Molecular and Cellular Neuroscience 41 (2009) 1–7

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Estrogen deficiency results in apoptosis in the frontal cortex of adult female aromatase knockout mice

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ARTICLE INFO

Article history:
Received 30 January 2008
Revised 30 November 2008
Accepted 23 December 2008
Available online 8 January 2009

Keywords:
Aromatase
Estrogen
Apoptosis
Frontal cortex
Female
Knockout mouse
Brain

ABSTRACT

The aromatase knockout (ArKO) mouse is completely estrogen deficient. We previously detected apoptosis in the hypothalamus of 1 year-old male ArKO mice. This study shows that 12 week-old female ArKO mice display spontaneous apoptosis of pyramidal neurons in the frontal cortex while wild-type (WT) littermates show no signs of apoptosis. Concomitantly, *bcl-2* related anti-apoptotic genes are down-regulated whereas the pro-apoptotic gene *TRADD* is up-regulated in the female ArKO frontal cortex. This phenotype can be rescued by 3-week replacement of 17β-estradiol. Furthermore, the apoptosis phenotype is exacerbated in 12–15 month-old female ArKO mice, which have 30% less neurons in the frontal cortex and lower brain weights than WT counterparts. These data show that estrogens are essential for the survival of female cortical neurons even in the absence of pathological conditions or external assaults. Our observations also demonstrate the sexually dimorphic susceptibility of neurons to estrogen deficiency.

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Introduction

The presence of aromatase (the enzyme that converts androgens to estrogens) and estrogen receptor (ER) mRNA and protein expression in several regions of the mammalian brain, including the cortex, suggests a strong functional role for estradiol in the brain (Balthazart et al., 1991; Foidart et al., 1995; Jakab et al., 1994). Indeed several studies have reported on the neuroprotective effects of estradiol including anti-apoptotic effects, protection from free radicals, anti-inflammatory effects, regulation of calcium channels and protection via increasing cerebral blood flow (see review Amantea et al., 2005). Such neuroprotective properties of estradiol have been implicated in the treatment of several neurological dysfunctions including cognitive and memory deficits, brain injury and stroke (Garcia-Segura et al., 2001; Wise et al., 2001) and in addition neurodegenerative diseases such as Alzheimer's disease (Tang et al., 1996; Xu et al., 2006) and Parkinson's disease (Cyr et al., 2002).

While in most cases, the neuroprotective actions of estradiol are ER mediated, non-genomic actions of estradiol are also beneficial — the unique phenolic A ringed structure of estradiol renders it an effective scavenger of free radicals (Moosmann and Behl, 1999). One mechanism of ER-mediated estrogenic neuroprotection is via regulation of anti- and pro-apoptotic genes. Several *in vitro* studies have demonstrated the anti-apoptotic effects of 17β-estradiol treatment via down-regulation of pro-apoptotic genes such as *BAD* (Gollapudi and Oblinger, 1999) and up-regulation of anti-apoptotic genes such as *Bcl-2* (Dubal et al., 1999; Zhao et al., 2004). In addition, neuroprotective effects of estradiol upon neurodegenerative disease processes such as Alzheimer's and Parkinson's may be mediated through the regulation of anti- and pro-apoptotic gene expression (Pike, 1999; Morissette et al., 2008). Clinically, the topic of estrogen replacement therapy in postmenopausal women has received much attention, and although the literature has been, for the most part, contradictory, it now seems evident that early in menopause, when neurons are still in a healthy state, estrogen therapy can be effective, however if this brief window of opportunity is missed, estradiol treatment is relatively ineffective (Amantea et al., 2005; Maki, 2005; Maki et al., 2007; Stephens et al., 2006). The use of animal models to effectively replicate problems such as hormone deficiency, neurological diseases, brain injury and stroke, is an effective tool for investigating the beneficial effects of estradiol on the brain. Studies using ovariectomy procedures followed by 17β-estradiol replacement have effectively demonstrated the positive effects that estradiol provides in cognitive performances and memory tasks (Gibbs, 2000; Vaucher et al., 2002).

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doi:10.1016/j.mcn.2008.12.009

Whereas the *in vivo* effects of estrogen on the brain were primarily investigated using gonadectomised animals (Fukuda et al., 2000; Miller et al., 1999), these animal models are not ideal as aromatase cytochrome P450 (the enzyme that synthesises estrogens from androgens) is known to be expressed in numerous regions of the brain (Lephart et al., 2001; Wagner and Morrell, 1997) such as hypothalamus, amygdala and hippocampus. In studies using aromatase inhibitors, complete blockage in all sites is uncertain. Therefore the total estrogen deficiency can be achieved by knocking out the aromatase gene. Our laboratory has developed an aromatase knockout (ArKO) mouse model of complete estrogen deficiency by deleting exon IX (Fisher et al., 1998) of the aromatase gene (*Cyp19A1*). Single point mutations in this region have resulted in total estrogen ablation in humans. Neurologically, female ArKO mice show significantly increased ischemic damage following insult in all brain areas, when compared to WT controls (McCullough et al., 2003). In addition, our laboratory has previously determined that 1 year-old male, but not female ArKO mice display apoptosis in the dopaminergic neurons of the arcuate nucleus and medial preoptic area (Hill et al., 2004), indicating that estradiol is not only neuroprotective upon brain injury, but is actually required for the maintenance and integrity of dopaminergic neurons in the hypothalamic region of male mice. Furthermore, we found that ERα agonist treatment prevented the appearance of apoptosis in the arcuate nucleus, while ERβ agonist treatment was neuroprotective in the MPO region (Hill et al., 2007). These data suggest that the role of estradiol in the regulation of dopaminergic neurons is ER mediated.

Another ArKO model was generated by deleting exons 1 and 2 of the *Cyp19A1*, this female ArKO mouse has been shown to exhibit abnormal sexual behaviours (Bakker et al., 2002), and in addition displays depressive-like behaviours that may not be reversed by estradiol treatment (Dalla et al., 2004), suggesting a developmental role for estradiol in the female brain. Furthermore, when crossed with the APP23 Alzheimer's disease model, the combined ArKO/APP23 mice exhibit earlier onset and increased β-amyloid peptide deposition (Yue et al., 2005), suggesting an essential role for estrogens as neuroprotectants from such insults as ischemia and amyloid deposition. This neuroprotective role for estradiol against neurotoxic insults also holds true in the male ArKO which shows enhanced hippocampal damage following domoic acid induced excitotoxicity when compared to WT (Azcoitia et al., 2001).

Herein, we present a comprehensive study on consequences of estrogen deficiency on the morphology of the brain of female mice.

Results

Histological studies

The brain tissues from ArKO mice were noticeably more friable than those of WT mice. Preliminary histological analysis by NeuN revealed there were less neurons in the frontal cortex in the 1 year-old female ArKO mouse (Fig. 1A) as compared to the 1 year-old female WT (Fig. 1B). No such differences were noticed in the younger animals (14 week-old). Subsequently, semi-quantitation of NeuN immunostained cells of the frontal cortex (Bregma 3.0 mm to 2.10 mm; Paxinos and Franklin 2004) showed that the 15 month-old female ArKO mouse has significantly less ($p<0.001$) neurons in the layerII/III, with an average of 196.8 ± 3.45 neurons/30 μm^2 (mean ± SEM) as compared to the WT counterpart (an average of 287.9 ± 3.94 neuron/30 μm^2) in the same region. We did not detect such differences in the 12 week-old animals (data not shown). In addition, by using TUNEL staining we detected extensive DNA breaks in the frontal cortex of 1 year-old female ArKO mice (Fig. 1C) but not in the WT counterparts (Fig. 1D). These DNA breaks could also be detected, although less frequently, in the young adult (10–12 week-old) female ArKO mouse frontal cortex (Fig. 1E). After 3 weeks of 17β-estradiol replacement, the level of TUNEL staining was greatly reduced (Fig. 1F), indicating that the frontal cortex DNA breaks phenotype could be rescued by 17β-estradiol replacement. However, MRI did not detect any difference between the 1 year-old ArKO and WT mice in volumes of the brain or apparent diffusion coefficients of water (data not shown). Interestingly, we did not detect any apoptosis in the hippocampus, the region associated with learning, in the female ArKO or WT mouse at all ages examined.

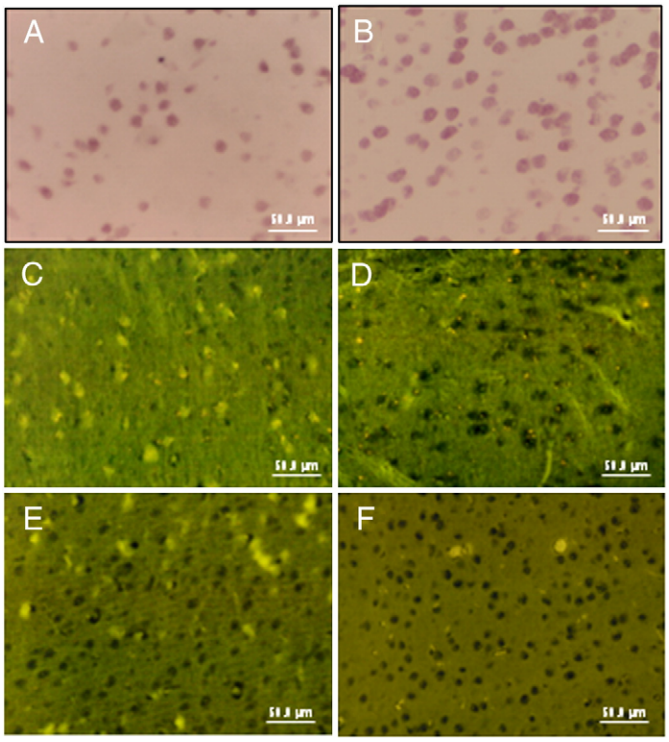

Fig. 1. Histological studies of the female WT and ArKO mice frontal cortex. Immunostaining of neurons in the frontal cortex in 1 year-old female (A) ArKO and (B) WT using a mouse anti-neuronal nuclei (NeuN) monoclonal antibody. Less neurons are present in the ArKO mice. (C) Fluorescent labelling of apoptotic cells by TUNEL staining in the frontal cortex in 1 year-old female ArKO. (D) Absence of TUNEL staining in the frontal cortex of 1 year-old female WT. (E) TUNEL staining in the frontal cortex of 10–12 week-old female ArKO. (F) Estrogen-replacement in 10–12 week-old female ArKO mice dramatically reduced TUNEL staining in the frontal cortex.

Immunohistochemistry for active caspase-3

In order to confirm that DNA strand breaks found by TUNEL labelling were due to the apoptotic process, immunohistochemistry for active caspase-3 (an end stage marker for apoptosis) was performed. While levels of active caspase-3 positive labelling were very low to none in WT female frontal cortex (Fig. 2A), a high expression of active caspase-3 was prominent in the layer II/III pyramidal cells of 1 year-old ArKO female mice (Fig. 2C) but no cells in layer V were labelled. We did not detect any TUNEL or active caspase 3 labelling in the same region in the male ArKO mice (data not shown).

Double immunohistochemistry for active caspase-3 and NeuN

To determine which cell type was undergoing apoptosis, double immunohistochemistry was performed against active caspase-3 and NeuN (a neuronal antibody). Confocal images shown in Figs. 3A and B demonstrate co-localisation of active caspase-3 with NeuN, indicating that the cell type undergoing apoptosis in the frontal cortex of 1 year-old female ArKO mice are neuronal.

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