Journal of Steroid Biochemistry & Molecular Biology 116 (2009) 15–20

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Central modifications of allopregnanolone and β-endorphin following subcutaneous administration of Nestorone

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ARTICLE INFO

Article history:
Received 17 November 2008
Received in revised form 2 April 2009
Accepted 8 April 2009

Keywords:
Nestorone
Allopregnanolone
β-Endorphin
Brain
Progestins

ABSTRACT

The aim of the present study was to evaluate the potential action of Nestorone (alone or in combination with estradiol valerate) on the level of allopregnanolone and of the opioid β-endorphin in selected brain areas.

Wistar ovariectomized rats were given 0.05 mg/(kg day) of estradiol valerate (E2V) or subcutaneous Nestorone at three dose levels: low dose (10 μg/(kg day)), antiovulatory dose (50 μg/(kg day)) and high dose (250 μg/(kg day)) with and without E2V.

E2V therapy reversed the reduction of allopregnanolone and β-endorphin induced by ovariectomy anywhere was analyzed except for the adrenal gland. Nestorone showed no effect on allopregnanolone concentration in serum or any part of the brain tissue when given alone while it had a synergistic increasing effect in allopregnanolone concentration in some parts of the brain (hippocampus, hypothalamus, anterior pituitary and serum) when given at high dose of 250 μg/(kg day) in combination with E2V. At lower doses it possesses a synergistic effect with E2V only in the hippocampus (at 50 μg/(kg day)) and in the anterior pituitary (at 10 and 50 μg/(kg day)).

Nestorone administered alone at any dose led to significant increase in β-endorphin levels in the hippocampus only while, in the high dose group, there was a significant increase in endorphin levels in anterior pituitary and hypothalamus in addition to hippocampus as compared to ovariectomized control rats. In addition, only the highest dose of Nestorone added to estrogen increased β-endorphin levels of hippocampus and plasma. Thus the lower doses of Nestorone alone or in combination with estrogen do not seem to exert any great effect on both allopregnanolone and β-endorphin. It is only the highest dose of Nestorone that increases allopregnanolone and β-endorphin levels in selected brain areas, which are the hippocampus, the hypothalamus, the anterior pituitary and serum/plasma. This suggests that Nestorone at the antiovulatory dose levels may not alter the positive effects of estrogen treatment on mood and behaviour.

1. Introduction

The widespread use of progestins, alone or in combination with estrogens, for female contraception and hormone replacement therapy, has generated renewed interest in the development of new progestins with high receptor selectivity indices [1–3]. Nestorone® (16-methylene-17α-acetoxy-19-nor-4-pregnene-3,20-dione), formerly called ST-1435, belongs to the 19-norprogesterone class of progestins. Upon oral administration it undergoes rapid metabolism and inactivation [3]. Nestorone showed very low affinity for androgen receptor but high affinity for progesterone receptor, which conferred high selectivity on this progestin. Nestorone showed no binding to SHBG [3,4].

Nestorone is a potent progestin when administered parenterally and does not appear to alter liver function, carbohydrate metabolism or lipid metabolism [3–5]. When given parenterally via sustained release formulations such as subdermal implant, vaginal ring or transdermal administration, a strong progestational activity combined with lack of androgenic, estrogenic and glucocorticoid-like activities confer special advantages to this steroid for use in contraception and hormone replacement therapy. Nestorone was found to be very effective in controlling fertility at low doses [3,6]. Kumar et al. found out that the dose of 10 μg/(rat day) in rats weighing 200–250 g inhibited complete ovulation when given by the subcutaneous route [3]. Its progestational activity is 10 times higher than that of levonorgestrel and 100 times greater than that of

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doi:10.1016/j.jsbmb.2009.04.004

progesterone and this was shown in rats treated with subcutaneous progestins too [3].

Clinical and experimental evidence suggest that progesterone and progestins affect central nervous system activity directly or through their conversion into neuroactive metabolites, such as allopregnanolone or through the activation or pathways that results in the production of β-endorphin. No studies on the possible central effect of the Nestorone molecule have yet been performed. Thus, the aim of the present study was to evaluate the potential action of Nestorone (alone or in combination with estradiol valerate) on the level of allopregnanolone and of the opioid β-endorphin in selected brain areas.

2. Animal model and study protocol

72 fertile female Wistar rats weighing 150–200 g, 3 months old, were used in the present study.

They were housed 8 per cage, provided with food and water *ad libitum* and kept in a climate-controlled room, with 14 h of illumination (light on at 6 a.m. and light off at 8 p.m.).

Bilateral ovariectomy was performed under tiletamine plus zolazepam anaesthesia (Zoletil, 1 mg/rat i.m.), with the only exception of the fertile control group. Rats were ovariectomized on the day of proestrus, as indicated by vaginal smear (evaluated daily). After a 2-week period of recuperation following gonadectomy, oral estradiol valerate (E2V, 0.05 mg/(kg day)), different doses of subcutaneous Nestorone (10, 50, 250 μg/(kg day)) or subcutaneous Nestorone plus oral estradiol valerate (10, 50, 250 μg/(kg day) + E2V 0.05 mg/(kg day)) were administered for 14 consecutive days to the castrated rats.

One group fertile and one group of OVX rats were used as controls, that is to say they did not receive active treatment. At the end of the treatment they were killed by decapitation as well as the OVX control group. The animals of the fertile control group were killed by decapitation too, in the morning of proestrus, as verified by a vaginal smear. The following organs, according to our previous studies, were then taken and weighed: the frontal and parietal lobes, the hippocampus and the hypothalamus, the anterior and neurointermediate pituitary, and the adrenal glands. All organs were weighed, collected in 2.5 ml solution of 4% acetic acid, and homogenized at ice-cold temperature. The homogenate was centrifuged at 1200 × *g* for 15 min at 4 °C and the supernatant was divided in two aliquots (1.25 ml each) and assayed in duplicate for allopregnanolone and β-endorphin. A blood specimen was immediately drawn after decapitation from each rat and collected into heparinised and non-heparinised plastic tubes. β-Endorphin levels were measured in the frontal and parietal lobes, the hippocampus, the hypothalamus, the anterior pituitary, the neurointermediate pituitary, and plasma, while allopregnanolone levels were measured in the frontal and parietal lobes, the hippocampus, the hypothalamus, the anterior pituitary, the adrenal glands, and serum. The differences in the choice of the areas to analyze are due to the fact that β-endorphin can also be detected in the neurointermediate lobe [7] and that allopregnanolone is also synthesized by the adrenal gland [8]. In each of the tissue selected and plasma or serum, β-endorphin and allopregnanolone were measured by radio immunological assays. The protocol was approved by the local Ethical Committee.

2.1. Allopregnanolone assay

The supernatant of tissue homogenates and serum was passed through a C-18 Sep-Pak cartridge, previously equilibrated with homogenizing buffer. The cartridge was sequentially washed with homogenizing buffer, 50% aqueous methanol, and the unconjugated steroid fraction was eluted with absolute methanol and brought to dryness under nitrogen. Analytical grade solvents were purchased from Merck (Darmstadt, Germany); C-18 Sep-Pak cartridges were obtained from Waters Corporation (Milford, USA). Allopregnanolone contents were measured by a radioimmunoassay method previously described, using an antiserum kindly provided by Dr RH Purdy (San Diego, CA, USA) [9,10]. The sensitivity of this assay was 20 pg/ml, the recovery after extraction and chromatography was 86.5 ± 12.7% (mean ± SEM), and the intra- and inter-assay coefficients of variation were 7% and 9%, respectively. In accordance with previously reported data, allopregnanolone levels were expressed in pg/mg of tissue in each tissue and in pg/ml in serum [9,11,12].

2.2. β-Endorphin assay

The supernatant of tissue homogenates and plasma was passed through a C-18 Sep-Pak cartridge, previously equilibrated with 50% aqueous methanol, and the unconjugated fraction was eluted with absolute methanol and brought to dryness under vacuum. β-Endorphin levels were measured by a previously described specific radioimmunoassay [13,14], using camel β-endorphin as standard (Sigma Chemicals, St. Louis, MO, USA).

The antiserum (supplied by Dr. P. Sacerdote, Milano, Italy) was used at the final dilution of 1:130,000. Analytical grade solvents were purchased from Merck (Darmstadt, Germany); C-18 Sep-Pak cartridges were obtained from Waters Corporation (Milford, USA). The sensitivity of this assay was 10 pg/ml, the recovery following acetic acid extraction and chromatography corresponded to 85 ± 11% of the total amount, and the intra- and inter-assay coefficients of variation were 6% and 8%, respectively. In accordance with previously reported data, β-endorphin levels were expressed in ng/organ in all tissues and in ng/ml in plasma [14–17].

2.3. Statistical analysis

All data are reported as mean ± standard deviation. Data obtained were analyzed by one-way analysis of variance, and the Bonferroni multiple comparison test was used to compare treatment groups.

3. Results (see Figs. 1 and 2)

3.1. Effects of ovariectomy

As reported earlier, ovariectomy led to a significant reduction of allopregnanolone in all the examined tissues (except for the adrenal) and in serum, and of β-endorphin in all analyzed areas and in plasma.

3.2. Effects of estradiol valerate (0.05 mg/(kg day)) administration on allopregnanolone

Estradiol valerate administration increased allopregnanolone levels in all inspected areas, with the exception of the adrenal gland, where it did not cause any significant change. E2V had a more significant effect ($p < 0.001$) in the parietal lobe, the hypothalamus, the anterior pituitary and serum. The E2V therapy raised allopregnanolone to levels in the range of fertile controls in the frontal and in the parietal lobes.

3.3. Effects of estradiol valerate (0.05 mg/(kg day)) administration on β-endorphin

Estradiol valerate administration increased β-endorphin levels in all inspected areas. E2V had a more significative effect ($p < 0.001$) in the parietal lobe, the anterior pituitary and the neurointermedi-

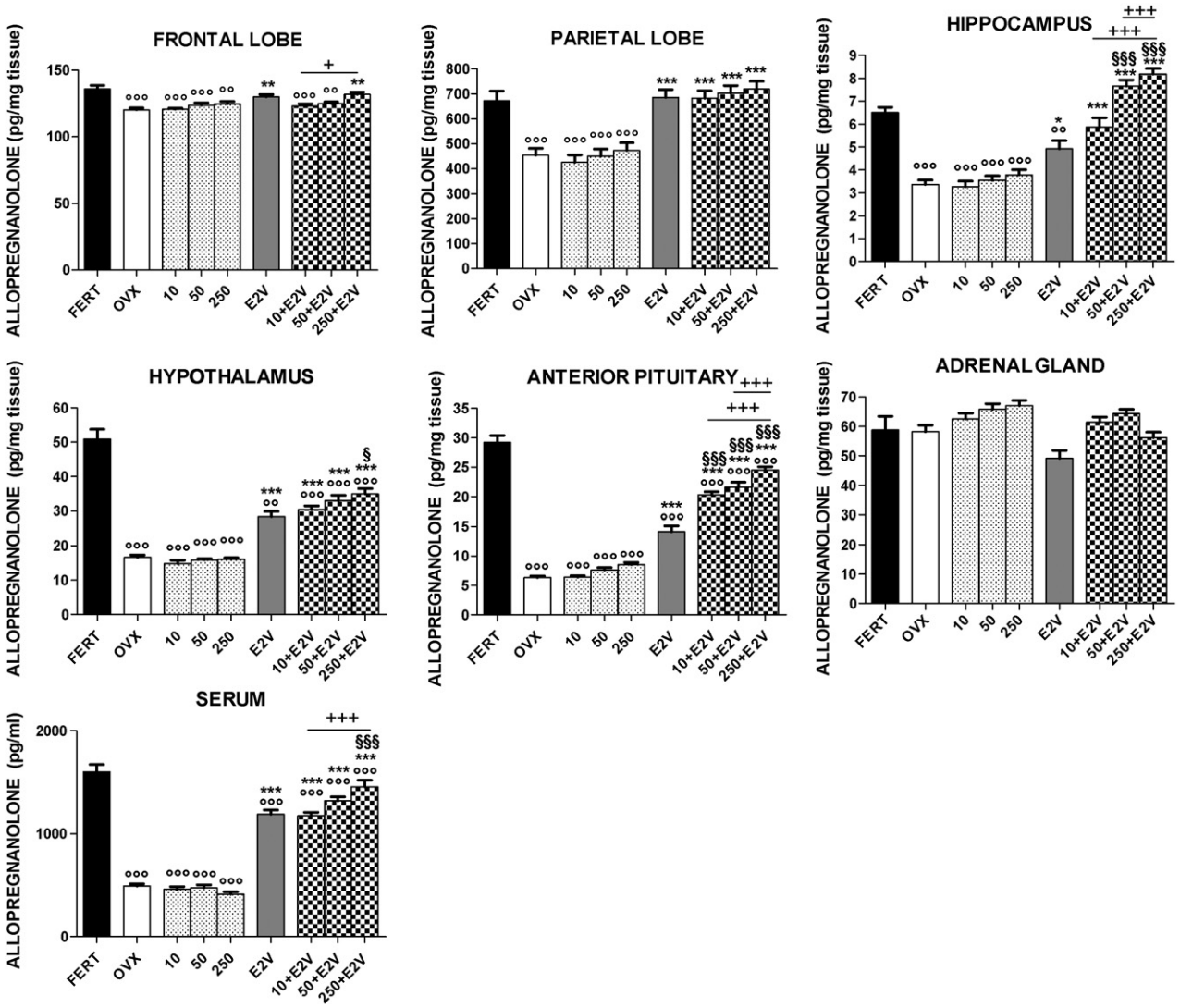

Fig. 1. "Central and peripheral levels of allopregnanolone induced by Nestorone with or without E2V".
Fert = fertile control group.
OVX = ovariectomized group.
(°) versus fertile rats (°$p < 0.05$, °°$p < 0.01$, °°°$p < 0.001$).
(*) versus ovariectomized untreated rats (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
(§) versus E2V treated rats (§$p < 0.05$, §§$p < 0.01$, §§§$p < 0.001$).
(+) between treatments (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$).

ate lobe. The E2V therapy increased β-endorphin to levels similar to the fertile controls in all the areas examined (and in plasma).

3.4. Effects of Nestorone administration (10, 50, 250 μg/(kg day)) without estradiol valerate on allopregnanolone

No significant change in the concentration of allopregnanolone was observed in the brain areas and adrenals after treating OVX rats with subcutaneous Nestorone at all three dose levels.

3.5. Effects of Nestorone administration (10, 50, 250 μg/(kg day)) plus estradiol valerate (0.05 mg/(kg day)) on allopregnanolone

10 μg/(kg day) of Nestorone administered with E2V augmented allopregnanolone ($p < 0.001$), if compared to E2V alone, only in the anterior pituitary; the combined administration of 50 μg/(kg day) of Nestorone and E2V further increased allopregnanolone ($p < 0.001$) in the hippocampus and in the anterior pituitary, without affecting the other areas. The addition of 250 μg/(kg day) of Nestorone significantly enhanced E2V's action in the hippocampus ($p < 0.001$), hypothalamus ($p < 0.05$), anterior pituitary ($p < 0.001$), and serum ($p < 0.001$). In the frontal lobe and in the parietal lobe the co-administration of the three dose levels of Nestorone and 0.05 mg/(kg day) of E2V did not significantly modify the content of allopregnanolone obtained after E2V administration. However, at these levels, a dose related increase of allopregnanolone was observed, even though it did not reach statistical significance. The adrenal content of allopregnanolone was not modified by the addition of any dose of Nestorone to 0.05 mg/(kg day) of E2V.

3.6. Effects of Nestorone administration (10, 50, 250 μg/(kg day)) without estradiol valerate on β-endorphin

10 and 50 μg/(kg day) dose of Nestorone increased ($p < 0.05$) only the hippocampal content of β-endorphin, while 250 μg/(kg day)

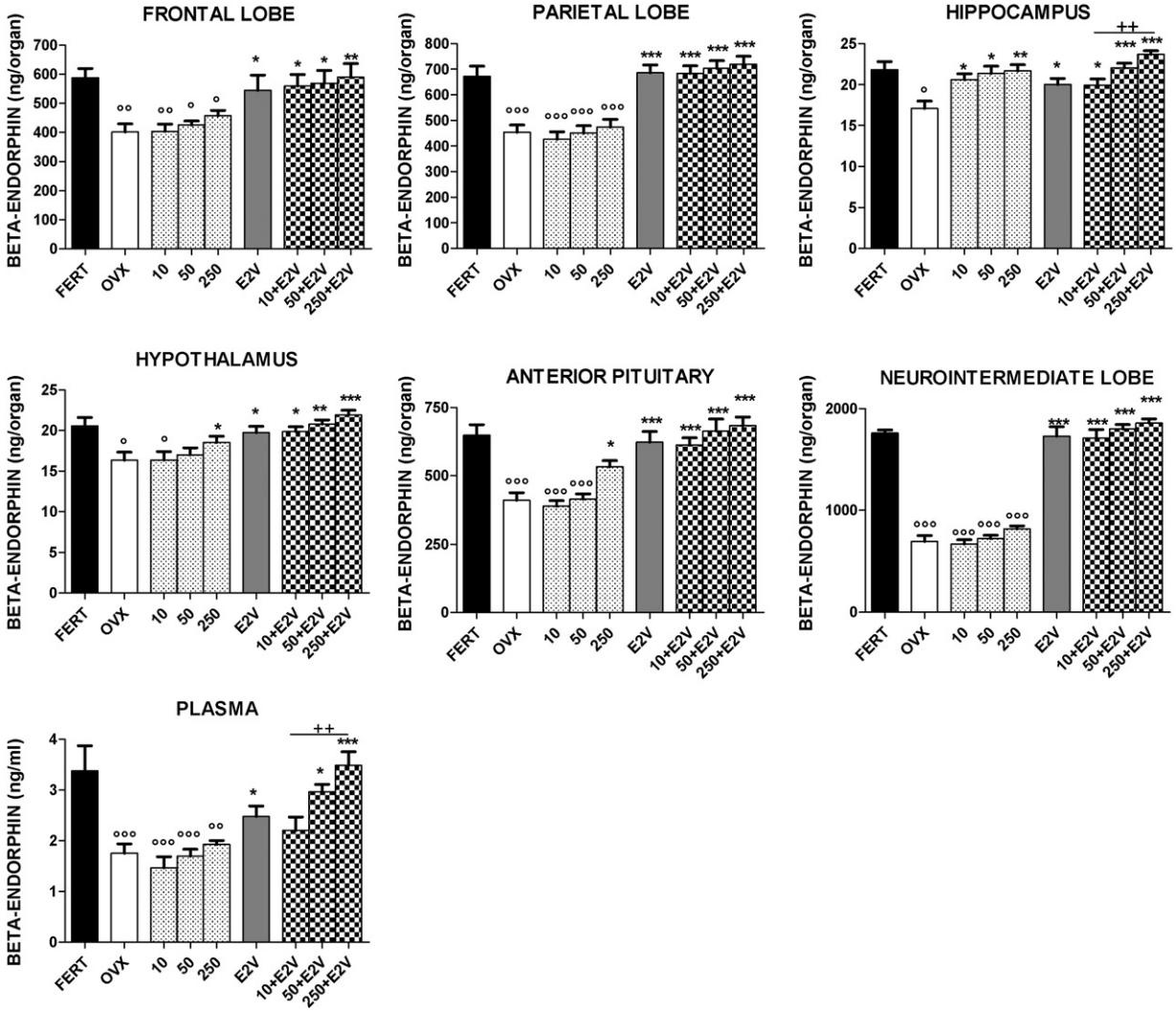

Fig. 2. "Central and peripheral levels of beta-endorphin induced by Nestorone with or without E2V".
Fert = fertile control group.
OVX = ovariectomized group.
(°) versus fertile rats (°$p < 0.05$, °°$p < 0.01$, °°°$p < 0.001$).
(*) versus ovariectomized untreated rats (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
(§) versus E2V treated rats (§$p < 0.05$, §§$p < 0.01$, §§§$p < 0.001$).
(+) between treatments ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$).

of nestorone increased the hippocampal ($p < 0.01$), the hypothalamic ($p < 0.05$) and the anterior pituitary ($p < 0.05$) content of β-endorphin without any effect on other areas examined.

3.7. *Effects of nestorone administration (10, 50, 250 μg/(kg day)) plus estradiol valerate (0.05 mg/(kg day)) on β-endorphin*

The addition of any dose of Nestorone to estradiol valerate did not alter the activity of E2V, but a dose related increasing trend (not statistically significant) could be observed in any investigated area, and this trend becomes statistically significant in the hippocampus (at the dose of 50 and 250 μg/(kg day)) and in plasma (at 250 μg/(kg day)).

4. Discussion

The administration of estradiol valerate confirming the results obtained in our previous studies, reversed the decline in the levels of both allopregnanolone and β-endorphin induced by ovariectomy in all analyzed brain areas except for the adrenal gland [18–22].

Treatment with Nestorone alone was inactive in inducing changes in allopregnanolone content. However, when given in combination with estradiol valerate, Nestorone led to a synergistic significant increase in allopregnanolone content in tissues analyzed except for the frontal and parietal lobes and the adrenal gland.

In contrast, treatment with Nestorone alone led to an increase in β-endorphin concentrations in the hippocampus at all three dose levels and in the hypothalamus and the anterior pituitary in the high dose group only. Combined treatment with Nestorone and E2V did not exert a synergistic effect on the β-endorphin concentration.

Estrogens given alone are known to affect brain levels of neurotransmitters that have positive influence on cognition and mood in women [23]. In contrast women who use sequential treatment of estrogen and progestins for contraception or HRT have reported altered mood changes that are mostly ascribed to the progestins [24,25]. Several different progestins are used in the oral contraceptives or HRT that may affect the mood alterations differently. An ideal progestin should have either beneficial central effects

Table 1
Effect of progestins on allopregnanolone and β-endorphin concentration in selected areas of brain tissue and serum/plasma.

	MP	MPA	NOMAc	NES
Allopregnanolone				
Frontal lobe	↑ 4–8 mg/(kg day)	↑ 0.2 mg/(kg day)	=	=
Parietal lobe	↑ 2–4–8 mg/(kg day)	↑ 0.2 mg/(kg day)	=	=
Hippocampus	↑ 8 mg/(kg day)	↑ 0.2 mg/(kg day)	↑ 0.5–1 mg/(kg day)	=
Hypothalamus	↑ 4–8 mg/(kg day)	↑ 0.2 mg/(kg day)	=	=
Anterior pituitary	↑ 8 mg/(kg day)	↑ 0.2 mg/(kg day)	=	=
Adrenal	=	=	↓ 1 mg/(kg day)	=
Serum	↑ 2–4–8 mg/(kg day)	=	=	=
β-Endorphin				
Frontal lobe	=	=	=	=
Parietal lobe	=	=	=	=
Hippocampus	=	↓	↑ 1 mg/(kg day)	↑ 0.01, 0.05, 0.25 mg/(kg day)
Hypothalamus	↑ 4–8 mg/(kg day)	=	↑ 0.5–1 mg/(kg day)	↑ 0.25 mg/(kg day)
Anterior pituitary	=	=	=	↑ 0.25 mg/(kg day)
Neurointermediate lobe	↑ 8 mg/(kg day)	↑ 0.2 mg/(kg day)	=	=
Plasma	↑ 2–4–8 mg/(kg day)	=	=	=

MP (micronized progesterone) 2–4–8 mg/(kg day) (see Ref. [19]), MPA (medroxyprogesterone acetate) 0.05–0.1–0.2 mg/(kg day) (see Ref. [19]), NOMAc (nomegestrol acetate) 0.05–0.1–0.2–0.5–1 mg/(kg day) (see Ref. [22]). =: no statistically significant modification versus OVX; ↑: statistically significant increase versus OVX; ↓: statistically significant decrease versus OVX.

on its own or it should not negate the beneficial effects of estrogens when co-administered for therapeutic purposes. Although the use of progestins and in particular MPA has been questioned for its effects on neuronal tissue, it is possible that progesterone or its 19-norprogesterone derivatives such as Nestorone when combined with estrogen might be beneficial. Indeed Nilsen and Brinton showed difference between progestins on estrogen-induced neuroprotection with a synergistic effect exerted by progesterone and 19-norprogesterone but an antagonism exerted by MPA [26].

In this study three dose levels of Nestorone (with and without E2V) were tested for its effects on the concentrations of allopregnanolone and β-endorphin in selected parts of brain tissue and blood: low dose (10 μg/(kg day)), antiovulatory dose (50 μg/(kg day)) and high dose (250 μg/(kg day)). Nestorone showed no effect on allopregnanolone concentration in serum or any part of the brain tissue when given alone while it had synergistic increase in allopregnanolone concentration in some parts of the brain (hippocampus, hypothalamus, anterior pituitary and serum) when given at high dose of 250 μg/(kg day) in combination with E2V. At lower doses it possesses a synergistic effect with E2V only in the hippocampus (at 50 μg/(kg day)) and in the anterior pituitary (at 10 and 50 μg/(kg day)).

Nestorone administered alone at any tested dose led to significant increase in β-endorphin levels in the hippocampus only while, at 250 μg/(kg day), there was a significant increase in endorphin levels in anterior pituitary and hypothalamus in addition to hippocampus as compared to ovariectomized control rats. In addition, Nestorone treatment together with estrogen did not alter the effect on β-endorphin levels in selected brain areas. Thus the lowest dose of Nestorone alone or in combination with estrogen does not seem to exert any effect on both allopregnanolone and β-endorphin (except in the hippocampus). Furthermore the antiovulatory dose of 50 μg/(kg day) of Nestorone has an impact only on hippocampus (regarding both allopregnanolone and β-endorphin) and anterior pituitary allopregnanolone. So, it is only the dose of 250 μg/(kg day) of Nestorone that increases allopregnanolone and β-endorphin levels in various brain areas, which are the hippocampus, the hypothalamus, and the anterior pituitary and in serum/plasma. This suggests that Nestorone at the lowest therapeutic dose levels may not alter the positive effects of estrogen treatment on mood and behaviour.

Our earlier study showed that MPA increased allopregnanolone content of frontal lobe, parietal lobe, hippocampus, hypothalamus and anterior pituitary at the highest dose administered alone

Table 2
Effect of progestins on concentration of allopregnanolone and β-endorphin induced by E2V in selected areas of brain tissue and serum/plasma.

	E2V	MP + E2V	MPA + E2V	NOMAc + E2V	NES + E2V
Allopregnanolone					
Frontal lobe	↑	▲ 8 mg/(kg day)	▲ 0.2 mg/(kg day)	–	–
Parietal lobe	↑	▲ 4–8 mg/(kg day)	▲ 0.2 mg/(kg day)	–	–
Hippocampus	↑	▲ 8 mg/(kg day)	–	▲ 1 mg/(kg day)	▲ 0.05, 0.25 mg/(kg day)
Hypothalamus	↑	▲ 8 mg/(kg day)	▲ 0.2 mg/(kg day)	▲ 1 mg/(kg day)	▲ 0.25 mg/(kg day)
Anterior pituitary	↑	▲ 8 mg/(kg day)	▲ 0.2 mg/(kg day)	▲ 1 mg/(kg day)	▲ 0.01, 0.05, 0.25 mg/(kg day)
Adrenal	=	–	▼ 0.2 mg/(kg day)	▼ 1mg/(kg day)	–
Serum	↑	▲ 2–4–8 mg/(kg day)	–	–	▲ 0.25 mg/(kg day)
β-Endorphin					
Frontal lobe	↑	▲ 4–8 mg/(kg day)	–	–	–
Parietal lobe	↑	▲ 4–8 mg/(kg day)	–	–	–
Hippocampus	↑	–	–	▲ 1 mg/(kg day)	–
Hypothalamus	↑	▲ 2–4–8 mg/(kg day)	–	▲ 1 mg/(kg day)	–
Anterior pituitary	↑	▲ 4–8 mg/(kg day)	▲ 0.2 mg/(kg day)	▲ 1 mg/(kg day)	–
Neurointermediate lobe	↑	▲ 2–4–8 mg/(kg day)	▲ 0.2 mg/(kg day)	–	–
Plasma	↑	▲ 8 mg/(kg day)	–	▲ 1 mg/(kg day)	–

E2V (estradiol valerate) 0.05 mg/(kg day) (see Refs. [18–22]), MP (micronized progesterone) 2–4–8 mg/(kg day) (see Ref. [19]), MPA (medroxyprogesterone acetate) 0.05–0.1–0.2 mg/(kg day) (see Ref. [19]), NOMAc (nomegestrol acetate) 0.05–0.1–0.2–0.5–1 mg/(kg day) (see Ref. [22]). =: no statistically significant modification versus OVX; ↑: statistically significant increase versus OVX; ▲: statistically significant increase versus E2V; ▼: statistically significant decrease versus E2V; –: no significant modification versus E2V.

whereas Nestorone alone showed no such effect. MPA had suppressing effect on the β-endorphin levels in hippocampus at all three doses tested while Nestorone increased the hippocampal levels at all doses tested [19]. Comparing the effects of MPA and Nestorone on estrogen induced increase in allopregnanalone, Nestorone at high dose seems to possess synergistic effect to the estrogen stimulated increase in hippocampus, hypothalamus, anterior pituitary and in serum/plasma while MPA at high dose had synergistic effect on the estrogen induced allopregnanolone levels in frontal and parietal lobes and hypothalamus and pituitary. However, these differences were observed only in the high dose group (see Tables 1 and 2).

Nestorone is a 19-norprogesterone derivative like trimegestone, nomegestrol acetate (NomAc) and promegestone. Our recent study showed that NomAc affected allopregnanolone content of hippocampus, hypothalamus and anterior pituitary. Regarding β-endorphin we found that NomAc increased hypothalamus and hippocampus by itself and also the anterior pituitary and plasma when associated with E2V (see Tables 1–2). These considerations stand for a selectivity of action of these two agents of the same class of the 19-norprogesterones for part of the limbic system and the anterior pituitary. An important difference might be the activity exerted on β-endorphin that for NomAc is obtained in part by itself and in part when combined with E2V, while Nestorone is active totally by itself although only at the higher pharmacological dose, with no interactions with the estrogen compound.

Further work is warranted to examine the effects in greater detail, especially in regard to neuroendocrine related effects.

In conclusion, this study shows that Nestorone at the lowest administered dose levels does not alter the concentrations of allopregnanolone and β-endorphin in the selected brain areas and blood in rats. The effect of Nestorone on these two parameters is different than that of progesterone or other progestins. Additional work needs to be done to understand the clinical significance of these effects of different progestins.

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