Brain Research 1663 (2017) 1–8

Contents lists available at ScienceDirect

Brain Research

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

Research report

Progesterone increased β-endorphin innervation of the *locus coeruleus*, but ovarian steroids had no effect on noradrenergic neurodegeneration

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ARTICLE INFO

Article history:
Received 16 December 2016
Received in revised form 3 March 2017
Accepted 6 March 2017
Available online 8 March 2017

Keywords:
Locus coeruleus
Rhesus monkeys
Cell death
Stress
Progesterone

ABSTRACT

With the decline of ovarian steroids levels at menopause, many women experience an increase in anxiety and stress sensitivity. The *locus coeruleus* (LC), a central source of noradrenaline (NE), is activated by stress and is inhibited by β-endorphin. Moreover, increased NE has been implicated in pathological anxiety syndromes. Hormone replacement therapy (HRT) in menopause appears to decrease anxiety and vulnerability to stress. Therefore, we questioned the effect of HRT on the inhibitory β-endorphin innervation of the LC. In addition, we found that progesterone protects serotoninergic neurons in monkeys, leading us to question whether ovarian steroids are also neuroprotective in LC neurons in monkeys. Adult Rhesus monkeys (*Macaca mulatta*) were ovariectomized, and either treated with Silastic capsules that contained estradiol, estradiol + progesterone, progesterone alone or that were empty (ovariectomized; control). After 1 month, the LC was obtained and processed for immunohistochemistry for β-endorphin and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL). The density of β-endorphin axons was determined with image analysis using ImageJ. The TUNEL-positive neurons were counted in the entire LC. Progesterone-alone significantly increased the density of the β-endorphin axons in the LC ($p < 0.01$). No significant differences between groups in the number of TUNEL-positive cells in the LC were found. In conclusion, we found that HRT increases the inhibitory influence of β-endorphin in the LC, which could, in turn, contribute to reduce anxiety and increase stress resilience. In addition, we did not find compelling evidence of neurodegeneration or neuroprotection by HRT in the LC of Rhesus monkeys.

1. Introduction

The noradrenergic (NE) neurons of the midbrain *locus coeruleus* (LC) respond to stress with an increase in activity. In addition, elevation of basal NE neurotransmission appears to increase anxiety [for review see (Myers et al., 2016)], and pathological anxiety is now largely treated with drugs that modulate the serotonergic and NE systems, called serotonin and NE reuptake inhibitors (SNRIs) [for review see (Ammar et al., 2014)].

While the serotonin system has been implicated in mood and affective disorders such as depression [for review see (Solomon and Herman, 2009; Warren, 2007)], the NE system is a crucial key to the regulation of anxiety and stress sensitivity. Besides its well-known participation in the stress response (Myers et al., 2016), NE projections from the LC innervate areas implicated in vigilance or arousal (Foote et al., 1991) and also fear and anxiety (Balaban, 2002). Thus, it has been proposed that the LC has a role as an initiator of anxiety responses [for review see (Pratt, 1992)] and an imbalance of this system could lead to exacerbated stress responses and anxiety disorders (Lipski and Grace, 2013). There are a significant number of studies showing that increased NE leads to increased anxiety and that SNRIs reduce anxiety by reducing NE. Administration of clonidine, an α2 antagonist, reduces NE, suggesting that the SNRI reduction in NE may involve an ultrashort loop feedback mechanism via α2 presynaptic receptors on NE neurons (Kuffel et al., 2014). Moreover, following a clonidine challenge, individuals with anxiety syndromes exhibit lower growth hormone (GH) secretion. This has been attributed to a downregulation of adrenergic receptors as a consequence of elevated NE (Abelson et al., 1991).

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http://dx.doi.org/10.1016/j.brainres.2017.03.008
0006-8993/

Experimental models of rodents have shown that estradiol and progesterone also modulate anxiety-like behaviors. Anxiety correlates with the estrous cycle, being increased during diestrus, when estradiol levels are low, compared to proestrus, when those levels are high (Frye and Walf, 2002; Marcondes et al., 2001). However, it is still not clear which hormone is involved in this anxiolytic behavior on proestrus since some authors ascribe this role to estradiol (Walf and Frye, 2013; Filova et al., 2010; Marcondes et al., 2001) while others to progesterone (Baykara et al., 2016; Frye and Walf, 2002).

A common situation that often brings an increase in anxiety is the transition into menopause (Bromberger et al., 2001; Freeman et al., 2005; Siegel and Mathews, 2015). It also leads to a decreased or altered ability to cope with stress, or decreased stress resilience (Kudielka et al., 1999; Villada et al., 2017). The accompanying decrease in estradiol and progesterone has been proposed as the driving factor of these changes [for review see (Brinton et al., 2015)]. However, the underlying neurobiology is not well defined in nonhuman primate (NHP) models of menopause. A few studies have shown that estrogen decreases anxiety in Rhesus monkeys (Mook et al., 2005) and that long-term ovariectomy increases anxiety in Japanese macaques (Coleman et al., 2011). Rhesus monkeys, like humans, exhibit a significant reduction in the serum concentration of ovarian steroids during menopause (Downs and Urbanski, 2006).This reduction can be to a certain degree simulated by ovariectomy in this animals (Bethea and Centeno, 2008), allowing for a reasonable model for studies on menopause.

LC of rats (Bloom et al., 1978a; Bloom et al., 1978b) and humans (Fodor et al., 1992) is densely innervated by β-endorphin axons from the hypothalamus, and LC neurons exhibit μ-opioid receptors (Reyes et al., 2007). In general, opioid peptides decrease the stress response by decreasing autonomic and neuroendocrine responses induced by stress (Drolet et al., 2001). Progesterone receptors are expressed in the β-endorphin neurons in the arcuate nucleus of monkeys, which indicates a direct action of progesterone on these neurons (Bethea and Widmann, 1996). In addition, during menopause there is a significant decrease of serum β-endorphin concentrations (Aleem and McIntosh, 1985). Together, these data suggest that estradiol and/or progesterone stimulate the β-endorphin system, which has been shown in primates (Wardlaw et al., 1982a).

We also found that progesterone alone is neuroprotective by decreasing the number of apoptotic cells in the dorsal raphe nucleus (DRN) of ovariectomized NHPs (Lima and Bethea, 2010). This observation correlated with the inhibition of pro-apoptotic genes expression and/or the stimulation of anti-apoptotic genes expression (Bethea et al., 2009; Bethea and Reddy, 2008; Tokuyama et al., 2008) as well as an increase in expression of DNA repair genes (Bethea and Reddy, 2015). There are no data in the literature regarding a neuroprotective action of ovarian steroids in the LC of monkeys.

Taking into account these considerations, we postulated two hypotheses: 1) that ovarian steroids could increase the β-endorphin innervation of the LC and thereby indirectly inhibit NE neurons; and 2) that ovarian steroids would have a neuroprotective role in the NE system of NHPs, as previously shown in the serotonergic system (Lima and Bethea, 2010).

2. Results

2.1. β-Beta-endorphin axon staining

Robust β-endorphin axon staining was observed in the LC. Fig. 1, panels A–D show β-endorphin axon staining in the LC from a representative animal of each group: ovariectomized (A), progesterone (B), estradiol (C) and estradiol + progesterone (D). Quantification of the β-endorphin axon density at 4 levels of the LC is shown in Fig. 1E. Progesterone-treated animals exhibited a significantly higher β-endorphin axon density compared to the other animal groups (ANOVA: $p = 0.0002$; post hoc comparisons: $p < 0.01$ for progesterone *vs* ovariectomized, progesterone *vs* estradiol and progesterone *vs* estradiol + progesterone). Estradiol treatment did not change β-endorphin axon density and prevented the increase induced by progesterone. The total area of LC examined was similar in all groups, with no statistical difference among them (data not shown).

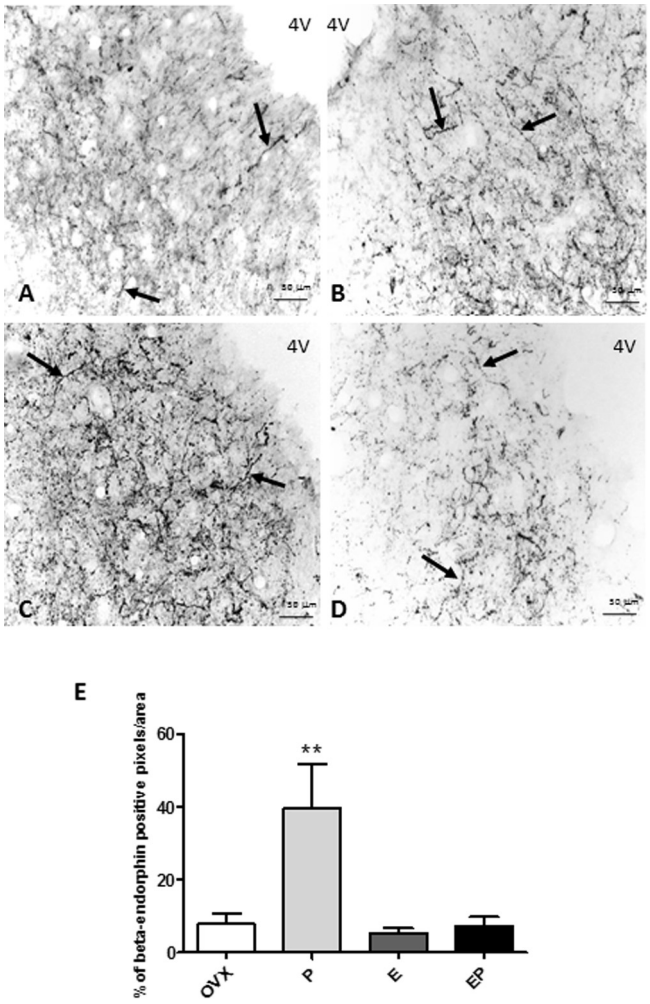

Fig. 1. Photomicrographs of β-endorphin immunoreactive fibers in the locus coeruleus (LC) of ovariectomized Rhesus monkeys treated with placebo (A), estradiol (B), progesterone (C) or estradiol + progesterone (D) (10× magnification), n = 5 animals/group. 4 V: 4th ventricle. (E). Overall mean ± SEM of β-endorphin positive pixel/area at 4 levels in the locus coeruleus (LC) of ovariectomized Rhesus monkeys (OVX), progesterone (P), estradiol (E) or estradiol + progesterone (EP), n = 5 animals/group. Test: One-way analysis of variance (ANOVA) followed by Newman-Keuls's post hoc comparison; *p = 0.0002*.

2.2. TUNEL staining

The TUNEL immunoreactive (ir) cells are scattered across the LC and the DNA fragmentation detected by the TUNEL assay occurred in two forms referred to as type I and type II (Piantadosi et al., 1997; Rink et al., 1995), as found in our previous study (Lima and Bethea, 2010). Type I is characterized by a complete dark staining of the nucleus, while type II presents peripheral staining in the perinuclear area. They may reflect different stages of the DNA fragmentation process that starts in the periphery and moves inward,