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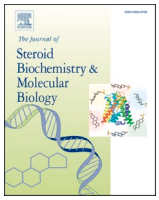

# Neuroactive steroids fluctuate with regional specificity in the central and peripheral nervous system across the rat estrous cycle

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ABSTRACT

Neuroactive steroids (i.e., sex steroid hormones and neurosteroids) are important physiological regulators of nervous function and potential neuroprotective agents for neurodegenerative and psychiatric disorders. Sex is an important component of such effects. However, even if fluctuations in sex steroid hormone level during the menstrual cycle are associated with neuropathological events in some women, the neuroactive steroid pattern in the brain across the ovarian cycle has been poorly explored. Therefore, we assessed the levels of pregnenolone, progesterone, and its metabolites (i.e., dihydroprogesterone, allopregnanolone and isoallopregnanolone), dehydroepiandrosterone, testosterone and its metabolites (i.e., dihydrotestosterone, 3α-diol and 17β-estradiol) across the rat ovarian cycle to determine whether their plasma fluctuations are similar to those occurring in the central (i.e., hippocampus and cerebral cortex) and peripheral (i.e., sciatic nerve) nervous system. Data obtained indicate that the plasma pattern of these molecules generally does not fully reflect the events occurring in the nervous system. In addition, for some neuroactive steroid levels, the pattern is not identical between the two brain regions and between the brain and peripheral nerves. Indeed, with the exception of progesterone, all other neuroactive steroids assessed here showed peculiar regional differences in their pattern of fluctuation in the nervous system during the estrous cycle. These observations may have important diagnostic and therapeutic consequences for neuropathological events influenced by the menstrual cycle.

## 1. Introduction

In the steroidogenic pathway, the first steroid synthesized from cholesterol is pregnenolone (PREG). This steroid is metabolized in progesterone (PROG), which in turn is converted in androgens, such as testosterone (T) and then in estrogens, such as 17β-estradiol (17β-E). PREG is also metabolized into dehydroepiandrosterone (DHEA), which represents a substrate for the synthesis of androgens and estrogens. PROG and T are also converted in reduced metabolites, such as dihydroprogesterone (DHP), allopregnanolone (ALLO) and isoallopregnanolone (ISOALLO) in case of PROG, and dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol) in case of T. All these steroidogenic steps occur in both the peripheral steroidogenic glands, with the formation of steroid hormones, and in the nervous system, with the formation of neurosteroids [1].

The term "neuroactive steroids" includes both steroid hormones and neurosteroids. Thus, this steroid family includes molecules that, with independence of their origin, are able to regulate the nervous system function, affecting for instance synaptic plasticity [2–10], cytoskeletal proteins, the morphology of neurons and astrocytes [2,9,11–14], adult neurogenesis [15–17] and cognition [6,7,9,14,18,19].

All of these mechanisms and processes are affected by sex, and neuroactive steroids contribute to these sex differences. Accordingly, sex differences in neuroactive steroid levels in the brain have been reported. For instance, the brain levels of PROG and its metabolites are higher in pseudopregnant female rats than in males [20] and neuroactive steroids show different levels in the brain and peripheral nerves of males and females [21]. In addition, in females, the levels of sex steroids and gonadotropins fluctuate in plasma across the ovarian cycle. This fluctuation, which is involved in different feedback neuroendocrine mechanisms, affects synaptic function in different regions of the nervous system, modulating mood, cognition, motivation, and behavior [22–26].

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Furthermore, the fluctuation of sex steroids during the menstrual cycle is temporally associated in some women with migraine, catamenial epilepsy, sleep alterations or mood and anxiety disorders [27–30], and impacts the condition of female patients affected by Parkinson's disease, multiple sclerosis, and stroke [27].

These actions of sex steroids in the nervous system may be in part mediated by direct actions on a variety of membrane and nuclear receptors expressed by neurons and glial cells. However, previous studies have shown that both gonadotropins and sex steroids produced by gonads affect central steroidogenesis and neuroactive steroid levels [31–34]. This suggests that the cyclic fluctuation of sex steroids and gonadotropins during the ovarian cycle may potentially alter the levels of neuroactive steroids in the nervous system.

Neuroactive steroids target a variety of receptors in neural cells, including neurotransmitter receptors, such as $GABA_A$ receptors [26,35]. Since some of these receptors are the target of medications that may be used for the treatment of menstrual cycle-associated mental and neurological disorders, it is important to determine the fluctuation of neurosteroids in the brain across the ovarian cycle. However, the pattern of change of these molecules in the brain across the ovarian cycle has been poorly explored, with limited information restricted to the hippocampus and the estrous cycle in rats [36,37], and nothing is known on the levels of neuroactive steroid levels across the ovarian cycle in the peripheral nerves.

To address this question, in the present study we have assessed the levels of selected neuroactive steroids across the ovarian cycle in rats to determine if their fluctuation in plasma is paralleled by similar changes in the brain and peripheral nerves. Previous studies have characterized the fluctuation of sex steroid levels in plasma across the rat estrous cycle [38–41], which consists in four consecutive phases: proestrus, estrus, diestrus 1 and diestrus 2, of 24 h each. These studies have shown that the main events in the fluctuation of sex steroids in plasma consist in a preovulatory elevation in 17β-E levels that peaks in the morning of proestrus, followed by two rises in PROG, one in the afternoon of proestrus and a second one in diestrus 1 [38–41]. In our study, in addition to the classical sex steroids related to the estrous cycle (i.e., 17β-E and PROG), we have investigated the levels of other neuroactive steroids, such as PREG, the PROG metabolites DHP, ALLO and ISOALLO, DHEA, T, and its metabolites DHT and 3α-diol. All these neuroactive steroids have been assessed by liquid chromatography tandem mass spectrometry in plasma, in two brain regions, the hippocampus and the cerebral cortex, and in the sciatic nerve, at the four phases of the rat estrous cycle (i.e., proestrus, estrus, diestrus 1, and diestrus 2).

## 2. Materials and methods

### 2.1. Study design and sample preparation

Adult female Sprague-Dawley rats, Crl:CD BR (Charles River, Calco, Italy) were housed in pairs in the animal care facility of the Dipartimento di Scienze Farmacologiche e Biomolecolari (Università degli Studi di Milano, Milan, Italy) with food and tap water available at libitum. The housing conditions were: controlled temperature (21 ±4°C), humidity (40–60 %), room ventilation (12.5 air changes per hours) and 12:12 h light / dark cycle (lights on 7 h A.M) and controlled humidity. The animals were handled following the European Union Normative (Council Directive 86 / 609 / EEC) and in accordance with national laws and policies (D.L. No. 26 and G.U. No. 61, both of 4 March 2014); all procedure have been approved by our Institutional Animal Use and Care Committees (OPBA) and by the Italian Ministry of Health (authorization number: 1083–2015-PR).

At 14 weeks of age, animals were sacrificed in proestrus (P; n=7), estrus (E; n=8), diestrus 1 (D1; n=7) and diestrus 2 (D2; n=8). Cyclicity of the animals have been checked by daily vaginal smears and only those demonstrating at least two consecutive 4-day cycles were used in the experiments described. All rats were euthanized during the morning under deep isoflurane anesthesia; blood was collected in tubes with EDTA 0.25 M pH 8 and plasma was obtained by centrifugation at 2500xg for 15 minutes at 4°C. After sacrifice, the brain was removed from the skull, hippocampus and cerebral cortex were dissected; similarly, sciatic nerves were dissected from the body, and all samples were rapidly frozen at −80°C.

### 2.2. Liquid chromatography–Tandem mass spectrometry analysis (LC–MS/MS)

The levels of pregnenolone (PREG), progesterone (PROG), dihydroprogesterone (DHP), allopregnanolone (ALLO), isoallopregnanolone (ISOALLO), dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-diol), and 17β-Estradiol (17β-E), were extracted and purified as previously described [21,34] from hippocampus (60 mg), cerebral cortex (60 mg), sciatic nerves (40–50 mg) and plasma (300 μL). For quantitative analysis, brain regions and nerves were homogenized in ice-cold methanol/acetic acid 1 % using a Tissue Lyzer (Qiagen, Milan, Italy), and plasma samples were diluted in acetonitrile. 17β-Estradiol-2,3,4-$^{13}C^3$($^{13}C_3$-17β-E) (2 ng/sample), progesterone-2,3,4,20,25-$^{13}C_5$ ($^{13}C_5$–PROG) (0.4 ng/sample), and pregnenolone-20,21-$^{13}C_2$-16,16 $D_2$ ($^{13}C_2D_2$–PREG) (10 ng/sample) were used as internal standards and added to all samples. After an overnight extraction at 4°C, the organic phase was purified using C18 SPE cartridges (HyperSep C18 SPE Columns 500 mg 3 mL; Microcolumn, Milano, Italy). The quantitative analysis was performed using liquid chromatography (LC) supplied by Surveyor LC Pump Plus and Surveyor Autosampler Plus (Thermo Fisher Scientific,Waltham, MA, USA) connected with a linear ion trap-mass spectrometer LTQ (Thermo Fisher Scientific,Waltham, MA, USA), operated in positive atmospheric pressure chemical ionization (APCI+) mode. The chromatographic separation was achieved with a Hypersil Gold column C18 (100 mm 2.1 mm, 3 m; Thermo Fisher Scientific, Waltham, MA, USA) maintained at 40°C. The mobile phases consisted of 0.1 % formic acid in water (phase A) and 0.1 % formic acid in methanol (phase B). Gradient elution was as follows: 0–1.50 min 70 % A; 1.50–2.00 min 55 % A; 2.00–3.00 min 55 % A; 3.00–35.00 min linear gradient to 36 % A; 35.00–40.00 min 25 % A; 41.00–45.00 min 1 % A; 45.00–45.20 min 70 % A; and 45.40–55.00 min equilibrate with 70 % A. The 25 μL sample was injected at a flow rate of 250 μL /min. The divert valve was set at 0–8 min to waste, 8–45 min to source, and 45–55 min to waste. The injector needle was washed with methanol/water 1:1 (v/v). LC–MS/MS data were acquired and processed using the software Excalibur® release 2.0 SR2 (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative analysis of neuroactive steroids was performed on the basis of calibration curves freshly prepared and extracted.

### 2.3. Statistical analysis

The Kolmogorov–Smirnov test was used to control the normal distribution of the data. All data were analyzed by one way ANOVA and considered significant when the p-value was $< 0.05$. Newman-Keuls multiple comparison post hoc test was performed to identify differences between the phases of the estrous cycle. Analyses were performed using Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

Levels of neuroactive steroids were evaluated by LC-MS/MS in plasma, hippocampus, cerebral cortex and sciatic nerve during proestrus, estrus, diestrus 1 and diestrus 2 phases of the rat estrous cycle. The results obtained are presented in Figs. 1–4. Fig. 1 shows the levels of PREG, PROG, and DHP; Fig. 2 the levels of ALLO and ISOALLO; Fig. 3 the levels of DHEA, T, and DHT and Fig. 4 the levels of 3α-diol and 17β-E.

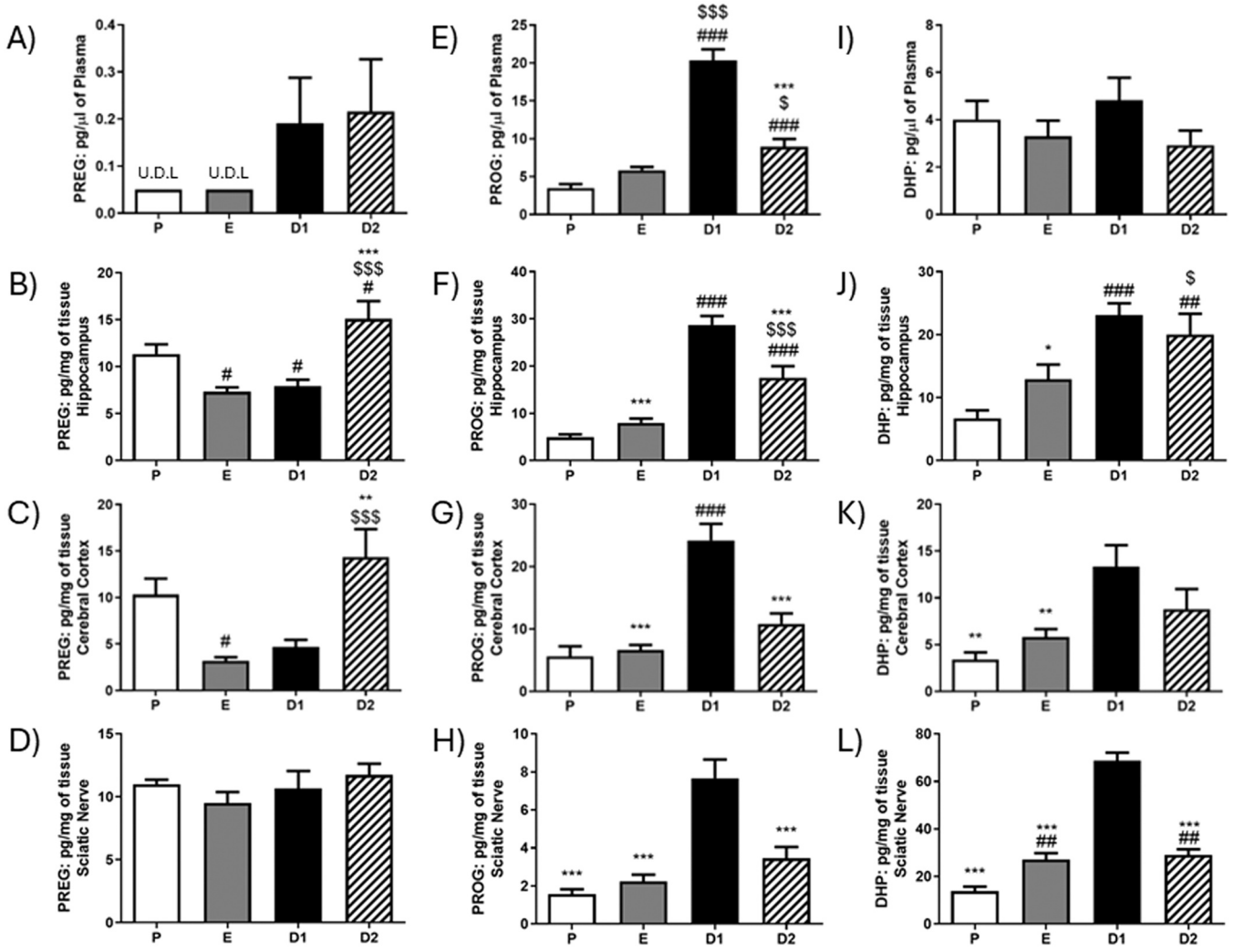


**Fig. 1.** Levels of pregnenolone (PREG, panels A, B, C and D), progesterone (PROG, panels E, F, G and H) and dihydroprogesterone (DHP, panels I, J, K and L) were assessed by liquid chromatography tandem mass spectrometry in plasma (panels A, E and I), hippocampus (panels B, F and J), cerebral cortex (C, G and K) and sciatic nerve (D, H and L) from adult female rats in the different phases of estrous cycle (P: proestrus; E: estrous; D1: diestrus 1; D2: diestrus 2). Data are expressed as pg/µL for plasma and pg/mg for tissues and are the mean ± SEM. U.D.L. = under detection limit. Detection limit was 0.05 pg/µL for PREG. n = 6–8 animals for each experimental group. The one-way ANOVA was used for statistical analysis followed by the multiple comparison Newman-Keuls post-hoc test. Statistical significance was indicated as follow: # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. P; ** $p < 0.01$ and *** $p < 0.001$ vs. D1; $ $p < 0.05$, and $$$ $p < 0.001$ vs. E.

### 3.1. Pregnenolone

As shown in Fig. 1A, plasma PREG levels did not show significant fluctuations during the different phases of the estrous cycle. PREG levels reached higher levels in brain (Fig. 1B,C) and peripheral nerve tissue (Fig. 1D) than in plasma (Fig. 1A). As in plasma, PREG levels remained stable across the estrous cycle phases in the sciatic nerve (Fig. 1D). In contrast, the levels of PREG fluctuated in the hippocampus (Fig. 1B) and cerebral cortex (Fig. 1C) according to the different estrous cycle phases. Thus, the levels of PREG show a significant decline from proestrus to estrus in both brain regions and rebounded from diestrus 1 to diestrus 2 to reach by diestrus 2 similar values to proestrus in the cerebral cortex, or even higher values than in proestrus in the hippocampus.

### 3.2. Progesterone and its metabolites: DHP, ALLO and ISOALLO

In contrast to PREG, the levels of PROG showed significant fluctuations during the estrous cycle in both plasma (Fig. 1E), brain (Fig. 1F, G), and peripheral nerve tissues (Fig. 1H), reaching the highest values in diestrus 1 in all samples.

A different pattern was observed for DHP. The plasma levels of this reduced PROG metabolite remained unchanged during the different phases of the estrous cycle (Fig. 1I). In contrast, DHP levels fluctuated during the estrous cycle in the hippocampus (Fig. 1J), cerebral cortex (Fig. 1K), and sciatic nerve (Fig. 1L), reaching the highest levels by diestrus 1 in all these structures, as observed for PROG.

Fig. 2 shows a different pattern of estrous cycle changes for the PROG metabolites ALLO and ISOALLO. Plasma ALLO levels (Fig. 2A) showed an abrupt increase from proestrus to estrus, remained elevated in diestrus 1, and returned to proestrus values by diestrus 2. Similar changes were detected in ALLO levels in the hippocampus (Fig. 2B), which showed an abrupt increase from proestrus to estrus. However, at difference with plasma, ALLO levels in the hippocampus remained elevated over proestrus values, not only in diestrus 1 but also in diestrus 2. In contrast to plasma and hippocampus, no significant fluctuation of ALLO levels was detected during the estrus cycle in the cerebral cortex (Fig. 2C) and the sciatic nerve (Fig. 2D).

ISOALLO levels remained basically unchanged in plasma (Fig. 2E) and in the brain (Fig. 2F, G) during the estrous cycle with only small fluctuations from estrus to diestrus 1 in plasma and from diestrus 2 to proestrus in the hippocampus (Fig. 2F). However, in contrast to ALLO, levels of ISOALLO showed a strong fluctuation during the estrous cycle

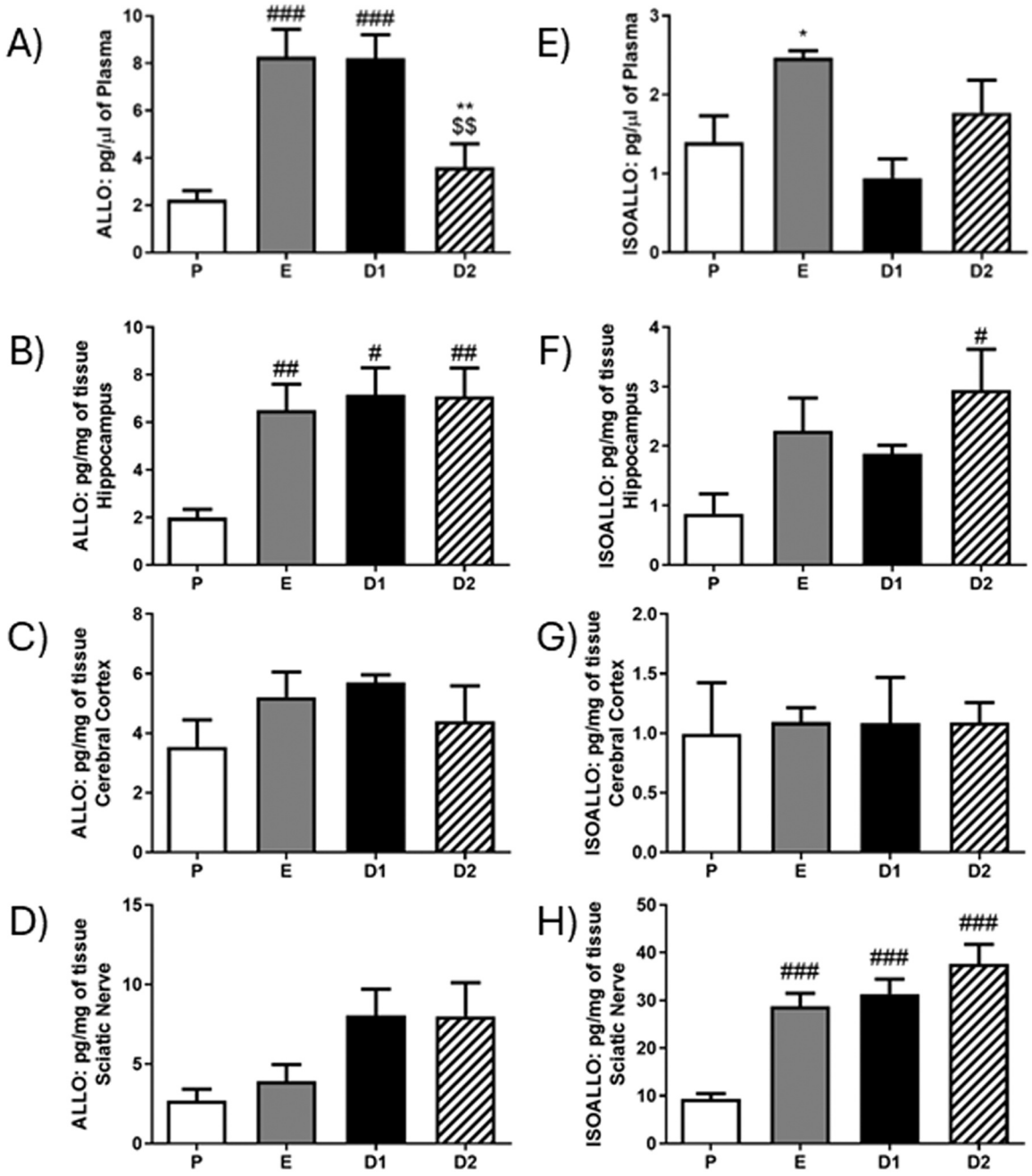


**Fig. 2.** Levels of allopregnanolone (ALLO, panels A, B, C and D), and isoallopregnanolone (ISOALLO, panels E, F, G and H) were assessed by liquid chromatography tandem mass spectrometry in plasma (panels A and E), hippocampus (panels B and F), cerebral cortex (C and G) and sciatic nerve (D and H) from adult female rats in the different phases of estrous cycle (P: proestrus; E: estrus; D1: diestrus 1; D2: diestrus 2). Data are expressed as pg/µL for plasma and pg/mg for tissues and are the mean ± SEM. n = 6–8 animals for each experimental group. The one-way ANOVA was used for statistical analysis followed by the multiple comparison Newman-Keuls post-hoc test. Statistical significance was indicated as follow: # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. P; * $p < 0.05$ and ** $p < 0.01$ vs. D1; $$ $p < 0.01$ vs. E.

in the sciatic nerve (Fig. 2H), with a marked increase from proestrus to estrus, remaining elevated over proestrus values during diestrus 1 and diestrus 2.

### 3.3. DHEA and testosterone

DHEA levels in plasma showed a significant peak in estrus compared to the other days of the estrous cycle (Fig. 3A). In contrast, DHEA remained unchanged across the estrous cycle in the hippocampus (Fig. 3B), the cerebral cortex (Fig. 3C) and the peripheral nerve (Fig. 3D).

No significant changes in T levels (Fig. 3E-H), were detected in plasma (Fig. 3E), hippocampus (Fig. 3F), and sciatic nerve (Fig. 3H) during the different phases of the estrous cycle. In contrast, T levels in the cerebral cortex (Fig. 3G) were significantly decreased in estrus, diestrus 1 and diestrus 2, compared to proestrus.

### 3.4. Testosterone metabolites: DHT, 3α-diol and 17β-estradiol

DHT levels remained basically unchanged during the estrous cycle in the four compartments analyzed (Fig. 3I-L), with only a small decrease in diestrus 2 in both plasma (Fig. 3I) and cerebral cortex (Fig. 3K).

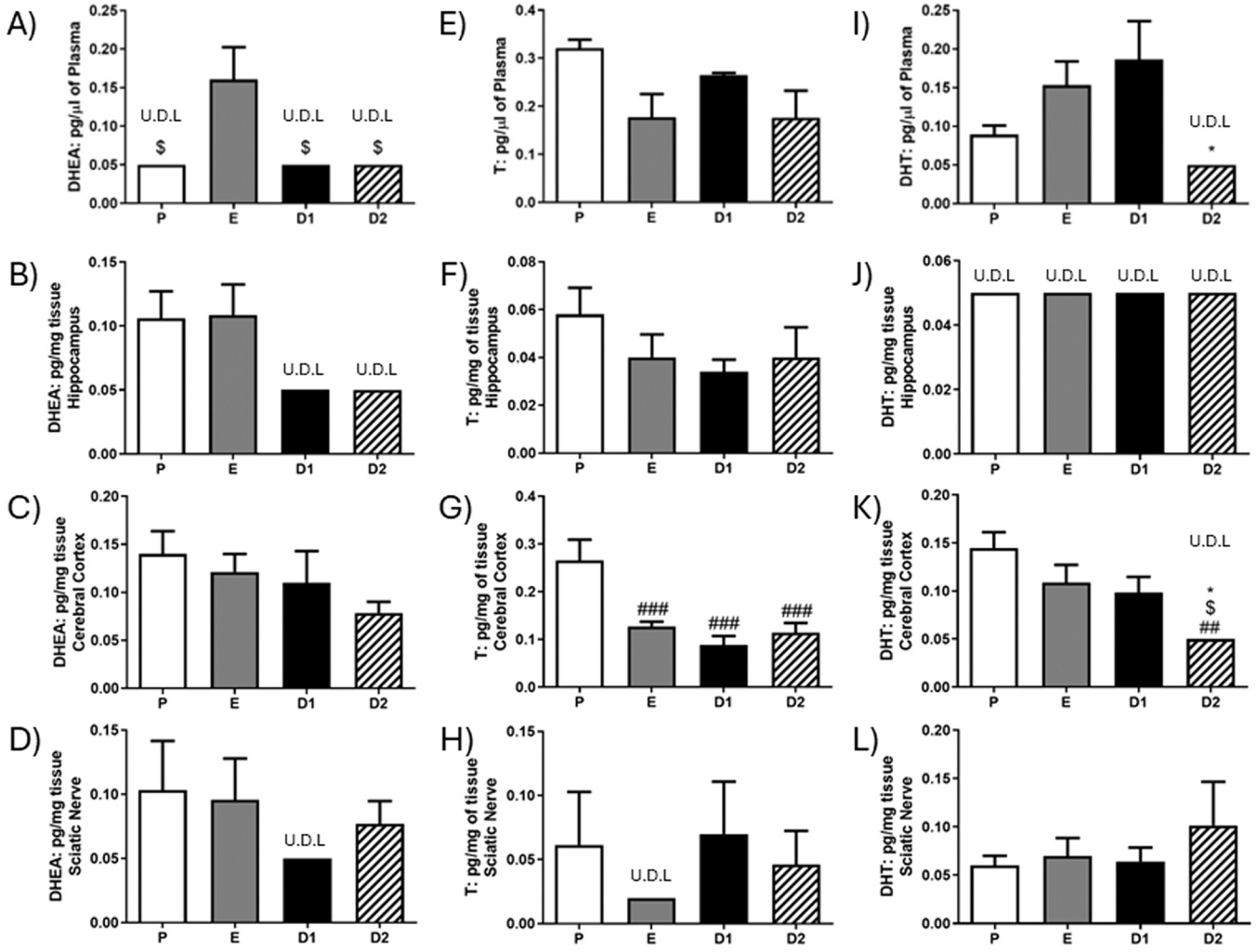


**Fig. 3.** Levels of dehydroepiandrosterone (DHEA, panels A, B, C and D), testosterone (T, panels E, F, G and H) and dihydrotestosterone (DHT, panels I, J, K and L) were assessed by liquid chromatography tandem mass spectrometry in plasma (panels A, E and I), hippocampus (panels B, F and J), cerebral cortex (C, G and K) and sciatic nerve (D, H and L) from adult female rats in the different phases of estrous cycle (P: proestrus; E: estrus; D1: diestrus 1; D2: diestrus 2). Data are expressed as pg/µL for plasma and pg/mg for tissues and are the mean ± SEM. U.D.L. = under detection limit. Detection limits were 0.05 pg/µL or pg/mg for DHEA an DHT, while 0.02 pg/mg for T. n = 6–8 animals for each experimental group. The one-way ANOVA was used for statistical analysis followed by the multiple comparison Newman-Keuls post-hoc test. Statistical significance was indicated as follow: ## $p < 0.01$ and ### $p < 0.001$ vs. P; * $p < 0.05$ vs. D1; $ $p < 0.05$ vs. E.

As shown in Fig. 4A-D, no significant differences in the levels of 3α-diol during the different phases of the estrous cycle were detected in plasma (Fig. 4A), brain (Fig. 4B, C), or peripheral nerve (Fig. 4D). In contrast, 17β-E levels showed an abrupt increase at proestrus in plasma (Fig. 4E) and hippocampus (Fig. 4F). Then, the levels of 17β-E decreased by estrus and remained under proestrus values during the rest of the estrous cycle in these two compartments (Fig. 4E, F). In contrast, in the cerebral cortex (Fig. 4G) and the sciatic nerve (Fig. 4H), 17β-E levels remained basically unchanged during the estrous cycle, although a small reduction in the cortical levels of 17β-E was detected by diestrus 1 compared to proestrus and estrus (Fig. 4G).

## 4. Discussion

Our findings show that the fluctuation of neuroactive steroids in the nervous system across the estrous cycle does not fully reflect the pattern in plasma. Within the brain, the fluctuation is not identical in two different cortical regions, the neocortex, and the hippocampus. Moreover, the fluctuation in these two brain regions is different to that observed in the sciatic nerve. Local steroidogenesis may contribute to these regional differences in the fluctuation of neuroactive steroids across the estrous cycle in neural tissue, because the brain and the peripheral nerves are known to produce PREG, which is the first steroid synthesized from cholesterol in the steroidogenic pathway, being the precursor of the other neuroactive steroids [42–44]. The importance of local steroidogenesis in determining brain steroid levels is emphasized by the higher levels of PREG in brain tissues compared to plasma detected in our study, in agreement with previous findings [42]. Moreover, it is known that PREG precursor cholesterol does not cross the blood brain barrier [45], indicating that the brain produces its own cholesterol for steroidogenesis.

In the nervous system, as in other tissues, the conversion of cholesterol in PREG is catalyzed by the P450scc (Cyp11a1) enzyme (Table 1). This step occurs in the mitochondria and depends on the transport of cholesterol from the outer to the inner mitochondrial membrane by StAR/STARD1 [46], which needs to be processed to be active [47]. Previous studies have shown that 17β-E, PROG, or the suppression of plasma gonadotropin levels, inhibit the processing of StAR in the brain [32], preventing the cholesterol transport to the mitochondria and the synthesis of PREG. In addition, LH induces PREG synthesis by direct actions on LH receptors in brain cells [48]. Moreover, 17β-E has been shown to regulate the metabolism of PREG and DHEA in brain astrocytes

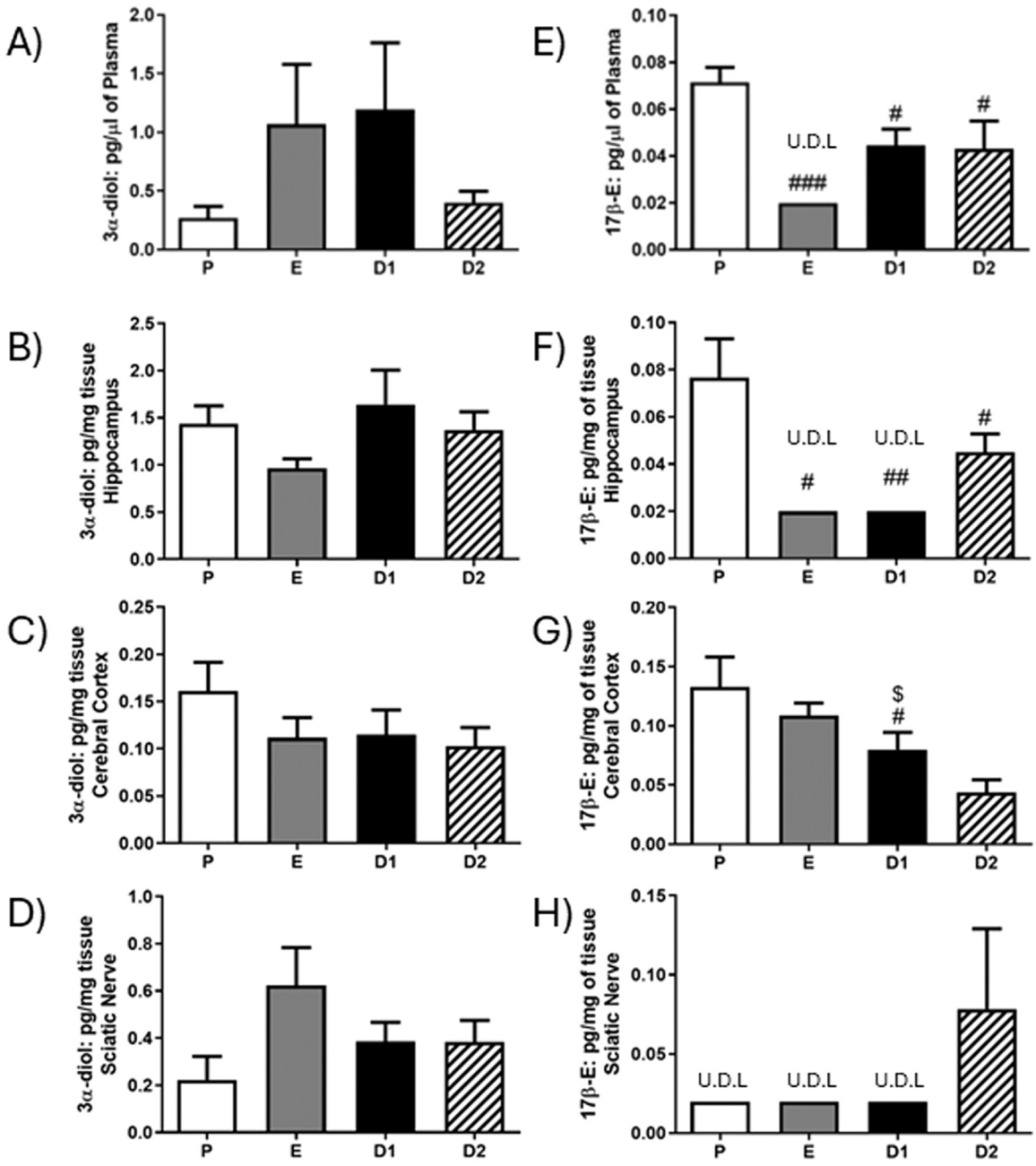


**Fig. 4.** Levels of 5α-androstane-3α,17β-diol (3α-diol, panels A, B, C and D), and 17β-Estradiol (17β-E, panels E, F, G and H) were assessed by liquid chromatography tandem mass spectrometry in plasma (panels A and E), hippocampus (panels B and F), cerebral cortex (C and G) and sciatic nerve (D and H) from adult female rats in the different phases of estrous cycle (P: proestrus; E: estrus; D1: diestrus 1; D2: diestrus 2). Data are expressed as pg/μL for plasma and pg/mg for tissues and are the mean ± SEM. U.D.L. = under detection limit. Detection limit was 0.02 pg/mg for 17β-E. n = 6–8 animals for each experimental group. The one-way ANOVA was used for statistical analysis followed by the multiple comparison Newman-Keuls post-hoc test. Statistical significance was indicated as follow: # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. P; $ $p < 0.05$ vs. E.

and sciatic nerve Schwann cells [49]. Therefore, it should be expected that modifications in PREG synthesis and metabolism by neural cells will occur across the rat estrous cycle following the fluctuating levels of 17β-E, PROG, GnRH, and LH. Our present observations suggest that this is the case, because we detected a significant decline in PREG levels from proestrus to estrus and a marked increase from diestrus 1 to diestrus 2 in the cerebral cortex and hippocampus, while its levels remain low and stable during all phases of the estrous cycle in plasma.

In the endoplasmic reticulum, PREG is metabolized by P45017α (Cyp17a1) enzyme in 17-OH-PREG and then in DHEA (Table 1). From the seminal studies of Baulieu and collaborators it is known that this route of DHEA synthesis from cholesterol operates in the rodent brain [42,51]. Brain DHEA synthesis and metabolism may explain the disparity in our study between DHEA levels in plasma, brain, and peripheral nerve across the estrous cycle. For instance, DHEA metabolism in astrocytes is known to be regulated by 17β-E [49,52] (Table 1). Therefore, fluctuating 17β-E levels across the estrous cycle in plasma may impact on brain DHEA levels.

The most characteristic fluctuation in the plasma levels of ovarian steroids during the rat estrous cycle is the preovulatory elevation of 17β-E, which peaks in the morning of proestrus [38–41,53]. In our study, the rise of 17β-E plasma levels on proestrus was associated with a similar increase in its levels in the hippocampus, in agreement with previous findings by Kawato's laboratory [36,37]. However, the rise of 17β-E in

**Table 1**
Summary of the steroids analyzed during the rat estrous cycle, enzymatic pathways involved in their synthesis, and possible regulating factors.

| Steroid | Pathway | suggested REGULATION |
|---|---|---|
| Pregnenolone **(PREG)** | **CHOL** $\longrightarrow^{P450scc:StAR}$ **PREG** | 17β-E, PROG, LH [32;48;49] |
| Progesterone **(PROG)** | **PREG** $\longrightarrow^{3\beta-HSD}$ **PROG** | |
| Dihydroprogesterone **(DHP)** | **PROG** $\longrightarrow^{5\alpha-R}$ **DHP** | |
| Allopregnanolone **(ALLO)** | **DHP** $\overset{3\alpha-HSOR}{\underset{\longleftarrow}{\longrightarrow}}$ **ALLO** | |
| Isoallopregnanolone **(ISOALLO)** | **DHP** $\overset{3\beta-HSOR}{\underset{\longleftarrow}{\longrightarrow}}$ **ISOALLO** | |
| Dehydroepiandrosterone **(DHEA)** | **PREG** $\longrightarrow^{P45017\alpha}$ **DHEA** | 17β-E [49; 51] |
| Testosterone **(T)** | **DHEA** $\longrightarrow^{3\beta-HSD}$ **T** | |
| Dihydrotestosterone **(DHT)** | **T** $\longrightarrow^{3\alpha-R}$ **DHT** | |
| 5α-androstane−3α,17β-diol (3α-diol) | **DHT** $\longrightarrow^{3\alpha-HSOR}$ **3α − diol** | |
| 17β-Estradiol **(17β-E)** | **T** $\longrightarrow^{ARO}$ **17β − E** | GnRH [31]; T, glutamate and neuronal activity [50] |

plasma was not accompanied by a similar change in the cerebral cortex or the sciatic nerve.

Previous studies have shown that GnRH regulates 17β-E synthesis in neurons [31] (Table 1), suggesting that the fluctuating levels of gonadotropins during the estrous cycle may impact on the levels of this neuroactive steroid in the nervous system. It is also known that adult brain neurons synthesize 17β-E from PREG [54]. As previously mentioned, PREG is metabolized to DHEA, the precursor of T (Table 1), which in turn is converted in 17β-E by the enzyme aromatase (ARO--Cyp19a1) (Table 1), which is expressed in the sciatic nerve [55] and in different regions of the brain, including the cerebral cortex and the hippocampus [56]. While in our study the levels of T remained stable in plasma, they showed a regional specific fluctuation across the estrous cycle in the nervous system, with a peak in proestrus in the cerebral cortex. This fluctuation of T may contribute to regional differences in 17β-E levels within the brain. It should be noted that T is not only the precursor of 17β-E, but is also one of the factors that exert a regional regulation of the activity of ARO in the nervous system, which is also regulated by other local factors, such as neuronal activity and glutamate levels [50] (Table 1). Therefore, local differences in T levels and ARO activity may contribute to the regional differences in the fluctuation of 17β-E across the estrous cycle within the nervous system.

In the case of progesterone, a clear parallelism between its levels in plasma, the hippocampus, the cerebral cortex, and the sciatic nerve was observed at the four periods analyzed of the estrous cycle. Thus, higher levels of progesterone at diestrus 1 compared to the other days of the cycle were detected in the four compartments analyzed, in agreement with previous findings in hippocampus [36]. The observed peak of progesterone at diestrus 1 in plasma also agrees with previous findings [38–41]. It should be noted that another peak of progesterone has been detected in rat plasma in the afternoon of proestrus, at the time of the LH surge [38–41]. This peak of progesterone has been observed also in mice [57]. However, it was not detected in our study because animals were killed in the morning and early afternoon of the proestrus day, before the rise in progesterone. In addition, since plasma progesterone levels are known to return to basal conditions in the morning of estrus, we detected similar levels of this steroid in our plasma samples from proestrus and estrus. Nevertheless, a significant increase in progesterone levels in estrus compared to proestrus was detected in the hippocampus, the cerebral cortex, and the sciatic nerve, which perhaps is the remnant of the rise in progesterone in plasma in the afternoon of proestrus.

The peak of progesterone in the afternoon of proestrus may be also the cause of the increase in the levels of DHP that we detected in the brain and peripheral nerve on the day of estrus. Indeed, while progesterone levels in the nervous system seem to reflect its plasma levels, the situation is different for the progesterone metabolite DHP. Thus, while no significant fluctuation in DHP levels was detected in plasma during the estrous cycle, this steroid showed a pattern of fluctuation in the hippocampus, the cerebral cortex, and the sciatic nerve that parallels that of plasma progesterone. Considering that the brain and peripheral nerve express 5α-reductase (5α-R), the enzyme involved in the conversion of progesterone to DHP [58] (Table 1), it is reasonable to postulate that progesterone metabolism within the nervous tissue is the cause of the fluctuation in the levels of DHP in the brain and peripheral nerve during the estrous cycle.

The nervous system also expresses the enzyme 3α-hydroxysteroid oxidoreductase (3α-HSOR), which converts DHP in ALLO, and the enzyme 3β-hydroxysteroid oxidoreductase (3β-HSOR), which converts DHP in ISOALLO [58] (Table 1). Plasma ALLO may drive the fluctuation of ALLO in the hippocampus, given that similar changes are observed in the two compartments during the estrous cycle. In contrast, there is no parallelism between the fluctuation in the levels of ALLO in plasma, the cerebral cortex, and the sciatic nerve. This is also the case for plasma ISOALLO and the fluctuation of this steroid in the brain and peripheral nerve. Regional differences in the metabolism of DHP to ALLO and ISOALLO may explain the discrepancies between plasma and tissue levels of these steroids.

The absence of a parallelism in the fluctuation of the levels of ALLO and ISOALLO in plasma and the brain is of relevance, considering the role attributed to these steroids in premenstrual syndrome and premenstrual dysphoric disorder, which occurs in the luteal phase of the menstrual cycle of affected women [59–62]. In addition, changes in ALLO levels in plasma during the menstrual cycle may also be linked to catamenial epilepsy [63].

ALLO modulates the activity of $GABA_A$ receptors, while ISOALLO antagonizes the GABAergic actions of ALLO [64–66]. These GABAergic actions participate in the modulation of estrous cycle-associated aggressive behavior in rats [66] and may be involved in the etiology of catamenial epilepsy and the premenstrual dysphoric disorder in women [61–63]. Although our findings in rats are not directly translatable to humans, the absence of a parallelism in the levels of GABAergic neurosteroids between the plasma and the brain is an important aspect to consider in the design of potential therapeutic strategies for women affected by these diseases.

GABAergic neurosteroids exert also different effects in the function of peripheral nerves [67], including the regulation of genes involved in the myelination program and the expression of myelin proteins. In this regard, the observed increase in the levels of ISOALLO from proestrus to estrus and the elevated levels of this steroid at diestrus 1 and diestrus 2 compared to proestrus in the peripheral nerve is intriguing. Previous studies have shown that ISOALLO levels are significantly higher in female peripheral nerves compared to males [68] and elevated plasma levels of ISOALLO have been detected in women with chronic fatigue syndrome [69]. However, the functional significance of the fluctuation in ISOALLO levels during the estrous cycle in peripheral nerves remains to be determined.

In summary, data here obtained indicate that the levels of several neuroactive steroids, such as those of PROG, ALLO, ISOALLO, DHT and 17β-E, change significantly across the different phases of the rat estrous cycle, not only in plasma, but also in the nervous system. However, the fluctuation of these neuroactive steroids in the nervous system is different to that observed in plasma. In addition, some neuroactive steroids, such as PREG, DHP and T, whose levels remain stable in plasma, present significant fluctuations in the brain. Furthermore, the fluctuation of neuroactive steroids in the nervous system across the estrous cycle show regional differences between two cortical regions, the hippocampus and the cerebral cortex, and between these two regions and the sciatic nerve. The regional fluctuation of neuroactive steroids in the nervous system across the ovarian cycle may have important diagnostic and possibly therapeutic consequences for women affected by

migraine, epilepsy, sleep alterations, premenstrual syndrome, premenstrual dysphoric disorder, Parkinson's disease, multiple sclerosis, and stroke, whose symptoms are affected by the menstrual cycle.

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## CRediT authorship contribution statement

**Lucia Cioffi:** Investigation. **Silvia Giatti:** Writing – review & editing, Investigation. **Luis Miguel Garcia-Segura:** Writing – original draft. **Roberto Cosimo Melcangi:** Writing – original draft, Funding acquisition. **Donatella Caruso:** Data curation. **Silvia Diviccaro:** Investigation. **Gabriela Chrostek:** Writing – review & editing.

## Declaration of Competing Interest

The authors report no declarations of interest

## Data Availability

Data will be made available on request.

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