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Progesterone Increased β -endorphin Innervation of the Locus Coeruleus, but Ovarian Steroids had no Effect on Noradrenergic Neurodegeneration

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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 serotonergic 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

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AUTHORSHIP CONTRIBUTIONS

Participated in the research design: F.B.L., C.L.B and J.A.A-F. Conducted experiments: F.B.L. and C.M.L. Wrote the paper: F.B.L. Contributed to discussion: F.B.L., C.L.B and J.A.A-F. All authors read and approved manuscript final format.

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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.

Graphical Abstract

Keywords

locus coeruleus; rhesus monkeys; cell death; stress; progesterone

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 & 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 & 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 α_2 antagonist, reduces NE, suggesting that the SNRI reduction in NE may involve an ultrashort loop feedback mechanism via α_2 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).

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 & 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 & Frye, 2013; Filova et al., 2010; Marcondes et al., 2001) while others to progesterone (Baykara et al., 2015; Frye & 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 & Matheus, 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 & Urbansky, 2006). This reduction can be to a certain degree simulated by ovariectomy in this animals (Bethea & 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 & Widmann, 1996). In addition, during menopause there is a significant decrease of serum β -endorphin concentrations (Aleem & 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., 1982).

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 & 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 & Reddy, 2008; Tokuyama et al., 2008) as well as an increase in expression of DNA repair genes (Bethea & 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 & Bethea, 2010).

2. RESULTS

2.1- β -Beta-Endorphin Axon Staining

Robust β -endorphin axon staining was observed in the LC. Figure 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 Figure 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).

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 & 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, or DNA leakage from the nucleus. Figure 2 illustrates TUNEL staining in the LC (A) or absence of TUNEL-ir cells (B). The single arrows in Figure 2C indicate type I staining and double arrows indicate type II staining. Figure 2D contains a photomicrograph of a section of the LC in which the Tdt enzyme was omitted during the TUNEL assay, thus this is a negative control section. Figures 2E and F contain photomicrographs of a section that was pretreated with DNase for 10 min before TUNEL staining and methyl green counterstaining (magnification 10x and 20x, respectively). This treatment digested the DNA and more nicks are available for labeling (positive control).

Figure 3, A and B illustrate TUNEL-ir neurons distributed in the LC of a monkey treated with progesterone. Panels C and D show apoptotic neurons from the LC of an estradiol-treated monkey, in different stages of DNA fragmentation. The single arrows indicate neurons in earlier stages of degradation and double-headed arrows indicate neurons in an advanced stage of degradation.

Figure 4 presents the comparison of all groups using the ANOVA test and showed no significant differences among the groups, neither when the total number of TUNEL-positive cells was computed ($p = 0.341$ - Fig. 4A) nor when the number of TUNEL positive cells per mm^3 was measured ($p = 0.338$ - Fig. 4B). Also, the total area analyzed did not differ between groups ($p = 0.851$ - Fig. 4C). It is noteworthy that there was individual variation between monkeys hindering analysis of variance across all treatment groups.

3- DISCUSSION

The present results found that treatment with progesterone-alone significantly increased the amount of positive β -endorphin axons innervating the LC compared to other treatments (placebo, estradiol or estradiol + progesterone). Estradiol alone and estradiol + progesterone had no effect on the β -endorphin axons in the LC. Since the expression of progesterone receptors would be reduced in the absence of estradiol (Helena et al., 2009; Bethea, 1994) these findings suggest that progesterone is being metabolized to allopregnenolone, which in turn could be acting through gamma-aminobutyric acid receptor-A (GABA-A receptors) (Callachan et al., 1987). Therefore, in this study, the increase of β -endorphin inputs to the LC observed in the progesterone-treated group is consistent with progesterone action through allopregnanolone. However, we cannot rule out the hypothesis that allopregnenolone could also directly inhibit LC neurons via GABA-A receptors as suggested earlier (Aston-Jones et al., 1991). Nonetheless, the intracellular crosstalk between allopregnanolone, GABA-A receptor and the hypothalamic β -endorphin neurons needs further analysis.

This result supports our hypothesis that part of the mechanism of HRT might be related to the stimulation of β -endorphinergic innervation to the LC neurons, which could be part of the mechanism that decreases anxiety and increases stress resilience. The endogenous opioid peptides, specially β -endorphin, control gonadal hormones secretion by inhibiting the GnRH release (Ferin & Vande, 1984). It has been shown in monkeys that castration decreases β -endorphin secretion in the portal blood, and the replacement of estradiol and progesterone reverts this, further indicating that ovarian hormones modulate the activity of hypothalamic β -endorphin neurons (Wardlaw et al., 1982; Wehrenberg et al., 1982). It is attractive to speculate that the arcuate β -endorphin neurons also send projections to the LC. A body of literature has shown a correlation between β -endorphin plasma levels and menopause. Menopausal women show lower circulating levels of β -endorphin, which is related to mechanisms controlling hot flushes (Genazzani et al., 1984) and mood variations (Adler, 1980). Also, the number of hypothalamic neurons expressing proopiomelanocortin (POMC), the precursor of β -endorphin, is reduced in women after menopause, suggesting that the decline of ovarian steroids might be responsible for this decrease (Abel & Rance, 1999). HRT increases plasma β -endorphin concentration, which in turn correlates with improved cognitive function, mood state and vasomotor activity (Akhan et al., 2002; Stomati et al., 1997). The specific effect of each ovarian steroid was suggested by Wardlaw & colleagues, (1982) who showed that treatment of ovariectomized rats with estradiol for three weeks decreased β -endorphin levels in hypothalamus, thalamus and mesencephalon. When progesterone was added to the estradiol treatment, it blocked the reduction of β -endorphin induced by estradiol. Therefore, these data suggest that progesterone stimulates while estradiol inhibits the β -endorphin system in the mesencephalic area. This observation is consistent with the results in the present study of NHPs.

The opioid system modulates stress responses by reducing autonomic and neuroendocrine responses induced by stress (Drolet et al., 2001; Kohno et al., 1983). The stress-induced increase in NE release in hypothalamus, amygdala and LC is attenuated by administration of β -endorphin (Tanaka et al., 2000). Also, β -endorphin inhibits NE release from rat LC cell culture (Raymon & Leslie, 1994).

In the present study, chronic replacement with progesterone, but not estradiol, increased the density of β -endorphin axons innervating the LC of rhesus monkeys, suggesting that progesterone might inhibit the activity of LC neurons, at least in part, through the β -endorphin neurotransmission. This inhibitory action seems to occur with chronic treatment, as used in this study. In contrast, we demonstrated in rats that one injection of progesterone in estradiol-primed females induces a marked increase in the electrical activity of LC neurons, and an increase in the NE release in the preoptic area (Szawka et al., 2013; Szawka et al., 2009). Thus, it seems that acute progesterone increases the firing rate of LC neurons while chronic progesterone induces the β -endorphin system, indirectly inhibiting LC neurons. Alternatively, these may be species differences. Results from the present study suggest that estradiol prevented the progesterone action on β -endorphin axon density. In rats, estradiol stimulates β -endorphin release in hypothalamus through GABA-B receptors (Micevych & Kevin Sinchak, 2013). Since allopregnenalone can act through GABA-A receptors, this difference may partially explain how estradiol and progesterone act in opposite ways on the β -endorphinergic innervation to the LC. Further studies are needed to assess the mechanisms behind these opposite actions. Nevertheless, our results suggest that chronic treatment with progesterone alone could be a key tool for the mechanism by which the general stress response can be reduced, via β -endorphin neurotransmission.

In order to evaluate if estradiol and progesterone would have neuroprotective actions on the LC of NHPs, we studied the number of apoptotic cells in the LC of ovariectomized monkeys treated with or without HRT. We initially hypothesized that the control-placebo treated animals would have a greater number of TUNEL-positive neurons than the progesterone or estradiol + progesterone groups. However, there was no difference in the number of TUNEL-positive neurons between the groups, and the hypothesis was null. The loss of NE neurons has been demonstrated in models of Alzheimer Disease (AD) (Manaye et al., 2013), and in several other neurodegenerative disorders [for review see (Marien et al., 2004)]. However, healthy brains do not appear to lose NE neurons during the natural aging process (Ohm et al., 1997). Manaye and colleagues (2011) described a neuroprotective role of estradiol in which this hormone attenuated the neuronal loss in the hypothalamus of ovariectomized rats in a rodent model of AD. Nonetheless, no studies have shown a neuroprotective effect of estradiol and progesterone in the LC, although there is evidence that the onset of menopause, characterized by the decline of ovarian steroids, can lead to anxiety and depression (Solomon & Herman, 2009; Warren, 2007), both related to alterations in NE neurotransmission.

Several studies have demonstrated the efficiency of ovarian steroids as neuroprotective factors (Singh, 2006; Suzuki et al., 2006; Simpkins et al., 2005). One of the main strategies for neuroprotection is the inhibition of apoptosis, a process characterized by chromatin condensation and DNA fragmentation, leading to the formation of apoptotic bodies (Peter et al., 1997). In the present study we have used the TUNEL assay to detect nuclear fragmentation and consequently apoptotic cells. Although the statistical analysis did not show a significant difference among groups, it is noteworthy that there was a considerable individual variation, which was a key factor in the statistical results. It is possible that animals used in the present study could have genetic predisposition to be more or less susceptible to the actions of ovariectomy on the DNA fragmentation. The variance of results

among the groups is large as is often the case with monkeys. We cannot rule out the hypothesis that the age variation (7–14 years old) could have contributed to the high variability found in the present study. This problem could be solved by increasing the number of animals, but the cost of primates is prohibitory. The progesterone-treated group exhibited the highest variability among all studied groups, which might be due to a variety of responses to progesterone and its receptors subtypes A (PRA) and B (PRB). Progesterone can interact with membrane receptors as PGRMC1, sigma 1 and GABA A through its metabolite allopregnenolone (Pluchino et al., 2009). Hence different efficacy of metabolism of and sensitivity to alloprenalone may play a role. Altogether, these confer distinct tissue specific responses that can elevate the variability of physiological responses.

There were a smaller number of apoptotic cells found in the LC compared to our previous data from the DRN of the same animals (Lima & Bethea, 2010). While the average of apoptotic neurons in the DRN was 60–100 neurons/mm³ (Lima & Bethea, 2010), the LC from the same animals had 1–2 neurons/mm³. This suggests that ovariectomy *per se* does not lead to a significant increase in the neuronal death in the LC. Also, it shows that the lack of neuron death in the LC is not directly related to animal's age, since both studies used the same animals. Therefore, the LC does not seem to be a target for apoptosis in ovariectomized animals without brain injury. These data are consistent with the finding that during normal brain development, the LC does not exhibit significant neuronal loss (Ohm et al., 1997). In our previous study we noted that the TUNEL staining was restricted to the dorsal and median raphe nuclei (Lima & Bethea, 2010). Tokuyama and colleagues (2008) showed that the expression of apoptosis-inducing factor (AIF) was restricted to the serotonergic neurons of the DRN, which is consistent with our observations. In sum, these data support the idea that there may be a difference in the sensitivity to apoptotic signals among amine neurons.

4- CONCLUSION

Altogether, the present data showed that HRT increases the inhibitory influence of β -endorphin in the LC, but has no effect on neurodegeneration or neuroprotection in the LC NE neurons. It should be noted that not all formulations of HRT are equally effective. Most importantly, the commonly used *medroxyprogesterone acetate* as the progestin component of HRT has major detrimental effects on neuronal survival and function compared to bioidentical progesterone, which was used in this study (Bethea, 2011).

5- EXPERIMENTAL PROCEDURES

The Oregon National Primate Research Center Institutional Animal Care and Use Committee approved this study.

5.1- Animals and treatments

Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized by the surgical personnel of ONPRC according to accepted veterinary surgical protocol. All animals were born in China and were aged between 7 and 14 years by dental exam, weighed 4 – 8 kg, and were in good health. Steroid hormone treatments were initiated 3–8 months after

ovariectomy. The ovariectomized control monkeys (n=5) received an empty (placebo) Silastic capsule. The estradiol-treated monkeys (n=5) were implanted with a 4.5-cm Silastic capsule (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI) filled with crystalline estradiol [1,3,5(10)-estratrien-3,17 β -diol; Steraloids, Wilton, NH] for 28 days. The estradiol + progesterone-treated monkeys (n=5) received an estradiol-filled capsule for 28 days supplemented with one 6-cm capsule filled with crystalline progesterone (4-pregnen-3,20 dione; Steraloids) on days 14–28. The progesterone only-treated monkeys (n=5) received a placebo capsule for 28 days supplemented with one 6-cm capsule filled with crystalline progesterone on days 14–28. All capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA). The monkeys were euthanized at the end of the treatment periods according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, given an overdose of pentobarbital (30 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

5.2- Tissue Preparation

Following euthanasia, the left ventricle of the heart was cannulated and the head of each animal was perfused with 1 liter of saline followed by 7 liters of 4% paraformaldehyde in 3.8% borate, pH 9.5 (both solutions made with DEPC-treated water (0.1% diethyl pyrocarbonate) to minimize RNase contamination). The brains were removed and dissected. Tissue blocks were post-fixed in 4% paraformaldehyde for 3 h, and then transferred to 0.02M potassium phosphate-buffered saline (KPBS), followed by 20% glycerol and 2% dimethyl sulfoxide at 4°C for 3 days to cryoprotect the tissue. After infiltration, the block was frozen in isopentene cooled to –55°C, and stored at –80°C until sectioning. Sections (25 μ m) were cut on a sliding microtome, and serial sections were collected in a cryoprotection buffer (30% ethylene glycol, 20% glycerol in 0.05 M PBS) and then stored at –20°C until processing for immunohistochemistry or TUNEL assay. Both protocols were performed with tissue from the same animals.

5.3- Immunohistochemistry for β -endorphin in the LC

Brain sections were removed from cryoprotectant and immediately washed with 0.02 M KPBS for 4 times with 15 min each (rinsed). Then, they were immersed in 0.6% H₂O₂ for 30 min, rinsed and incubated with the following blocking solutions: 0.6% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) for 1 hour; 3% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) for 1 hour; avidin for 20 min and biotin for 20 min (Vector Laboratories, Burlingame, CA, USA). Sections were then incubated at 4°C for 72 h in antibody to β -endorphin (MAB5276, Millipore, Billerica, MA, USA), diluted 1/2000 in 0.6% NGS, 0.4% triton and KPBS. After blocking, the sections were rinsed and incubated in Vector biotinylated antibody diluted 1:400, for 1 hour, rinsed, incubated with Vector ABC reagent for 1 hour, rinsed, incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.2 mg/ml; Sigma) in KPBS plus nickel sulfate (25 mg/mL) and 3% hydrogen peroxide for approximately 2 min, rinsed and dehydrated through a graded series of ethanols, xylene, and Histoclear. Sections were mounted under glass with DPX.

5.4- Quantitative Analysis of β -endorphin staining

Sections were anatomically matched between animals using anatomical reference points. Each section was examined throughout the rostro-caudal extent of the LC (four levels, 120 μ m intervals between sections) with an Olympus, BH-2 Brightfield microscope. For each anatomical level, the largest representation of the LC was chosen from amongst all monkeys. A rectangle outline was placed over the chosen area and the exact dimensions were recorded (pixels and square micrometer total area). The same size rectangle was then used for all of the monkeys at that anatomical level. The operator chose a gray level threshold above which the pixel was considered positive and then the image was segmented into positive and negative pixels by Image-J (NIH, USA). Next, images were examined at high magnification to verify that only specific-immunostained signal concentrated in axons was highlighted. The positive pixel area was obtained by operator thresholding of signal to background (set to consistently reflect the signal from slide to slide). The positive pixel area was computed as percent of the total area in pixels. The photographs and analysis were conducted at 50x magnification. The positive pixel area was obtained from each morphological level and the results of the 4 levels from each animal were averaged.

5.5- TUNEL assay

For the *in situ* analysis of DNA fragmentation, TUNEL staining was performed using a commercial kit (ApopTag Kit-S7100, Chemicon, Temecula, CA). The manufacturer's instructions were followed. Briefly, frozen sections were post-fixed with 1% paraformaldehyde for 15 min followed by a cold solution of ethanol: acetic acid (2:1) for 5 min. Then the slides were washed 2 times with 50 mM phosphate buffer saline (PBS), immersed in 0.5% triton for 15 min, washed 2 times, digested with proteinase K (20 μ g/ml) for 15 min and endogenous peroxidase was quenched with 3% H_2O_2 for 20 min. After 2 washings, the sections were incubated with TdT enzyme at 37°C for 90 min. This was followed by incubation with anti-digoxigenin-peroxidase for 60 min at room temperature, and color development with H_2O_2 -3,3'-diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan) for 20 min, followed by 3 washes in milli-Q water. Sections were dehydrated in butanol followed by xylene and then mounted on Superfrost Plus slides (Fischer, Santa Clara, CA) under glass with DPX. Some of the slides were counterstained with 0.5% methyl green. For positive controls, specimens of mammary gland tissue from rats were provided in the Apop Tag kit. Extensive apoptosis occurs 3–5 days after weaning of rat pups so mammary gland tissue exhibits marked chromatin fragmentation labeled with TUNEL. Negative controls were performed by omission of TdT enzyme from the incubation buffers. The region of the LC was analyzed and the morphometrical evaluation was made at a magnification of 1,000 $\times$ with careful registration of the morphological features as previously described (Lima & Bethea, 2010).

5.6- Quantitative Analysis of TUNEL staining

Sections were anatomically matched between animals using anatomical reference points. Each section was examined throughout the rostro-caudal extent of the LC (five levels, 120 μ m interval between sections). Images were captured with an Olympus, BH-2 Brightfield microscope. An area was defined and measured (μ^2 total area) using the Image J software

(NIH, USA). For cell counting, the area of interest was outlined and TUNEL stained cells were identified and counted by hand. All cell counts refer to detectable neurons only. The number of TUNEL-positive neurons in the designated area of the five sections was summed, generating one value for each animal. The individual sums were averaged to obtain the group mean. In addition, the thickness of each section ($20\text{ }\mu\text{m} \times 5\text{ sections} = 100\text{ }\mu\text{m}$) plus the intervals between the sections ($120\text{ }\mu\text{m} \times 4\text{ intervals} = 480\text{ }\mu\text{m}$) in microns were used as the length of the region ($100 + 480 = 580\text{ }\mu\text{m}$). The average area of the region for each animal (μ^2 outlined by Image J), was multiplied by the length to obtain the volume of the region ($\mu^3/10^9 = \text{mm}^3$). The total number of TUNEL-positive neurons in each animal was divided by the volume of the area examined to obtain the number of positive neurons per cubic millimeter for each animal. The individual numbers of TUNEL-positive neurons/ mm^3 were then averaged to obtain the group means.

5.7- Statistical Analysis

The results are expressed as the mean $\pm$ SEM of the indicated number of experiments. The symmetry of the data was tested by Kolmogorov-Smirnov normality test. One-way analysis of variance (ANOVA) for unpaired groups followed by Newman-Keuls's post hoc was used for multiple comparisons of parametric data. Statistical analyses were conducted using the Prism Statistical software 5.0 (GraphPad Software Inc., San Diego, Calif., USA). $p < 0.05$ was considered statistically significant.

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Highlights

- Progesterone increases the beta-endorphin inhibitory input to *locus coeruleus*.
- The absence of ovarian steroids does not lead to an increased cell death in the *locus coeruleus*.
- Progesterone, but not estrogen, could facilitate the β -endorphin neuronal suppression of stress, mediated by *locus coeruleus*.

Figure 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) (10x magnification), $n=5$ animals/group. 4V: 4th ventricle. (E). Overall mean $\pm$ 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; $p = 0.0002$.

Figure 2.

Illustration of DNA fragmentation and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) with methyl green counterstaining in the locus coeruleus (LC) of rhesus monkeys treated with progesterone (A) or absence of positive staining in rhesus monkeys treated with estradiol (B), $n=5$ animals/group, (5x magnification). In (C), there are two staining patterns observed in an ovariectomized animal: type I staining (single arrows) and type II staining (double headed arrow), (20x magnification). Photomicrograph of a counterstained section of the LC of an ovariectomized animal, in which the Tdt enzyme was omitted during the TUNEL assay (negative control section), with no apparent DNA fragmentation detected (10x magnification) (D). Photomicrographs of a section that was exposed to DNase for 10 min before TUNEL staining and methyl green counterstaining, in an ovariectomized animal, used as a positive control. (10x magnification) (E) and (20 x magnification) (F).

Figure 3.

Illustration of terminal deoxynucleotidyl transferase nick end labeling (TUNEL) with methyl green counterstaining distributed in the locus coeruleus (LC) of ovariectomized rhesus monkeys, treated with progesterone, n=5 animals/group, (10x magnification) (**A**) and (**B**). Photomicrograph of a counterstained section of the LC showing apoptotic neurons in different degrees of DNA fragmentation. Neurons from the LC of an estradiol-treated monkey, the single arrows indicate in earlier stages of degradation and double-headed arrows indicate neurons in an advanced stage of degradation (**C**) and (**D**) (20x magnification).

Figure 4.

Number of terminal deoxynucleotidyl transferase nick end labeling (TUNEL)-positive neurons in the locus coeruleus (LC) of ovariectomized rhesus macaques treated with placebo (OVX), estradiol (E), progesterone (P) or estradiol + progesterone (EP). Total number of TUNEL positive cells in the four levels of the LC in each group (n= 5 animals/group) are presented as average $\pm$ SEM. Total number of TUNEL-positive cells (A). Number of TUNEL-positive cells per mm³ (B). The total area did not differ between groups (C). ANOVA *n.s.* (non highly).