were performed in birds, with few studies in other species. Thus, future studies using other species are needed. Functionally, the elevation of aromatase and BDE₂ after brain injury has been implicated to exert important neuroprotective and anti-inflammatory actions to help protect and repair the injured brain, which will be discussed in detail in a subsequent section (Arevalo et al., 2015; Brocca and Garcia-Segura, 2019; Garcia-Segura et al., 2003, 1999; Wang et al., 2020).

Interestingly, in addition to trauma and ischemic brain injury, there is evidence that aromatase and local E₂ are altered in other types of neurodegenerative disorders, although the number of studies is relatively small. For instance, aromatase expression was found to be increased in astrocytes at disease onset in an animal model of amyotrophic lateral sclerosis, followed by decreased expression as the disease progressed (Eisenman, 1988). In addition, examination of brain tissue samples from Alzheimer's disease (AD) subjects revealed increased aromatase immunoreactivity in the hippocampal CA4 region, but

contrastingly aromatase immunoreactivity was decreased in the brains of 5xFADD mice versus wild type controls (Prange-Kiel et al., 2016). Increased aromatase mRNA and immunoreactive protein levels were also demonstrated in the prefrontal cortex of the late-stage human AD brain, with the aromatase increase occurring primarily in astrocytes (Luchetti et al., 2011). Unfortunately, local E₂ levels were not determined in these two studies on AD. A subsequent study did measure brain E₂ levels in postmortem brain samples from women with AD, as well as aromatase expression and immunoreactivity (Yue et al., 2005). This study found that aromatase expression, aromatase immunoreactivity, and E₂ levels were significantly decreased in frontal cortex and cerebellum of AD subjects, as compared to normal controls (Yue et al., 2005). In addition, there was a significant negative correlation between the reduced aromatase expression and amyloid plaque density in the cortex in AD subjects, suggesting that BDE₂ may protect the AD brain by regulating plaque formation (Yue et al., 2005). In support of this possibility, APP23/Ar^{+/-} mice, which are transgenic mice that overexpress amyloid precursor protein (APP) and lack aromatase, were found to have increased BACE activity as well as early onset and increased A β deposition in the brain (Yue et al., 2005). Finally, seizure activity in a rat model of status epilepticus was found to stimulate *de novo* synthesis of E₂ in the hippocampus (Sato and Woolley, 2016). Furthermore, systemic or intra-hippocampal administration of the AI, fadrozole in gonadectomized rats suppressed kainic acid-induced seizures (Sato and Woolley, 2016). These findings suggest that over-production of hippocampal-derived E₂ may have a role in promoting seizures and that aromatase inhibitors may have therapeutic utility for status epilepticus.

4. Role of brain aromatase and BDE₂ in sexual differentiation, reproduction and socio-sexual behavior

The above studies demonstrated aromatase localization and BDE₂ production in many brain regions in multiple species, and showed regulation occurs by multiple processes and factors, often in a tissue- or cell-specific manner. In the following sections, we will review and discuss the evidence supporting multiple important functional roles implicated for BDE₂ in the brain.

4.1. Regulation of sexual differentiation

In many species, including rodents, birds, ruminants and carnivores, neuronal aromatase and BDE₂ have been implicated to play a key role in masculinization of the brain (Gorski, 1985), although the evidence for a similar role in other mammalian species such as monkeys and humans is less clear. As discussed previously, aromatase is highly expressed in the hypothalamus and sexual dimorphic regions of the POA (Sasano et al., 1998; Selmanoff et al., 1977). During the perinatal period, aromatase in these and other brain regions acts to convert testosterone to estrogen, which then contributes significantly to sexual differentiation (masculinization) of the brain (McCarthy, 2008). Masculinization of the brain is characterized by expression of male-typical sexual behavior, male-type pattern of gonadotropin secretion, and aggression (Negri-Cesi et al., 2004). In addition, there is a second process of defeminization, in which male ability to exhibit female typical behaviors in adulthood is significantly attenuated (Negri-Cesi et al., 2004). After exposure to testosterone from the developing testes, the mammalian brain develops as male, while in the absence of such exposure it develops as female (Gorski, 1985). In females, the lack of early exposure to testosterone is essential for sexual behavior and expression of the ovulatory surge of gonadotropins (Gorski, 1985; Negri-Cesi et al., 2004).

In support of a key role for aromatase and BDE₂ in sexual differentiation of the brain in rodents, early work showed that estrogen administration neonatally in castrate male rats suppressed the female pattern of gonadotropin secretion and behavior, while inducing the male pattern of sexual behavior (Booth, 1977). This finding suggested that sexual differentiation of the brain involves aromatization of testicular

androgens to estrogens in the brain. In further support of this suggestion, administration of an aromatase inhibitor perinatally to male rats resulted in reduced development of the sexual dimorphic nucleus in the POA and reduced masculine sexual behavior (Houtsmuller et al., 1994). Injection of an aromatase inhibitor later on day 12 postnatally had no significant effect in male rats, indicating that BDE₂ acts in the early postnatal period to induce sexual differentiation (Gonzalez and Leret, 1994). Furthermore, BDE₂ actions appear to be mediated by estrogen receptors as postnatal administration of the estrogen receptor antagonist, tamoxifen inhibited sexual differentiation of the brain (Dohler et al., 1984). Further work using estrogen receptor knockout mouse models has suggested that ER α mediates masculinization, while ER β is critical for the defeminization effects of BDE₂ (Kudwa et al., 2006). BDE₂ appears to regulate sexual differentiation through multiple processes including regulation of apoptosis, neurite outgrowth, and synaptic patterning in various brain regions (McCarthy, 2008; Tsukahara and Morishita, 2020). Additional work indicates that BDE₂ increases excitatory inputs to the POA in order to promote male sexual behavior in adulthood, and there is evidence this effect involves an increase in PGE₂ that promotes anchoring of glutamate receptors in dendritic spines (Wright et al., 2010). There is also evidence that BDE₂ enhances glutamate release in the hypothalamus as a mechanism to facilitate defeminization (Schwarz et al., 2008).

4.2. Regulation of reproduction

In addition to regulation of sexual differentiation, there is increasing evidence that BDE₂ has an important role in adult animals to regulate GnRH and gonadotropin secretion, as well as puberty and socio-sexual behavior. With respect to a role in regulation of gonadotropin secretion, recent work has demonstrated that systemic letrozole administration strongly attenuated the exogenous E₂-induced LH surge in ovariectomized monkeys (Kenealy et al., 2017). This effect appears to be due to actions in the hypothalamus to regulate factors that control LH release, as letrozole administration in the median eminence of the hypothalamus of ovariectomized monkeys significantly attenuated the exogenous E₂-induced surges of kisspeptin and GnRH, which are the key hypothalamic factors responsible for the LH surge (Kenealy et al., 2013; Terasawa, 2018). Estradiol benzoate administered into the median eminence of ovariectomized monkeys was also found to rapidly stimulate release of pulsatile GnRH and E₂ and this effect was blocked by letrozole treatment. The investigators also found that excitation of the mediobasal hypothalamus through electrical stimulation stimulated both GnRH and E₂ release, suggesting an activity-dependent regulation of BDE₂ in the hypothalamus (Kenealy et al., 2013; Terasawa, 2018). Additional work revealed that direct administration of letrozole into the median eminence of ovariectomized monkeys suppressed spontaneous GnRH release and estradiol benzoate-induced release of GnRH and E₂ (Kenealy et al., 2013; Terasawa, 2018). Collectively, these studies provide evidence that BDE₂ is involved in regulation of pulsatile GnRH release and is necessary for full expression of the GnRH and LH surge. In addition to these effects, there is also evidence that BDE₂ may contribute to negative feedback regulation of gonadotropin secretion. In support of this suggestion, chronic daily letrozole treatment of ovariectomized female monkeys resulted in a suprahypogonadotropic phenotype one month after treatment was initiated (Kraynak et al., 2017). A potential role for BDE₂ in negative feedback control in males was also suggested based on the finding that treatment of male monkeys with the aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD) reduced hypothalamic aromatase activity by 80–90 % and resulted in elevation of LH (Ellinwood et al., 1984). Likewise central administration of the aromatase inhibitor fadrozole in male sheep increased LH pulse frequency, suggesting that central aromatization controls pulsatile LH secretion (Sharma et al., 1999).

The above finding that BDE₂ may exert negative feedback control over gonadotropin secretion is intriguing and raises the possibility it

could participate in restraining GnRH pulsatility until the time of puberty. During the infantile period after birth, there is strong GnRH pulsatility, which becomes quiescent during childhood, and then recommences at the time of puberty. This finding has led to the suggestion that there is a “brake” or inhibitory signal that restrains GnRH pulsatility, which is released at puberty. Intriguingly, Lephart and Ojeda (Lephart and Ojeda (1990)) found that in both male and female rats, hypothalamic aromatase activity is significantly decreased at the time of puberty. Furthermore, hypothalamic aromatase mRNA and protein levels were also shown to be decreased at the onset of true precocious puberty in female rats (Tian et al., 2004). Additional work revealed that stalk-median eminence levels of E_1 and E_2 levels are higher at the prepubertal phase in female monkeys than at later early pubertal phase when GnRH release begins to increase at night (Kenealy et al., 2016). This finding suggests that the elevated E_1 and E_2 are hypothalamic in origin as circulating E_1 and E_2 are low in prepubertal and early pubertal monkeys. Collectively, these findings raise the possibility that BDE_2 may play a role in central inhibition of GnRH before the onset of puberty. This intriguing hypothesis awaits further testing.

4.3. Regulation of socio-sexual behavior

Over the past several decades, there has emerged abundant evidence that BDE_2 regulates socio-sexual behavior. A large part of this work has been performed in male quail, although some work has also been performed in other species. Early work in adult male quail found that exogenously administered testosterone must be aromatized to activate copulation (Watson and Adkins-Regan, 1989). In the study, administration of an aromatase inhibitor blocked copulatory behavior in castrate males that had testosterone implants in the POA. Subsequent work demonstrated that systemic administration of the aromatase inhibitor vorozole rapidly inhibited sexual motivation in male quail (Cornil et al., 2006). Additional support for a role of BDE_2 in regulation of sexual behavior came from the finding that central administration of vorozole rapidly inhibited sexual motivation but not sexual performance in male quail (Seredynski et al., 2013). Interestingly, central administration of E_2 or membrane-impermeable E_2 analogs was also able to enhance sexual motivation in estrogen-deplete male quail (Seredynski et al., 2013). This suggests that the effects of BDE_2 may involve mediation by membrane estrogen receptors. In support of this suggestion, administration of estrogen receptor antagonists have been shown to decrease sexual motivation (Seredynski et al., 2013). BDE_2 may also regulate aggressive behavior in males. In support of this possibility, high aromatase activity in the anterior hypothalamus/POA was shown to be correlated with aggressive behavior in male quail and male song sparrows (Schlinger and Callard, 1989; Soma et al., 2003). Furthermore, administration of an aromatase inhibitor inhibited aggression in reproductively active male quail (Schlinger and Callard, 1990) and in nonbreeding male song sparrows in winter (Soma, 2006), and blocked testosterone-induced aggression in reproductively inactive male quail (Schlinger and Callard, 1990).

Studies in mice also support a role for brain aromatase in sexual behavior. Whole body aromatase knockout in male mice resulted in deficits in motivational and consummatory sexual behavior (Bakker et al., 2002). In addition, a brain-specific aromatase knockout mouse was recently generated by crossing floxed aromatase mice with nestin-cre mice and used to examine the role of BDE_2 in male sexual behavior (Brooks et al., 2020). The brain-specific aromatase male mice exhibited a 50 % decrease in number of mounts and intromissions, which could be rescued by testosterone and E_2 replacement. The mice had elevated testosterone levels which the authors postulated could be due to a defect in negative feedback upon gonadotropin secretion (Brooks et al., 2020). However, they were unable to demonstrate any significant effect on gonadotropin levels in the knockout mice versus wild type mice.

Finally, there have been contradictory results on whether

aromatization is necessary for male sexual behavior in humans. One study reported that in men who received testosterone, treatment with an aromatase inhibitor was associated with significant decreases in sexual desire and erectile function (Finkelstein et al., 2013). In contrast, another study reported that administration of an aromatase inhibitor or an estrogen receptor antagonist had no effect upon sexual function in men (Gooren, 1985). Likewise, it was shown that male sexual function could be maintained without aromatization in healthy men who received the non-aromatizable androgen, 5 α -dihydro-testosterone (DHT) (Sartorius et al., 2014). The study found no effect of DHT on 33 measures of sexual function except for a mild decrease in sexual desire. Taken as a whole, the above studies suggest that brain aromatization is necessary for normal sexual behavior in quail and rodents, but the case for a similar role in humans is unclear and requires additional study.

5. Role of brain aromatase and BDE_2 in synaptic plasticity and cognitive function

5.1. Synaptic plasticity

BDE_2 has been implicated to also play an important role in regulating synaptic plasticity in the brain in a variety of species. Rune and co-workers were the first to report that letrozole treatment of rat hippocampal slices cultured *in vitro* decreased E_2 release and the density of spines and spine-synapses, as well as decreased levels of the presynaptic protein synaptophysin and the postsynaptic protein spinophilin (Kretz et al., 2004). A decrease of spine synapses in rat hippocampal neurons cultured *in vitro* was also observed following letrozole treatment (Kretz et al., 2004). Furthermore, letrozole treatment in mouse hippocampal slice cultures caused decreases in mitochondrial volume, dendritic spine density and synaptic proteins, and exacerbated $A\beta^{1-42}$ -induced mitochondrial and synaptic plasticity defects (Chang et al., 2013). *In vivo* letrozole treatment of adult female mice decreased spine synapse density in the hippocampal CA1 region and dentate gyrus molecular layer of the hippocampus; with no significant effect observed in the prefrontal cortex or cerebellum (Bender et al., 2010; Fester et al., 2012; Prange-Kiel et al., 2013). Similar to the *in vitro* findings, *in vivo* letrozole treatment also decreased spinophilin and synaptophysin expression levels in the female mouse hippocampus, as well as the glutamate receptor, NMDAR1 (Zhou et al., 2010). Interestingly, Rune and coworkers also reported a sex difference in the letrozole effect on synaptic plasticity, as *in vivo* peripheral letrozole treatment for 4-weeks decreased hippocampal spine synapses in cycling and ovariectomized female mice (Zhou et al., 2010), as well as cycling female rats, but not in male rats (Fester et al., 2012). In contrast to this report, Zhou et al. found that *in vivo* letrozole treatment did not significantly decrease hippocampal spines, synapses and post-synaptic density (PSD) proteins in male mice (Zhao et al., 2018). They also found decreased PSD thickness and increased actin depolymerization in the letrozole-treated male mice. Regulation of actin polymerization by BDE_2 may help explain the changes in spine density observed in the letrozole-treated mice, as the actin cytoskeleton plays an important role in spine formation, elimination and morphology (Harris, 1999; Matus, 2000). It is not clear why the two studies yielded divergent results with regards to the sex-specific effect of letrozole on synaptic plasticity. While both groups used 4-weeks of daily intraperitoneal administration of letrozole, Zhou et al. used a two-fold higher dose of letrozole as compared to Rune's group (e.g., 80 μ g/kg BW versus 40 μ g/kg BW). Interestingly, some studies have found that E_2 levels in the male hippocampus are higher than that observed in females (Oishi et al., 2012). Therefore, a higher dose of letrozole might be required to effectively reduce hippocampal E_2 levels in males and observe an inhibitory effect on synaptic plasticity.

Long-term potentiation (LTP) is widely considered a synaptic mechanism for memory (Bliss and Collingridge, 1993; Nicoll, 2017). Over the past decade, a number of groups have examined the role of aromatase and local-derived E_2 in LTP in several brain regions. Grassi

and coworkers were the first to demonstrate that local-derived E_2 is essential for induction of LTP in the brainstem (Grassi et al., 2009). Using male rat brainstem slices, they showed that letrozole had no effect on baseline glutamatergic synaptic responses but prevented LTP produced by high frequency tetanic stimulation (HFS) (Grassi et al., 2009). HFS administered at 5 min after letrozole administration did not induce LTP (Grassi et al., 2009). This finding would appear to rule out relevant storage of E_2 and raises the possibility that HFS enhances aromatase activation to rapidly induce E_2 . In support of this possibility, HFS has been shown to induce E_2 release in spinal cord slices, which could be blocked by aromatase inhibitor treatment (Zhang et al., 2012). These findings suggest that activity-dependent regulation of local E_2 synthesis and release is a key mechanism mediating HFS-induced LTP.

Subsequent work demonstrated that local-derived E_2 similarly mediates LTP in the hippocampus (Grassi et al., 2011). Using male rat hippocampal slices, the investigators found that letrozole reduced the amplitude of LTP by 60 % while not affecting baseline responses. A full LTP response could be rescued by exogenous E_2 treatment in letrozole-treated slices (Di Mauro et al., 2017). In additional studies, inhibition of E_2 synthesis by letrozole also prevented LTP in medial spiny neurons and cholinergic interneurons in the dorsal striatum of male rats, and this effect could be rescued by exogenous E_2 (Tozzi et al., 2015). Letrozole treatment also prevented LTP at the cerebellar parallel fiber-Purkinje cell synapse, which was correlated with impairment of both gain increases and decreases adaption of the vestibulo-ocular reflex (Dieni et al., 2018a, b). BDE_2 likely acts through estrogen receptors to mediate its synaptic plasticity effect, as administration of an estrogen receptor antagonist also significantly reduced LTP amplitude in hippocampal slices (Grassi et al., 2011). Interestingly, letrozole treatment had no effect upon maintenance of LTP or on pair pulse facilitation, a type of presynaptic plasticity that involves a change in neurotransmitter release probability (Grassi et al., 2011). Further work found that letrozole also had no effect upon induction of long-term depression (LTD) in hippocampal and cerebellar slices, which indicates that BDE_2 only regulates induction of LTP and not LTD in the hippocampus and cerebellum (Di Mauro et al., 2017, 2015; Dieni et al., 2018a). Letrozole can lead to an increase of androgens due to inhibition of their conversion to estrogen. This raises the possibility that androgens may mediate the effect of letrozole on LTP. However, the fact that E_2 replacement rescued LTP would seem to argue against this possibility. Furthermore, Tozzi et al. (Tozzi et al., 2019) showed that androgen receptor antagonists had no effect on LTP in male rat hippocampal slices. Thus, it appears that activation of estrogen receptors, but not androgen receptors, is critical for inducing LTP in hippocampal pyramidal neurons. The finding of a role of BDE_2 in LTP also extends to mice and to females, as 7-day *in vivo* letrozole treatment reduced LTP amplitude in hippocampal slices from ovariectomized mice by 50 %, and by 20 % in male mice (Vierk et al., 2012). It is not clear why the magnitude of inhibition of LTP in this study in male mice was less than that observed in the above studies for male rats (e.g. 20 % versus 60 % inhibition). It could be due to the different species used, different *in vitro* versus *in vivo* letrozole treatment paradigms, and/or different dosing schedules employed in the studies.

Finally, to more specifically explore the roles and function of neuron-derived E_2 (NDE₂) in the regulation of synaptic plasticity in the brain, our group generated a forebrain-neuron-specific aromatase knock-out (FBN-ARO-KO) mouse model to specifically deplete NDE₂ in the forebrain (Lu et al., 2019). *Cyp19a1-Cre/LoxP* conditional KO mice were created, under the control of the CaMKII α promoter, which expresses *Cre* exclusively in forebrain excitatory neurons. Characterization of the mice using PCR, Western blot, IHC and ELISA analysis demonstrated a profound loss of aromatase in hippocampal and cortical neurons with an associated 65–70 % decrease of E_2 levels in the hippocampus and cortex of both male and ovariectomized female FBN-ARO-KO mice, as compared to FLOX mice (Lu et al., 2019). Aromatase expression was unchanged in the hindbrain and ovary and there was no change in serum E_2 levels in FBN-ARO-KO versus FLOX mice, demonstrating that the

knockout was specific for forebrain neurons. Furthermore, the postnatal deletion of the aromatase gene in excitatory forebrain neurons did not result in any apparent defects in brain structure in FBN-ARO-KO mice (Lu et al., 2019).

Interestingly, Golgi analysis revealed that spine density is significantly decreased in the hippocampal CA1 region and cortex of adult male and ovariectomized female FBN-ARO-KO mice, with thin spines showing the greatest decrease in males, and mushroom spines showing the greatest decrease in females (Lu et al., 2019). This finding indicates that NDE₂ is an important regulator of spine formation in the cerebral cortex and hippocampal CA1 region in both male and female mice. Examination of synapse number by examining synaptophysin and PSD95 contacts revealed a 33 % decrease of synapse density in the hippocampal CA1 and cortex of male FBN-ARO-KO mice, and a 55–58 % decrease in female FBN-ARO-KO mice (Lu et al., 2019). Thus, this result suggests that NDE₂ also regulates forebrain synaptic density in both male and female mice.

We also examined the role of NDE₂ in regulation of synaptic transmission and LTP in our FBN-ARO-KO mouse model (Lu et al., 2019). The results revealed a significant defect in the efficiency of synaptic transmission in hippocampal slices of both male and ovariectomized female FBN-ARO-KO mice, which was fully rescued by exogenous E_2 (Lu et al., 2019). Examination of LTP in hippocampal slices from FLOX and FBN-ARO-KO mice revealed a 57 % decrease in the amplitude of LTP in male FBN-ARO-KO mice versus a 91 % decrease in female FBN-ARO-KO mice (Lu et al., 2019). As seen in the effect upon synaptic density above and here for LTP, depletion of forebrain E_2 in FBN-ARO-KO mice significantly decreased synaptic density and LTP amplitude in both sexes, but a greater percent decrease was observed in ovariectomized females as compared to males. Nevertheless, the effects in both sexes appear functionally significant, as cognitive function was robustly and significantly attenuated in both sexes of FBN-ARO-KO mice (e.g., significantly decreased in ovariectomized females, intact females and intact males) (Lu et al., 2019), as discussed in the section below. It is not entirely clear which comes first following NDE₂ depletion, loss of spines and synapses or loss of LTP. It is possible that the loss of spines and synapses could drive a diminishment of LTP in the forebrain. However, in argument against this, Vierk et al. (Vierk et al., 2012) found that LTP impairment preceded the loss of spines and synapses in the hippocampus of letrozole-treated female mice. Furthermore, we found that LTP in hippocampal slices from FBN-ARO-KO mice could be rapidly rescued within minutes by exogenous E_2 (Lu et al., 2019), an effect that would seem too fast for induction of spine synapses to have occurred. Interestingly, LTP has been reported to induce spines and synapses in the brain (Toni et al., 1999), further supporting that loss of LTP could drive the loss of spine and synapses following BDE_2 depletion. Nevertheless, before definitive conclusions can be reached, more studies are needed to understand the precise temporal dynamics underlying the spine and synapse loss and LTP impairment observed in the FBN-ARO-KO mice.

Mechanistically, both non-genomic and genomic signaling mechanisms may contribute to NDE₂ regulation of synaptic plasticity (Lai et al., 2017). In support of a non-genomic mechanism, we found that E_2 replacement *in vitro* could rapidly rescue the decrease in synaptic transmission efficiency and LTP amplitude in hippocampal slices from both male and female FBN-ARO-KO mice (Lu et al., 2019). Furthermore, the E_2 rescue appeared to involve rapid mitogen-activated protein kinase (MAPK) signaling as it was blocked by co-administration of the MAPK inhibitor, U0126 (Lu et al., 2019). In further support of a critical role for NDE₂ in regulating rapid kinase signaling in the forebrain, both AKT and ERK activation were found to be significantly attenuated in the hippocampus and cortex of both male and female FBN-ARO-KO mice, and this effect could be rescued by exogenous E_2 replacement (Lu et al., 2019). Functionally, both AKT and ERK signaling have been implicated to mediate LTP and synaptic plasticity in the brain (Levenga et al., 2017; Mao and Wang, 2016; Sweatt, 2001). Furthermore, exogenous E_2 rapidly enhances induction of both signaling pathways in the

hippocampus and cortex (Ogiue-Ikeda et al., 2008; Singh, 2001; Singh et al., 1999), and inhibitors to these pathways block exogenous E₂-induced LTP in hippocampal slices (Hasegawa et al., 2015). The rapid signaling effects of NDE₂ on kinase signaling are proposed to be mediated by estrogen receptors localized at the cell membrane. Indeed, ER α and ER β , in addition to being localized in the cytoplasm and nucleus, have also been reported to be localized at the cell membrane in neurons (Evinger and Levin, 2005; McEwen et al., 2001; Milner et al., 2005, 2001; Raz et al., 2008), and the newest proposed member of the estrogen receptor family, G-protein coupled estrogen receptor-1 (GPER1), has also been shown to be localized at the membrane in various cell types including neurons (Kumar and Foster, 2020; Raz et al., 2008; Revankar et al., 2005; Waters et al., 2015). Furthermore, all three receptors have been implicated in mediating both the rapid signaling and plasticity effects of E₂ in the brain (Fester et al., 2013; Kumar et al., 2015; Kumar and Foster, 2020; Liu et al., 2008; Mukai et al., 2010; Raz et al., 2008; Sellers et al., 2015; Smejkalova and Woolley, 2010; Spencer-Segal et al., 2012). While we did not examine which estrogen receptor mediates NDE₂ effects in our studies, future studies are planned to test selective ligands for the three estrogen receptors in our FBN-ARO-KO mice to help determine which receptor(s) mediates rescue of the plasticity and memory defects. Testing of membrane impermeable E₂-BSA or E₂-dendrimer conjugates to further confirm the role of membrane localized estrogen receptors and cytoplasmic estrogen receptors would also be important. Interestingly, the results of our study did suggest a possible role for NDE₂ in differential regulation of estrogen receptor levels in the forebrain, as we found ER β is up-regulated in the hippocampus and cortex of FBN-ARO-KO mice, while ER α is down-regulated (Lu et al., 2019). Similar to our results in FBN-ARO-KO mice, letrozole treatment was found to also decrease ER α while increasing ER β in mouse hippocampal slices (Fester et al., 2013). Since neither study examined GPER1 expression, it is not clear whether GPER1 is also regulated by BDE₂. Therefore, future studies are needed to address this question. It should be pointed out that since exogenous E₂ was able to rescue synaptic plasticity and memory defects in both FBN-ARO-KO mice (Lu et al.,

2019) and aromatase inhibitor-treated animals (Tozzi et al., 2015; Tuscher et al., 2016) it suggests that local E₂ synthesis may not be necessary for exogenous E₂ effects to enhance plasticity and memory.

Finally, we also examined cAMP response element binding protein (CREB) activation and brain-derived neurotrophic factor (BDNF) expression in the forebrain of FBN-ARO-KO mice, as both factors are also known to be key regulators of synaptic plasticity and memory (Benito and Barco, 2010; Kida, 2012; Miranda et al., 2019). Interestingly, CREB is a transcription factor activated by phosphorylation that can regulate expression of many genes, including BDNF (Kida, 2012; Shieh et al., 1998). Conversely, BDNF can regulate activation of CREB through tropomyosin receptor kinase (Trk) B receptors (Finkbeiner et al., 1997). Examination of intact male and ovariectomized female FBN-ARO-KO mice revealed a significant decrease in CREB phosphorylation (pCREB) and BDNF expression in the hippocampus and cortex of FBN-ARO-KO mice, as compared to FLOX controls (Lu et al., 2019). This effect appeared to be due to loss of forebrain E₂, as reinstatement of E₂ levels in the forebrain by exogenous E₂ replacement was able to rescue CREB activation and BDNF expression in FBN-ARO-KO mice (Lu et al., 2019). Thus, in addition to regulating rapid kinase signaling, BDE₂ appears to be a key regulator of CREB and BDNF in the forebrain, which is proposed to contribute to its plasticity and memory regulatory effects.

Based on our results from the FBN-ARO-KO mice (Lu et al., 2019), a proposed mechanism for NDE₂ effects on synaptic plasticity and LTP in the forebrain is illustrated in Fig. 5. As shown in Fig. 5, NDE₂ regulation of spines, synapses and LTP is proposed to involve membrane estrogen receptor-mediated rapid PI3K/AKT and MEK/ERK kinase signaling, which is capable of quickly modulating synaptic plasticity. As also shown in Fig. 5, the activated intracellular kinase signaling can in turn phosphorylate the important transcriptional factor, CREB which translocates into the nucleus to facilitate the expression of CREB target genes that regulate synaptic plasticity, including the neurotrophic factor BDNF and the synaptic protein PSD95. Furthermore, BDNF can also regulate synaptic plasticity by coupling to the rapid intracellular kinases. BDNF signaling can also activate cofilin, which is required for F-actin assembly

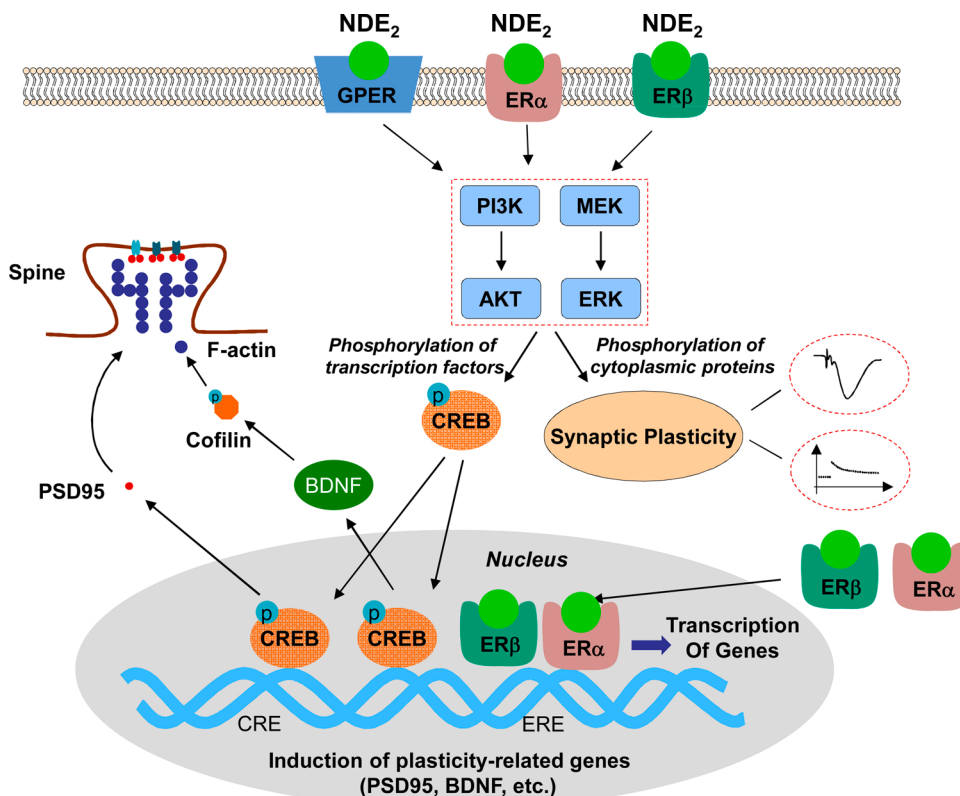


Fig. 5. Schematic illustration of the potential mechanisms underlying neuron-derived E₂ (NDE₂) regulation of synaptic plasticity. It is proposed that neuron-derived E₂ (NDE₂) regulates synaptic plasticity via both rapid and genomic signaling mechanisms. 1) Membrane localized estrogen receptors (estrogen receptor- α and β , ER α and ER β , and G-protein coupled estrogen receptor-1, GPER1) can bind NDE₂ and 2) the receptor bound NDE₂ then induces rapid PI3K/AKT and MEK/ERK kinase signaling, which is capable of quickly shaping synaptic plasticity. In addition, the activated intracellular kinase signaling also phosphorylates the important transcriptional factor, CREB which further translocates into nucleus to facilitate the expressions of neurotrophic factor BDNF and synaptic protein PSD95. BDNF can also regulate synaptic plasticity by coupling to the rapid intracellular kinases. Moreover, BDNF signaling activates cofilin which is required for F-actin assembly and dendritic spine formation. Intracellular ER α and ER β act in the genomic signaling pathway by transactivating estrogen response elements (ERE) in regulated genes and promoting genes transcription. CRE = cAMP response element.

and dendritic spine formation (Briz et al., 2015). Classical nuclear ER α - and ER β -mediated genomic signaling may also contribute to NDE₂ regulation of synaptic plasticity by transactivating estrogen response elements (ERE) in plasticity-related genes and promoting their transcription.

5.2. Cognitive function

BDE₂ regulation of synaptic plasticity and LTP in the forebrain suggests it may regulate memory. Indeed, there is now a large body of evidence in a variety of species implicating an important role of BDE₂ in the regulation of cognitive function. For instance, a number of clinical studies in humans have found that aromatase inhibitor treatment in breast cancer patients is associated with a variety of memory defects, including defects in verbal and visual learning/memory, executive function, and processing speed, which were reversible after cessation of aromatase inhibitor therapy (Bender et al., 2007; Phillips et al., 2011; Rocha-Cadman et al., 2012; Underwood et al., 2018), although there are dissenting studies (Hurria et al., 2014). Furthermore, Bayer and co-workers performed cognitive testing and MRI analysis on postmenopausal women with breast cancer and a control group to examine the effect of aromatase inhibition on cognitive function (Bayer et al., 2015). The study participants underwent cognitive testing twice before the start of letrozole therapy and then again at least 3 months after start of treatment (Bayer et al., 2015). The results revealed that letrozole treatment impaired hippocampal-dependent memory, and MRI analysis revealed this effect was associated with decreased hippocampal activity during encoding (Bayer et al., 2015). Collectively, the above studies provide evidence that BDE₂ may be important for cognitive function in humans.

Basic science studies in animals have also confirmed a critical role for BDE₂ in cognitive function. One of the first studies to examine this question was a study that used adult castrate male zebra finches (songbirds) with implants of vehicle, an aromatizable androgen (testosterone), a non-aromatizable androgen (5 α -DHT), or E₂ and measured the effect upon spatial memory (Oberlander et al., 2004). Testosterone- and E₂-treated birds, but not 5 α -DHT-treated birds, learned the spatial memory task. The investigators concluded that brain aromatization enhances spatial memory in songbirds (Oberlander et al., 2004). Subsequent work utilized aromatase inhibitors to confirm the suggested role of BDE₂ in cognitive function. These elegant studies demonstrated that aromatase inhibition in male songbirds disrupts auditory association learning and neural memory for previously heard songs (Macedo-Lima and Remage-Healey, 2020; Yoder et al., 2012). Acute aromatase inhibition was also shown to reduce motivation to sing in male canaries, as well as song acoustic stereotypy, a measure of consistency over song renditions (Alward et al., 2016). Further work showed that local-derived E₂ is important for auditory processing in the NCM, a part of the songbird auditory association cortex important for song learning, memorization and perception (Tremere et al., 2009). The study found that aromatase inhibitor infusion in the NCM markedly decreased neuronal firing rates in a dose-dependent manner in birds stimulated with conspecific songs (Tremere et al., 2009). Aromatase inhibition in the NCM was also associated with reduced induction of MAPK-dependent genes, which have been implicated to mediate synaptic plasticity and memory in the songbird (Tremere et al., 2009). In addition to the NCM, there is evidence that local E₂ also acts in the hippocampus to regulate spatial memory. For instance, central aromatase inhibition in the male zebra finch attenuated PSD95 levels in the hippocampus and impaired spatial memory in a food-finding task (Bailey et al., 2013, 2017; Bailey and Saldanha, 2015).

A role for BDE₂ in cognitive function has also been implicated in rodents. For instance, intracerebroventricular administration of letrozole for 14 days in intact male and female rats decreased hippocampal E₂ levels and hippocampal pyramidal neuron firing and caused dose-dependent defects in working memory and novel object recognition

memory (Marbouti et al., 2020a). Similarly, letrozole treatment in male and female mice resulted in spatial memory impairment (Liu et al., 2019; Zhao et al., 2018). Furthermore, infusion of letrozole bilaterally into the hippocampus impaired hippocampal memory consolidation in ovariectomized female mice, and this defect could be rescued by exogenous E₂ replacement (Tuscher et al., 2016). This finding suggests that BDE₂ has a critical role in hippocampal memory consolidation.

To confirm and extend these pharmacological studies, we used the genetic FBN-ARO-KO mouse model to specifically deplete forebrain NDE₂ and examined its role in cognitive function (Lu et al., 2019). As shown in Fig. 6, we conducted cognitive behavioral testing on intact male, intact female and ovariectomized female FLOX and FBN-ARO-KO mice using standard behavioral testing paradigms for rodents, including the Barnes maze for testing hippocampal-dependent spatial reference learning and memory, the novel object recognition test for testing hippocampal-dependent spatial recognition memory, the forced swim test for testing for depressive-like behavior, and the fear conditioning test to examine long-term fear memory. The results revealed that the adult intact male, intact female and ovariectomized female FBN-ARO-KO mice all had significant deficits in hippocampal-dependent spatial reference learning and memory, hippocampal-dependent recognition memory, and hippocampal-dependent contextual fear memory (Lu et al., 2019). These differences were not due to differences in locomotor function, as FBN-ARO-KO mice had normal locomotor function and anxiety, as measured by the open field test. Interestingly, ovariectomized female FBN-ARO-KO mice, but not intact male and intact female FBN-ARO-KO mice, exhibited depressive-like behavior in the forced swim test (Lu et al., 2019). The behavior defects in the FBN-ARO-KO mice appear to be due to the loss of forebrain E₂ as reinstatement of forebrain E₂ levels by exogenous E₂ replacement was able to fully rescue the hippocampal-dependent spatial recognition memory and reference memory in ovariectomized female FBN-ARO-KO mice (Lu et al., 2019). We did not examine E₂ rescue of fear memory or depressive behavior, and thus future studies are needed to examine this issue.

Finally, examination of male and ovariectomized female astrocyte-specific aromatase knockout (GFAP-ARO-KO) mice revealed no defects in hippocampal-dependent spatial reference learning and memory, hippocampal-dependent recognition memory or long-term fear memory Wang et al. (2020). This finding indicates that only NDE₂ regulates cognitive function in the basal (non-injured) state. This finding is not unexpected as aromatase is low to undetectable in astrocytes basally and is only induced by stress, injury or ischemia. However, as will be discussed in the next section, GFAP-ARO-KO mice do exhibit enhanced cognitive deficits after GCI, which appears to be due to a loss of a neuroprotective function of ADE₂ in the GFAP-ARO-KO mice Wang et al. (2020). Taken as a whole, the above studies indicate that NDE₂ has a critical role in regulating synaptic plasticity, LTP and memory in a variety of species.

6. Role of brain aromatase and BDE₂ in neuroprotection

6.1. Aromatase inhibitor and knockdown studies

As discussed previously, numerous studies have demonstrated that brain injury induces aromatase in astrocytes, which leads to a local increase of BDE₂ in the brain. This finding has led to the suggestion that elevation of BDE₂ may serve as a protective mechanism in response to brain injury. Azcoitia and coworkers were the first to examine this question in detail (Azcoitia et al., 2001). Based on their previous work showing that aromatase was induced in brain astrocytes after excitotoxin injury (Garcia-Segura et al., 1999), the investigators examined the potential neuroprotective effect of an aromatizable androgen (testosterone) and non-aromatizable androgen (5 α -DHT), as well as E₂ in the hippocampus. They found that testosterone and E₂, but not 5 α -DHT, was neuroprotective against the excitotoxin, domoic acid in castrate male

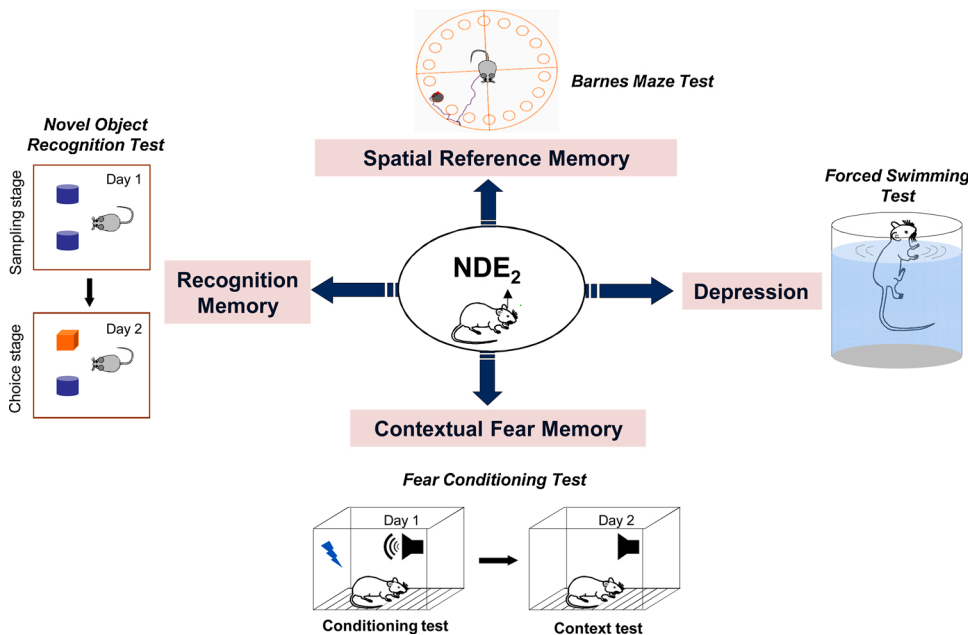


Fig. 6. Cognitive functions regulated by neuron-derived E_2 (NDE_2) and the commonly used behavioral tests for rodent studies. Neuron-derived E_2 (NDE_2) is critical for hippocampus-dependent spatial reference memory, which is often tested using the Barnes Maze test. Hippocampus-dependent recognition memory is another important cognitive function that is regulated by NDE_2 and which can be assessed using the Novel Object Recognition Test (NORT). In addition, NDE_2 was also demonstrated to regulate contextual, but not cued fear memory, which can be evaluated using Fearing Conditioning Test. Finally, NDE_2 is essential to prevent depressive-like behavior in female mice, which can be tested with the Forced Swimming Test.

rats (Azcoitia et al., 2001). The ability of testosterone to exert neuroprotection was suggested to be due to aromatization to E_2 as testosterone's neuroprotective effect was blocked by administration of the aromatase inhibitor, fadrozole (Azcoitia et al., 2001). Furthermore, a neuroprotective role for aromatase and hippocampal BDE_2 was implicated by the finding that systemic or intracerebroventricular administration of fadrozole in intact male rats protected the hippocampus from neuronal damage induced by kainic acid (Azcoitia et al., 2001). In a subsequent study, it was found that the upstream precursors of E_2 , pregnenolone and dehydroepiandrosterone (DHEA), also protected hippocampal neurons from kainic acid neurotoxicity, and their neuroprotective effect was blocked by fadrozole (Veiga et al., 2003). This finding suggests that the neuroprotective effects of pregnenolone and DHEA are mediated by their conversion to E_2 . In addition, a neuroprotective role for BDE_2 in the hippocampus was further supported by the finding that inhibition of E_2 synthesis by letrozole treatment enhanced methyl-mercury-induced neurotoxicity in male rat hippocampal slices *in vitro*, and this effect could be rescued by E_2 replacement (Yamazaki et al., 2013). An additional interesting study examined a potential neuroprotective role of local E_2 synthesis in the male rat striatum in the 6-hydroxydopamine-lesioned model of Parkinson's disease model (McArthur et al., 2007). In this study, central administration of the aromatase inhibitor anastrozole was found to exacerbate 6-hydroxydopamine-induced striatal lesions, suggesting that BDE_2 is neuroprotective in the striatum (McArthur et al., 2007). Further work has demonstrated that the neuroprotective effect of aromatase and BDE_2 extends to other types of brain injury in addition to excitotoxic injury. For instance, male zebra finches treated with fadrozole and subjected to a penetrating brain injury were found to have a significantly larger lesion size with a greater number of apoptotic nuclei (Wynne and Saldanha, 2004; Wynne et al., 2008). The investigators proposed that E_2 from glial cells mediates the neuroprotection after penetrating brain injury. Additional work showed that E_2 replacement was able to rescue the defects, which further supports that the loss of local-derived E_2 underlies the increased neuronal damage in fadrozole-treated birds (Wynne and Saldanha, 2004; Wynne et al., 2008).

While most studies have examined the neuroprotective effect of aromatase and BDE_2 in male animals, a few studies have utilized female animals and demonstrated a similar neuroprotective effect for BDE_2 as was observed in males. For instance, early work using a global whole-body knockout of aromatase in female mice demonstrated increased

infarct damage in the cortex and striatum of the knockout mice, and the effect in the knockout mice was greater than observed in ovariectomized mice, suggesting that extragonadal E_2 has a neuroprotective role (McCullough et al., 2003). Similarly, letrozole treatment significantly increased infarct volume, neuronal damage, apoptosis and cognitive deficits in intact female rats following focal cerebral ischemia (Zhang et al., 2017b). To further confirm the role of BDE_2 in females, subsequent studies were conducted in ovariectomized animals so as to remove the potential confound of contributions by gonadal-derived E_2 . The first studies examined protection of the brain from excitotoxicity and revealed that administration of a low dose of kainic acid, which did not produce neuronal damage in control ovariectomized rats, led to highly significant neuronal damage in the hippocampus of fadrozole-treated ovariectomized female rats (Veiga et al., 2003). In addition, work by our group found that antisense oligonucleotide knockdown of aromatase in the hippocampus of ovariectomized female rats led to significantly enhanced neuronal damage following global cerebral ischemia (GCI), further supporting a neuroprotective role of BDE_2 in female animals (Zhang et al., 2014). Furthermore, aromatase knockdown animals had higher microglial activation in the hippocampus following GCI, suggesting that BDE_2 exerts an anti-inflammatory effect after cerebral ischemia. In support of an anti-inflammatory role of BDE_2 after brain injury, another study found that central fadrozole administration to male and female zebra finches that received a penetrating brain injury caused sustained elevation of tumor necrosis factor- α (TNF- α), COX-2 and PGE $_2$ in the injured lobe in both sexes, with females also exhibiting enhanced elevation of IL-1 β (Pedersen et al., 2016). Central E_2 replacement reversed these effects. These findings suggest that BDE_2 may suppress inflammation in part by acting on microglia, which are known to play a key role in inflammation, and which express estrogen receptors and can be regulated by E_2 (Villa et al., 2016).

6.2. Conditional knockout studies - role of NDE_2 in neuroprotection

Since aromatase is induced in astrocytes after brain injury, it has been inferred that ADE_2 mediates neuroprotection of the brain following ischemia or injury. However, since neurons also make E_2 constitutively, then NDE_2 could also potentially exert a neuroprotective role. In addition, astrocyte and neuronal aromatase are both inhibited by aromatase inhibitors; thus, the inhibitor effects could be due to depletion of either NDE_2 or ADE_2 , or both. Indeed, *in vitro* studies have found that NDE_2 can

exert neuroprotection similar to ADE₂. For instance, anastrozole treatment in H19-7 hippocampal neurons suppressed E₂ production and led to enhanced neuronal cell death following H₂O₂ treatment (Chamniansawat and Chongthammakun, 2012). Furthermore, morphine treatment of hippocampal neurons was shown to increase release of E₂, and this effect was correlated with morphine-induced neuroprotection from A β neurotoxicity (Cui et al., 2011). A critical role for hippocampal NDE₂ in the morphine-induced neuroprotection was shown by the finding that siRNA knockdown of aromatase blocked morphine neuroprotection from A β neurotoxicity. To more specifically determine the role of NDE₂ in neuroprotection, we utilized our FBN-ARO-KO mice in which forebrain NDE₂ is specifically depleted and performed GCI (Lu et al., 2020). We utilized intact males and ovariectomized females and examined hippocampal neuronal damage, cognitive function, astrocyte reactivity, and astrocyte polarization. The studies revealed that both ovariectomized female and intact male FBN-ARO-KO mice had significantly greater neuronal damage and decreased neuronal structural integrity in the hippocampal CA1 region after GCI, as compared to FLOX controls (Lu et al., 2020). Barnes maze testing revealed that intact male and ovariectomized female FBN-ARO-KO mice both exhibited significantly greater impairment of hippocampal-dependent spatial reference memory after GCI (Lu et al., 2020). These findings indicate that NDE₂ exerts a neuroprotective effect in the hippocampus following ischemic brain injury. Global transcriptome analysis coupled with qRT-PCR confirmation further revealed down-regulation of genes involved in reactive astrogliosis, neuroprotection, and neuroinflammation in the hippocampus of FBN-ARO-KO mice as compared to FLOX mice after GCI (Lu et al., 2020). Examination of reactive astrogliosis using immunohistochemistry and Western blot analysis for the astrocyte markers, GFAP or vimentin at 3, 7 and 14 days after GCI revealed that reactive astrogliosis in the hippocampus was significantly compromised in both intact male and ovariectomized female FBN-ARO-KO mice as compared to FLOX mice (Lu et al., 2020). The reduced reactive gliosis may explain the enhanced neuronal damage and worse cognitive outcome following GCI in FBN-ARO-KO mice. In support of this possibility, GFAP^{-/-}Vm^{-/-} mice were found to have compromised reactive astrogliosis and GLT-1-mediated glutamate transport after cerebral ischemia, as well as enhanced neuronal damage (Li et al., 2008). Reactive astrocytes can enhance neuroprotection by increasing uptake of excess glutamate by GLT-1 and releasing neuroprotective factors, such as BDNF, insulin-like growth factor-1 (IGF-1), and even E₂ itself (Liu and Chopp, 2016). We thus examined each of these astrocytic neuroprotective factors in FBN-ARO-KO mice after GCI and found that expression of each of these factors (GLT-1, BDNF, IGF-1, aromatase) was significantly decreased in FBN-ARO-KO astrocytes after GCI. Elevation of E₂ in the FBN-ARO-KO hippocampus after GCI was also diminished (Lu et al., 2020). These findings indicate that the neuroprotective functions of astrocytes after GCI are significantly impaired by depletion of NDE₂, and accordingly, reinstatement of forebrain E₂ levels reversed all of the molecular and function defects (Lu et al., 2020).

Our study also assessed how NDE₂ regulates reactive astrogliosis. Previous work has shown that fibroblast growth factor-2 (FGF2) is an important factor produced by neurons that functions to suppress reactive astrogliosis (Kang et al., 2014; Zhang et al., 2017a). Interestingly, our RNASeq analysis found that FGF2 was upregulated in the hippocampus of FBN-ARO-KO mice after GCI. Further examination by double immunohistochemistry confirmed that FGF2 was increased in hippocampal neurons in FBN-ARO-KO mice after GCI, and we additionally found increased expression of its major receptor FGFR3 in reactive astrocytes in FBN-ARO-KO mice after GCI, as compared to FLOX mice (Lu et al., 2020). These findings suggest that the attenuated reactive astrogliosis in FBN-ARO-KO mice after GCI may be due to enhanced neuronal FGF2 signaling. This suggestion is supported by our group's finding that blocking FGF2 signaling by central infusion of a FGFR3-neutralizing antibody rescued reactive astrogliosis after GCI in FBN-ARO-KO mice (Lu et al., 2020). Furthermore, astrocytic expression of BDNF, aromatase

and GLT-1 expression were also rescued, which correlated with a significant reduction of neuronal damage after GCI (Lu et al., 2020).

Interestingly, our findings also suggest that NDE₂ is important for enhancing polarization of the reactive astrocytes toward an A2 neuroprotective phenotype. Previous transcriptome analysis by the Barres lab (Zamanian et al., 2012) has implicated at least two major types of reactive astrocytes: A1 and A2 astrocytes. A1-type astrocytes are pro-inflammatory and neurotoxic and are induced by inflammatory agents, such as LPS, while A2-type astrocytes are neuroprotective and are induced following cerebral ischemia (Liddelow et al., 2017). We thus examined astrocyte phenotype in FBN-ARO-KO mouse hippocampal astrocytes in our study using qRT-PCR, Western blot analysis and immunohistochemistry for A1 and A2 genes/markers. The results revealed a significant downregulation of A2 astrocyte phenotype in the hippocampus of FBN-ARO-KO mice after GCI (Lu et al., 2020). We did not detect A1 astrocyte induction after GCI, a finding in agreement with the Barres' group previous results. These results indicate that NDE₂ is critical for induction of the neuroprotective A2 reactive astrocyte phenotype following GCI.

Based on our findings in the FBN-ARO-KO mouse described above, we have schematically illustrated a summary of the proposed mechanisms that underlie NDE₂ neuroprotection following GCI (see Fig. 7). As shown in Fig. 7, we propose that following GCI, NDE₂: 1) suppresses neuronal FGF2 signaling, a negative regulator of reactive astrogliosis, 2) enhances reactive astrogliosis after GCI, 3) enhances the A2 neuroprotective astrocyte phenotype, and 4) increases production of the astrocytic neuroprotective factors, GLT-1, BDNF and IGF-1, as well as ADE₂ itself. Collectively, this leads to enhanced neuroprotection and preserved cognitive function following GCI. Furthermore, since NDE₂ can be released from neurons, we cannot exclude the possibility that NDE₂ also acts on estrogen receptors on neighboring neurons as an additional mechanism to facilitate neuroprotection.

6.3. Conditional knockout studies - role of ADE₂ in neuroprotection

To further determine the role of ADE₂ in neuroprotection and neuroinflammation in ischemic brain injury, we utilized our GFAP-ARO-KO mice that have aromatase and E₂ specifically depleted in astrocytes (Wang et al., 2020). GFAP-ARO-KO mice were viable and fertile, with normal gross brain structure, normal aromatase expression in neurons, normal basal forebrain E₂ levels, normal astrocyte morphology, intensity and distribution, and normal cognitive function basally (Wang et al., 2020). The lack of a phenotype basally is not unexpected, as aromatase is not expressed basally in astrocytes and is only induced following stress, brain injury or ischemia. Note that the knockout of aromatase was specific for astrocytes, as neuronal aromatase and basal forebrain E₂ levels were unchanged in the GFAP-ARO-KO mice. In contrast, after GCI, we found that intact male, intact female and ovariectomized female GFAP-ARO-KO mice: 1) failed to exhibit the normal elevation of astrocyte aromatase and hippocampal E₂ levels after ischemia; 2) exhibited a profound reduction of ischemia-induced reactive astrogliosis; 3) had increased hippocampal neuronal damage, 4) exhibited enhanced microglia activation and reduced expression of microglial homeostatic genes, and 5) had enhanced cognitive deficits after GCI (Wang et al., 2020). RNA-Seq analysis further revealed that the normal upregulation of the A2 panel of reactive astrocyte genes was significantly attenuated in the ischemic GFAP-ARO-KO mouse hippocampus, suggesting that ADE₂ is critical for induction of the A2 astrocyte phenotype after ischemic brain injury (Wang et al., 2020).

Our study also examined the molecular pathways and mechanisms that might underlie ADE₂ regulation of reactive astrogliosis after GCI. Along these lines, RNA-seq analysis revealed that the IL-6/Janus kinase (JAK)/signal transducer and activator of transcription3 (STAT3) signaling pathway was negatively correlated with GFAP-ARO-KO after GCI (Wang et al., 2020). This is intriguing as the JAK-STAT3 pathway plays a critical role in mediating reactive astrogliosis after ischemia and

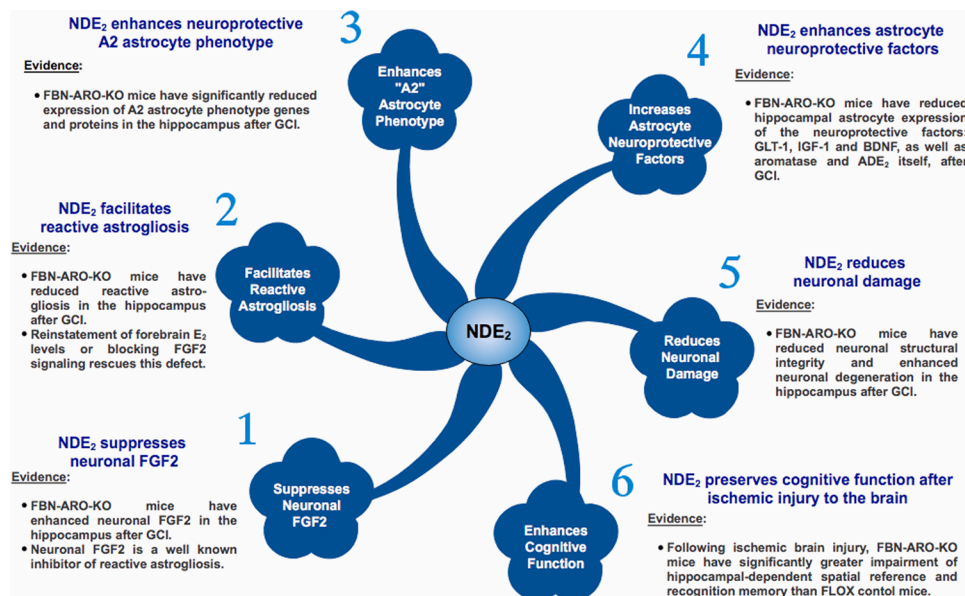


Fig. 7. Proposed mechanisms underlying neuron-derived E₂ (NDE₂) neuroprotection in the ischemic brain. See text for full description. FBN-ARO-KO: forebrain neuron-specific aromatase knockout.

injury (Ceyzeriat et al., 2016; Liddelaw and Barres, 2017). Our study also found that STAT3 activation in the hippocampus after GCI is significantly suppressed in GFAP-ARO-KO astrocytes (Wang et al., 2020), which may explain the reduced reactive astroglia in our GFAP-ARO-KO mice after GCI. In support of this possibility, previous work demonstrated a failure of reactive astroglia in mice with conditional deletion of STAT3 following spinal cord injury (Okada et al., 2006), and this effect was associated with increased lesion volume and attenuated functional recovery. Interestingly, we also found that induction of leukemia inhibitory factor (LIF), a member of the IL-6 cytokine family and an upstream regulator of STAT3 (Murakami et al., 2019), was strongly attenuated in GFAP-ARO-KO astrocytes, as compared with FLOX astrocytes after GCI (Wang et al., 2020). Finally, E₂ replacement fully rescued the defects in JAK-STAT3 signaling and

reactive astroglia and was able to reverse the enhanced neuronal damage and microglial activation in GFAP-ARO-KO mice after GCI (Wang et al., 2020). Fig. 8 summarizes the mechanisms that are proposed to underlie ADE₂ neuroprotection after GCI. Based on the findings discussed above, we propose that ADE₂ has several key roles following GCI, including 1) being critical for reactive astroglia in the hippocampal CA1 region, 2) inducing JAK-STAT3 signaling in astrocytes, 3) facilitating induction of the A2 panel of reactive astrocyte genes, 4) suppressing microglia activation, and 5) reducing neuronal damage and preserving hippocampal-dependent cognitive functions.

7. Future directions and conclusions

Despite the remarkable progress described above in elucidating BDE₂

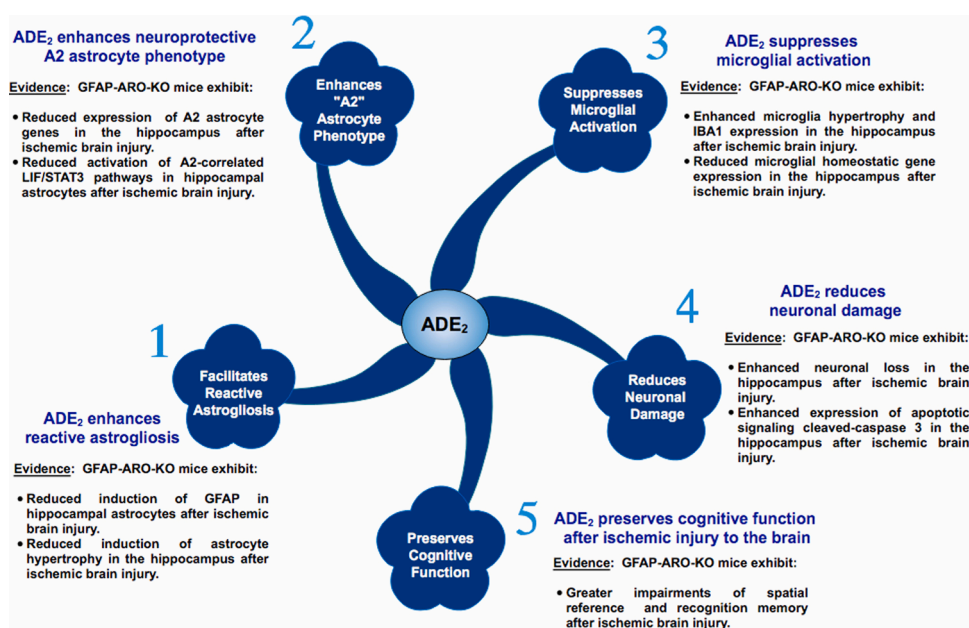


Fig. 8. Proposed mechanisms underlying astrocyte-derived E₂ (ADE₂) neuroprotection in the ischemic brain. See text for full description. GFAP-ARO-KO: astrocyte-specific aromatase knockout.

functions in the brain, there remain many unanswered questions that require further studies. Below, we have summarized key questions and future directions that could help advance this important research area and provide clarity to areas where controversy currently exists.

7.1. Development of new animal models

An important caveat of the FBN-ARO-KO mouse studies is that deletion of aromatase was specifically targeted in forebrain excitatory glutamatergic neurons. While these constitute the major excitatory neurons in the brain, other types of neurons also express aromatase, including inhibitory neurons (Hoyk et al., 2014; Yague et al., 2010, 2008). To address the role of NDE₂ in these neurons, future studies are needed to develop genetic models that ablate aromatase specifically in these other neuronal types. Furthermore, in addition to the forebrain, aromatase is also expressed in the midbrain and hindbrain. Therefore, future studies are needed to develop genetic models for targeted deletion in these brain areas to help elucidate the function of BDE₂ in these brain areas.

An intriguing finding of our studies was that even though neuronal aromatase and NDE₂ appear uncompromised in the GFAP-ARO-KO mice, NDE₂ was unable to compensate for the loss of ADE₂. One explanation for this could be that the effects of NDE₂ and ADE₂ are additive and loss of one or the other sources of local E₂ will yield a similar phenotype. To address this question, we have generated double aromatase knockout mice by crossing the FBN-ARO-KO and GFAP-ARO-KO mice and will use these mice in future studies to determine if the double knockout mice have greater defects than the single knockout mice after GCI. Alternatively, NDE₂ neuroprotective effects may require ADE₂ mediation. In potential support of this possibility, NDE₂ was shown to regulate reactive astrogliosis and ADE₂ generation by suppressing neuronal FGF2 signaling, which serves as an inhibitory “brake” to astrocyte activation following ischemia (Lu et al., 2020).

It should also be mentioned that BDE₂ effects on astrocyte activation may be injury- or context-specific. For instance, contrary to the reduced reactive astrogliosis observed in our knockout mice after GCI, aromatase inhibitor treatment in male zebra finches with a penetrating wound injury was associated with increased reactive gliosis around the wound area (Wynne et al., 2008). This discrepancy could be due to differences in the injury model (ischemic versus traumatic injury), differences in approaches (chronic knockout versus an acute pharmacological knock-down of aromatase), or to species differences. To rule out that the differences were due to different approaches, we examined the effect of pharmacological inhibition of aromatase by letrozole in our GCI model. The results revealed that letrozole treatment in wild type male mice yielded a similar reduced reactive astrogliosis in the hippocampus after GCI as observed in the GFAP-ARO-KO mice (Wang et al., 2020). Thus, the difference in results does not appear to be due to different approaches, but rather appears to be due to different injury models and/or species differences. To provide greater clarity on this issue, in the future, we plan to use our knockout mice to study the role of NDE₂ and ADE₂ following different types of brain injury, including penetrating brain injury, traumatic brain injury, and excitotoxic injury. Furthermore, we have also generated inducible conditional astrocyte-specific knockout mice using tamoxifen-inducible *Gfap-cre/ERT2* and *Aldh1l1-cre/ERT2* mouse lines that we plan to utilize as an additional tool in the future to further interrogate and enhance our understanding of the roles and actions of ADE₂ in brain function.

7.2. Sex differences

There are conflicting reports on sex differences in BDE₂ actions in the brain especially regarding effects on synaptic plasticity. Some studies found evidence of a role in females but not in males, while others indicate a significant role in both sexes. Thus, future studies on BDE₂ actions and functions in the brain should include both sexes and do

direct comparisons where possible so as to better clarify sex differences in BDE₂ effects and actions in the brain. We also plan to more thoroughly interrogate for sex differences using our conditional aromatase knockout models. While we observed similar gross phenotypes in both sexes in our knockout mice, we did not do comprehensive direct comparisons of the two sexes that are needed to reveal specific sex differences in effects, magnitude, and/or temporal patterns of the processes under study.

7.3. Additional clinical studies

While animal studies have shown that aromatase is upregulated in astrocytes in the brain after stroke and trauma, it is unclear whether a similar upregulation occurs in humans. Future studies using post-mortem human brain samples could examine this question. Since NDE₂ has been implicated to regulate memory and cognition, approaches that increase NDE₂ in the brain could potentially have efficacy at memory improvement/enhancement in humans. Intriguingly, one such approach to increase NDE₂ could be exercise, as exercise has been reported to enhance E₂ levels in the hippocampus in ovariectomized rats, and this effect was correlated with enhanced cognitive function (Kaidah et al., 2016). Thus, it would be interesting to determine whether exercise can similarly upregulate aromatase/E₂ in the brain in humans. It would also be intriguing to determine whether exercise enhancement of cognition may involve NDE₂ mediation, especially since NDE₂ has been shown to regulate BDNF, spine and synapse density, and LTP – all key regulators and/or processes that underlie memory. Studies are already underway in our laboratory to address this interesting question.

7.4. Microglial regulation

Few studies have examined BDE₂ for regulatory effects upon microglia. This is surprising as microglia are well known to possess estrogen receptors and exogenously administered E₂ can regulate microglia function. Our study using GFAP-ARO-KO mice did provide evidence that ADE₂ regulates microglial activation and microglial homeostatic gene expression in the hippocampus after GCI (Wang et al., 2020). However, follow up studies are needed to further explore the functional consequences of such regulation, the mechanisms underlying them, whether they extend to other types of brain injuries and neurodegenerative disorders, and whether NDE₂ can exert similar regulation of microglial activation and function.

In conclusion, the findings described in this review provide substantial evidence of aromatase expression and local E₂ production in many different brain areas of almost all species studied to date. E₂ is produced in neurons basally, while production in astrocytes is induced by stress, brain injury or ischemia. A strength of this body of work is that many different techniques were used in studying the localization of aromatase and E₂ production in the brain, and many different approaches were used to confirm the roles of BDE₂ in the brain, including aromatase inhibitor and knockdown studies, as well as global aromatase and brain cell-specific aromatase knockout animal models. A role for NDE₂ is implicated in a diverse spectrum of functions in the brain, including regulation of sexual differentiation, reproduction, socio-sexual behavior, synaptic plasticity, LTP, memory and cognition, auditory processing, injury-induced reactive gliosis and astrocyte phenotype, neuroprotection, and anti-inflammatory effects. Following its induction after brain injury or ischemia, ADE₂ has been implicated in the regulation of reactive gliosis, astrocyte phenotype, neuroprotection, cognitive preservation, and anti-inflammatory effects. Collectively, these findings demonstrate a critical role for BDE₂ in many different brain functions in both physiological and pathological conditions.

Authorship contribution statement

D.W.B. drafting and writing of manuscript, editing and revising manuscript, funding acquisition, and prepared figures; R.K.V. writing,

editing and revising manuscript, and funding acquisition; Y.L. helped prepare figures, edited and revised manuscript; J.W. edited and revised manuscript and helped prepare figures; Q.Z. edited and revised manuscript; R.T. prepared table, edited and revised manuscript; G.S. edited and revised manuscript; U.P. edited and revised manuscript; R.R.T. edited and revised manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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