ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

Chronic treatment with hormonal contraceptives alters hippocampal BDNF and histone H3 post-translational modifications but not learning and memory in female rats

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Chronic treatment with hormonal contraceptives alters hippocampal BDNF and histone H3 post-translational modifications but not learning and memory in female rats / Laura Boi, Sabrina Petralla, Barbara Monti, Giuseppe Talani, Enrico Sanna, Maria Giuseppina Pisu, Giulia Calderisi, Elisabetta Maciocco, Mariangela Serra, Alessandra Concas, Patrizia Porcu. - In: HORMONES AND BEHAVIOR. - ISSN 0018-506X. - ELETTRONICO. - 144:(2022), pp. 105218.1-105218.12. [10.1016/j.yhbeh.2022.105218]

Availability:
This version is available at: https://hdl.handle.net/11585/895011 since: 2022-09-29

Published:
DOI: http://doi.org/10.1016/j.yhbeh.2022.105218

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/).
When citing, please refer to the published version.

(Article begins on next page)

23 April 2024

This is the final peer-reviewed accepted manuscript of:

Chronic treatment with hormonal contraceptives alters hippocampal BDNF and histone H3 post-translational modifications but not learning and memory in female rats.

Boi L, Petralla S, **Monti B**, Talani G, Sanna E, Pisu MG, Calderisi G, Maciocco E, Serra M, Concas A, Porcu P.Horm Behav. 2022 Aug;144:105218.

The final published version is available online at: doi: 10.1016/j.yhbeh.2022.105218.

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/)

When citing, please refer to the published version.

Long-term administration of hormonal contraceptives alters hippocampal BDNF and histone H3 post-translational modifications but not learning and memory in female rats

Laura Boi[a], Sabrina Petralla[b], Barbara Monti[b], Giuseppe Talani[c], Enrico Sanna[a,c], Maria Giuseppina Pisu[c], Giulia Calderisi[a], Elisabetta Maciocco[c], Mariangela Serra[a], Alessandra Concas[a], Patrizia Porcu[c]

[a]*Department of Life and Environment Sciences, Section of Neuroscience and Anthropology, University of Cagliari, Cagliari, Italy*

[b]*Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy*

[c]*Neuroscience Institute, CNR - National Research Council of Italy, Cagliari, Italy*

Corresponding Author:
Patrizia Porcu, Ph.D.
Neuroscience Institute
CNR - National Research Council of Italy
Cittadella Universitaria, 09042 Monserrato, Cagliari, Italy
Phone: +39-070-6754163
Email: patrizia.porcu@in.cnr.it

Highlights

- Hormonal contraceptives decrease hippocampal BDNF protein in female rats
- Hormonal contraceptives alter histone H3 post-translational modifications
- Hormonal contraceptives do not alter long-term plasticity at glutamatergic synapses
- Hormonal contraceptives do not alter learning, memory and behavioral flexibility

Abstract

Hormonal contraceptives prevent ovulation with subsequent reduction in endogenous levels of estradiol, progesterone and its neuroactive metabolite allopregnanolone. These neurosteroids modulate several brain functions, including neuronal plasticity, cognition and memory. We hypothesized that hormonal contraceptives might affect synaptic plasticity, learning and memory, as a consequence of suppressed endogenous hormones levels. Female rats were orally treated with a combination of ethinyl estradiol (EE, 0.020 mg) and levonorgestrel (LNG, 0.060 mg) once daily for four weeks. Long-term EE-LNG treatment decreased hippocampal brain-derived neurotrophic factor (BDNF) levels and induced histone H3 post-translational modifications (PTMs) that persist up to 14 days after drug discontinuation. These effects are not accompanied by alterations in long-term plasticity at glutamatergic synapses, recognition memory in the novel object and novel place location tests, or spatial learning, memory, and behavioral flexibility in the Morris water maze test. Thus, decreased BDNF content does not affect synaptic plasticity and cognitive performance; rather it might be relevant for the occurrence of certain psychiatric symptoms, reported by some women using hormonal contraceptives. These results provide the first evidence of hippocampal epigenetic changes induced by hormonal contraceptives and complement previous studies on the neurobiological actions of hormonal contraceptives; the finding that effects of long-term EE-LNG treatment on BDNF content and histone PTMs persist up to 14 days after drug discontinuation warrants further investigation to better understand the implications of such long-term consequences for women’s health.

Keywords

Hormonal contraceptives; BDNF; Synaptic plasticity; Learning and memory; Female rats.

1. Introduction

More than 150 million women worldwide use hormonal contraceptives, mostly for birth control, but also for treatment of endometriosis, polycystic ovarian syndrome and menstrual cycle-related symptoms (Levin et al., 2018). They are available from almost 60 years as synthetic estrogen and progestin formulations, and it is now accepted that they affect the central nervous system. In fact, they alter the volume and structure of several brain regions with subsequent functional changes that in some instances persist after discontinuation (Pletzer et al., 2010; Pletzer and Kerschbaum, 2014). Hormonal contraceptives prevent ovulation by inhibiting the hypothalamic-pituitary-ovarian axis, thus reducing endogenous progesterone and estradiol levels (Levin et al., 2018). We showed that long-term treatment with ethinyl estradiol (EE) and levonorgestrel (LNG), two of the synthetic steroids commonly used in hormonal contraceptive pills, markedly decreased brain and plasma levels of progesterone and its neuroactive metabolite allopregnanolone in female rats (Follesa et al., 2002; Porcu et al., 2012; Santoru et al., 2014; Sassoe-Pognetto et al., 2007). Likewise, in women hormonal contraceptives prevent plasma allopregnanolone fluctuations during the menstrual cycle (Follesa et al., 2002; Paoletti et al., 2004; Rapkin et al., 2006).

Estradiol, progesterone and allopregnanolone act on the central nervous system to regulate reproduction, sexual and social behaviors, as well as neuronal plasticity and cognition (Porcu et al., 2016; Sundström-Poromaa et al., 2020; Taxier et al., 2020). Indeed, hormonal fluctuations strongly affect cognitive function, with estrogen exerting beneficial effects (Luine, 2014), while allopregnanolone worsens learning and memory in preclinical models (Johansson et al., 2002; Mayo et al., 1993; Rabinowitz et al., 2014), and in women (Kask et al., 2008). We thus hypothesized that hormonal contraceptives, by suppressing endogenous levels of such hormones, might affect cognition.

Studies on the effects of hormonal contraceptives on women's cognitive performance are inconclusive, likely due to different cognitive domains analyzed (verbal and spatial abilities, executive function, attention, working memory, etc.), type of progestin administered, different dose of EE used, and/or duration of treatment (Beltz et al., 2015; Gogos et al., 2014; Pletzer and Kerschbaum, 2014; Warren et al., 2014; Wharton et al., 2008). Either improvement (Gurvich et al., 2020; Plamberger et al., 2021; Rosenberg and Park, 2002), no difference (Gogos, 2013; Gravelsins et al., 2021; Islam et al., 2008; Mihalik et al., 2009; Mordecai et al., 2008; Rosenberg and Park, 2002), or deterioration (Griksiene et al., 2018; Griksiene and Ruksenas, 2011) in cognitive abilities, have been reported in hormonal contraceptives' users compared to naturally cycling women.

In animal models, long-term EE-LNG treatment did not alter spatial learning and memory in the Morris water maze test (Santoru et al., 2014), or the novel object recognition (NOR) and novel context recognition tests (Simone et al., 2015) in female rats. However, the 30 μg EE dose increased preference for the novel object, indicating improved recognition memory, but lower doses of EE (10 μg), LNG (20 μg), or their combination impaired recognition memory in the NOR test, an effect associated with decreased hippocampal brain-derived neurotrophic factor (BDNF) mRNA expression (Simone et al., 2015).

BDNF plays a crucial role in learning and memory processes by facilitating synaptic plasticity and long-term potentiation (LTP) in the hippocampus (Bekinschtein et al., 2014). Interestingly, BDNF expression is regulated by steroid hormones; specifically, estradiol directly promotes its expression through an estrogen response element in the BDNF gene (Sohrabji et al., 1995), but also through its signaling cascade involving ERK-mediated phosphorylation of cAMP-response element-binding protein (CREB) and epigenetic modifications such as histone acetylation, which both increase BDNF gene expression (Taxier et al., 2020). Likewise, pregnenolone and allopregnanolone also affect BDNF protein in several brain regions, including hippocampus (Naert et al., 2007).

In female rats we assessed the effects of long-term treatment with EE-LNG and its discontinuation on hippocampal BDNF and on some of the transcriptional and epigenetic mechanisms regulating its expression, i.e. phospho-CREB and histone H3 post-translational modifications (PTMs). We also evaluated hippocampal long-term plasticity at glutamatergic synapses, as well as cognitive performance, by assessing recognition memory in the NOR and novel place location (NPL) tests, and spatial learning, memory and behavioral flexibility in the Morris water maze test. We hypothesized that long-term treatment with hormonal contraceptives might alter expression of BDNF, phospho-CREB and histone H3 PTMs, as well as synaptic plasticity and cognitive performance.

2. Material and Methods

2.1. Animals

Female Sprague-Dawley rats (Charles River, Italy) were bred in our colony and maintained on an artificial 12-hour-light, 12-hour-dark cycle (light on from 8.00 to 20.00 hours) at a constant temperature of 22 ± 2°C and a relative humidity of 65%. Food and water were available ad libitum. Adequate measures were taken to minimize pain or discomfort of animals whose care and handling throughout

the experimental procedures were in accordance with the European Parliament and the Council Directive of 22 September 2010 (2010/63/EU), the Italian Legislative decree n. 26, 4 March 2014, and were approved by the Italian Ministry of Health.

2.2. EE-LNG treatment

Female rats were orally treated with a combination of ethinyl estradiol (EE, 0.020 mg, Sigma-Aldrich E4876) and levonorgestrel (LNG, 0.060 mg, Sigma-Aldrich N2260) once a day (08:30 hours) for 4 weeks. EE and LNG were dissolved in a 0.4% aqueous solution of sodium carboxymethylcellulose (Sigma-Aldrich C5678) and were administered in a volume of 1 ml through a feeding needle. Controls received the same volume of vehicle. Animals were euthanized or were subjected to the behavioral tests 24 hours or 14 days after the last drug treatment. Stage of the estrous cycle was determined by daily vaginal smears taken between 9:00 and 10:00 hours (approximately 30-45 minutes after drug treatment) starting from the second week of treatment and up to sacrifice or behavioral tests.

2.3. Expression of BDNF, phospho-CREB and histone H3 post-translational modifications (PTMs)

For measurement of BDNF, phospho-CREB and histone H3 PTMs, rats were sacrificed by decapitation, the brain was rapidly removed, and the hippocampus dissected on ice and frozen at −80°C until nucleic and cytosolic proteins extraction according to Caruccio and Banerjee (1999).

2.3.1. Nucleic and cytosolic extract preparation

Tissues from hippocampus of vehicle and EE-LNG-treated rats were homogenized in 500 µl of extraction buffer with low salt (20 mM HEPES pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 10 µl/ml protease and phosphatase inhibitor cocktails, all from Sigma Aldrich) and left 15 minutes on ice. After a centrifugation at 800×g for 5 minutes at 4°C, the supernatant (cytoplasmic fraction) was collected in an Eppendorf tube while the nuclei were washed with 200 µl of a washing buffer (20 mM HEPES pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 10 µl/ml protease and phosphatase inhibitor cocktails, all from Sigma-Aldrich) to remove detergent NP-40 and centrifuged at 800×g for 5 minutes at 4°C. The pelleted nuclei were then resuspended into 200 µl of an extraction buffer with high salt (20 mM HEPES pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 10 µl/ml protease and phosphatase inhibitor cocktails, all from Sigma-Aldrich) and left for 45 minutes on ice with periodic mixing. At the cytoplasmic fraction, 1/3 volume of a cytoplasmic extraction clarification buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 0.2 mM

EDTA, 40% glycerol, 1 mM DTT, 10 µl/ml protease and phosphatase inhibitor cocktails) was further added and left for 50 minutes at 4°C to equilibrate the cytoplasmic proteins with NaCl. After adding 1% SDS, all samples were sonicated and total protein content was determined (Lowry et al., 1951).

2.3.2. Western Blot

50 µg of each sample with Laemli loading buffer (1M Tris-HCl pH 6.8; 20% sodium dodecyl sulfate; 0.4 µl/ml glycerol; 2 g/l bromophenol blue and 2M DTT; all from Sigma-Aldrich), were loaded per lane for western blot analysis and resolved in SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences) and non-specific sites were blocked 1 hour at room temperature with PBS 0.1% Tween-20 (Sigma-Aldrich) and 5% nonfat dried milk. Membranes were incubated overnight with primary antibodies against Acetyl-Histone H3 (Lys9/Lys14) (rabbit polyclonal IgG, Cell Signaling Technology); BDNF (rabbit polyclonal IgG, Santa Cruz Biotechnology); CREB (48H2) (rabbit monoclonal IgG, Cell Signaling Technology); GAPDH (mouse monoclonal IgG, Santa Cruz Biotechnology); Histone H3 (rabbit polyclonal IgG, Santa Cruz Biotechnology); Methyl-Histone H3 (Lys9) (rabbit polyclonal IgG, Cell Signaling Technology); Phospho-CREB (Ser133) (rabbit monoclonal IgG, Cell Signaling Technology) and then with HRP-linked secondary antibodies goat anti-rabbit (Santa Cruz Biotechnology), goat anti-mouse (Santa Cruz Biotechnology) for 90 minutes at room temperature and visualized by Clarity ECL (Enhanced ChemiLuminescence; Bio-Rad). All primary antibodies were diluted 1:1000, except GAPDH 1:20000, while HRP-linked secondary antibodies were diluted 1:2000 in PBS-0.1% Tween 20. Images were acquired with a Bio-Rad ChemiDoc imager. Densitometric analysis was performed by using Bio-Rad Image Lab software (Version 5.1).

2.3.3. Real-Time Polymerase Chain Reaction

BDNF messenger RNA (mRNA) expression was quantified in hippocampus of vehicle and EE-LNG-treated rats. For total RNA extraction, 10 µl/mg of tissue of Tri reagent (Sigma-Aldrich) was added, following the manufacturer's protocol. RNA pellets were resuspended in deionized water treated with diethyl pyrocarbonate (DEPC; Sigma-Aldrich) and RNA levels quantified using a NanoDrop UV spectrophotometer (NanoDrop Products; Thermo Scientific). For each sample, 1 µg of DNase-treated RNA (Fermentas) was retrotranscribed using the Superscript III First-Strand Synthesis SuperMix for quantitative Real-Time Polymerase Chain Reaction (RT-PCR) kit (Life Technologies, Italy) according to the manufacturer's instruction.

The following primer pairs for rat BDNF (accession number NM_001270630.1) and rat B-actin (accession number 1.NM_007393.5) (Sigma Genosys) were used:

- <u>Rat BDNF</u>: forward 5′ AGCTGAGCGTGTGTGACAGTAT 3′ (Tm = 63.9°C); reverse 5′ TGATACCGGGACTTTCTCCA 3′ (Tm = 64.4°C);
- <u>Rat B-actin</u>: forward 5′ AGCAGATGTGGATCAGCAAG 3′ (Tm = 63.1°C); reverse 5′ AACAGTCCGCCTAGAAGCAT 3′ (Tm = 62.7°C)

Complementary DNA samples were diluted to a concentration of 10 pg/µL. A master mix of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Srl) was mixed with adequate primers (final concentration, 0.2 µmol/L), complementary DNA (20 pg per replicate), and deionized water. Quantitative RT-PCR was performed using Multimode Plate Reader EnSpire (Perkin Elmer), and RT-PCR measurements with 4 biologic replicates were carried out in duplicate. Messenger RNA relative expression levels were normalized using B-actin as internal control.

2.4. Electrophysiology

2.4.1. Preparation of brain slices

Coronal brain slices containing the hippocampus were prepared as previously described (Talani et al., 2016). Briefly, after reaching deep anesthesia with vapors of 5% isoflurane, rats of all the experimental groups were euthanized and their brains rapidly removed from the skull and transferred to a modified artificial cerebro-spinal fluid (aCSF) containing (in mM): 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2). Brain slices (thickness of 250 µm) containing the dorsal hippocampus were cut using a vibratome (Leica, Germany) and then immediately transferred to a nylon net submerged for at least 40 min at a controlled temperature of 35°C in standard aCSF containing (in mM): 126 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, and 10 D-glucose (pH 7.4, set by aeration with 95% O2 and 5% CO2). After subsequent incubation for at least 1 h at room temperature, a hemi-slice was transferred to a recording chamber with a constant flow rate of ~2 ml/min of aCSF.

2.4.2. Electrophysiology experiments

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed in the stratum radiatum of the CA1 hippocampal subregion through stimulation of the Schaffer collateral afferents, as previously described (Talani et al., 2011). Responses were recorded by filling the recording electrode with KCl (3 M) (resistance ranged from 4.5 to 6.0 MΩ) and were triggered digitally every 20 s by application of a constant current pulse of 0.2-0.4 mA with a duration of 60 µs, which usually yielded a half-maximal response, using a bipolar concentric electrode (FHC) and a Grass stimulator (Digitimer Ltd).

To evaluate the effect of the treatment on long-term plasticity, a specific stimulating protocol for LTP induction was applied. After 10 min of stable baseline, where fEPSPs were evoked every 20 s by a stimulation which yielded a half-maximal response (EC_{50}), a single high-frequency stimulation (HFS, 100 stimuli at 250 Hz) was applied, after which fEPSPs were recorded, every 20 s, for an additional 60 min. The slope values of fEPSPs recorded between 50 and 60 min after HFS were calculated and averaged. To include only stable recordings, if the variation of the fEPSP slope during baseline varied more than ± 5% the recording was excluded from the analysis. Analysis of fEPSP slope was performed using Clampfit 10.7 software (Axon Instruments).

2.5. Behavioral testing

2.5.1. Recognition memory in the NOR and NPL tests

The NOR and NPL tests were performed in a black PVC open field apparatus (width x length x height: 100x100x40 cm) located in the center of a room dedicated to behavioral tests, illuminated (120 lux) by four light bulbs aimed at the ceiling to avoid light reflections in the apparatus. A video-camera positioned above the apparatus recorded behavior for subsequent manual score by an observer blind to the experimental groups. The objects were plastic bottles of different shape and dimensions, weighted so that rats would not move them around, and available in triplicate copies. Both apparatus and objects were thoroughly cleaned with a 20% methanol solution to avoid olfactory cues between each trial. Tests took place over five days in the following order: on days 1 and 2, rats familiarized with the open field for 10 minutes each day; on day 3 they were subjected to the NOR test, and on day 5 they were subjected to the NPL test.

The ability to discriminate novel objects in the NOR test assesses recognition memory (Ennaceur and Delacour, 1988). The test consisted of a sample phase in which the rat was allowed to explore two identical objects. The rat was then returned to its home cage, and after a 60 min delay it was reintroduced in the apparatus for the choice phase, in which two new objects were located in the same place as the sample phase; one was identical to the objects explored in the sample phase and the other was novel. Objects were located 20 cm away from the back corners of the apparatus, and the rat was placed facing away from the objects. Rats were allowed to explore the objects for 3 minutes; time and frequency of exploration were recorded for each object and each phase. Exploration was recorded only when the rat touched or sniffed the objects at a distance less than 2 cm; turning around or sitting near or on the objects was not considered exploratory behavior. Preference for the novel object was

estimated as a percentage of time (and frequency) of exploration: [novel object/(novel object + old object)]x100.

The NPL assesses recognition memory of object location (Ennaceur et al., 2005); it was performed identically to the NOR test, except for the test phase in which identical copies of the sample objects were used, but one was moved to a new location (from the back to the front corner of the apparatus).

Two separate groups of 12 EE-LNG-treated rats each were tested 24 hours and 14 days from the last drug administration, respectively; 29 vehicle-treated rats were monitored for the estrus cycle and 24 of them, showing a regular cycle, were tested in the NOR and NPL on the days of proestrus and/or diestrus 1. All tests were performed in the morning (9:00 to 13:00 hours).

2.5.2. Spatial learning, memory and behavioral flexibility in the Morris water maze test

Spatial learning and memory as well as behavioral flexibility were assessed in the Morris water maze test as previously described (Pisu et al., 2019; Santoru et al., 2014). The water maze consisted of a circular pool (150 cm in diameter, 60 cm in depth), whose interior is painted black, located in the center of a room dedicated to measurement of this behavioral paradigm. The room had visual cues on the walls and was illuminated (120 lux) by four light bulbs aimed at the ceiling to avoid light reflections in the water. The water temperature was maintained at 25 ± 2°C with the use of a submersible digital water-heating system. The pool was divided into four virtual quadrants (south, west, north, east), and a removable circular escape platform (10 cm in diameter, 32 cm in height) was introduced into one of the quadrants (target quadrant) at a depth of 2 cm below the water surface. Assessment of spatial learning occurred on four consecutive days (days 1-4), followed by a probe trial on day 5 to assess memory performance. 48 hours later, behavioral flexibility was assessed by performing four additional days of training in the reversal learning test (days 8-11). For spatial learning, each rat was subjected to four training trials on each of four consecutive days (days 1-4); the rat was released in the maze from one of the four different quadrants (starting from the south one) in order to locate the escape platform on quadrant east. Once the animal had climbed onto the platform, it was allowed to remain there for 15 seconds before the next trial; if it had not found the escape platform by 120 seconds, it was gently guided to the platform and allowed to rest there for 15 seconds. The time elapsed (latency) before the animal climbed onto the platform and distance travelled during each trial were recorded; animals that did not climb onto the platform before the end of the trial period were assigned a latency value of 120 seconds. Swim speed and cumulative search error (i.e. the corrected cumulative distance to the platform) were also recorded on training trials. On day 5, 24 hours after the last training trial, each rat

was subjected to a probe trial, in which the escape platform was removed from the pool and the animal was released from the quadrant (west) opposite to the original platform location and allowed to freely swim for 60 seconds. On days 6 and 7, rats were allowed to rest; then, starting from day 8, behavioral flexibility was assessed in the reversal learning test. Rats were subjected to a new four-day training session in which the platform was moved to the opposite side than the learning sessions (i.e. from the east to the west quadrant). Rats had to relearn the new platform location. Performance in the reversal learning test was indexed by cumulative search error and latency to reach the platform. On day 12, 24 hours after the last training trial of the reversal learning, each rat was subjected to a probe trial, in which the escape platform was removed from the pool and the animal was released from the east quadrant, opposite to the west one where the platform was located during the reversal learning, and allowed to freely swim for 60 seconds. Behavioral data from the training and probe tests were acquired and analyzed using an automated tracking system (Ethovision XT 5.0, Noldus, Wageningen, The Netherlands).

Twelve rats from each experimental group were examined. They were treated with EE-LNG or vehicle once a day for 4 weeks before starting the behavioral test. The EE-LNG 24 hours group continued to receive treatment immediately after each of the test days (days 1-11). To test the effects of EE-LNG discontinuation, rats were treated with EE-LNG or vehicle once a day for 4 weeks and the behavioral test started 14 days after the last drug administration. All tests were performed in the morning (9:00 to 13:00 hours). Given the repeated daily testing over 11 consecutive days, the estrus cycle phase was not taken into account as a variable.

2.6. Statistical analysis

Statistical analysis was performed using commercially available statistical programs (GraphPad Prism, version 7, GraphPad Software, San Diego, CA, USA). Data are presented as means ± SEM. Statistical significance was assessed by Chi-square test, or by one- or two-way analysis of variance (ANOVA), followed by Bonferroni post-hoc comparison test. Effect size estimates for ANOVAs were computed by η^2. A p value <0.05 was considered statistically significant.

3. Results

3.1. EE-LNG treatment alters the estrus cycle

We previously reported that long-term EE-LNG treatment (0.020-0.060 mg/rat, respectively) decreased plasma allopregnanolone levels by 43%, from 17.6 ng/ml in vehicle-treated rats to 10.1 ng/ml in EE-LNG-treated rats (Porcu et al., 2019), suggestive of a blunted hypothalamic-pituitary-ovarian axis. Such alteration disrupts the estrus cycle. Indeed, a regular cycle was observed in most of the vehicle-treated animals in the present experiments, with all the phases alternating over 4-5 days (Chi-square=0.4, p=0.94, Figure 1A). In contrast, none of the EE-LNG-treated rats showed a regular estrous cycle during treatment. In the EE-LNG 24 hours group we observed an 80% occurrence of a diestrus phase (sometimes resembling a diestrus 1, sometimes a diestrus 2), followed by occasional estrus (14% occurrence) and rare proestrus (6% occurrence) (Chi-square = 37.3, $p<0.0001$, Figure 1B). Ovarian function is still disrupted 14 days after treatment discontinuation; in fact, in the EE-LNG 14 days group we observed a 63% occurrence of a diestrus phase, followed by a 25% occurrence of estrus and 12% occurrence of proestrus (Chi-square = 10.1, p=0.006, Figure 1C), which might indicate a slow return to normal cycling.

3.2. BDNF and transcription factors expression following EE-LNG treatment

BDNF contributes to cognitive function, acting in long-term potentiation and in the formation of long-term memory (Bekinschtein et al., 2014), and previous evidence showed that contraceptive hormones altered BDNF gene and/or protein expression (Simone et al., 2015). A significant decrease in BDNF protein expression was observed in cytosolic extracts from hippocampus of long-term EE-LNG-treated rats 14 days after drug discontinuation compared to proestrus controls (-58%, $p<0.05$; Figure 2), but no other differences were found ($F_{3,20}=2.63$, p=0.078, $\eta^2=0.283$); however, hippocampal BDNF mRNA expression, obtained from RT-PCR analysis, was not altered among the experimental groups examined ($F_{3,12}=1.57$, p=0.248, $\eta^2=0.282$, Table 1).

Since BDNF expression is regulated by transcriptional mechanisms and, especially at the hippocampal level, by the transcription factor CREB (Nibuya et al., 1996), we evaluated the expression of activated CREB through its phosphorylation on serine 133. No difference in phospho-CREB expression in nuclear extracts from hippocampus of vehicle and EE-LNG-treated rats was observed ($F_{3,20}=1.99$, p=0.148, $\eta^2=0.229$, Figure 3).

3.3. EE-LNG treatment alters histone H3 PTMs

Epigenetic mechanisms related to histone modification may also contribute to regulate BDNF expression (Tsankova et al., 2006). Therefore, we evaluated histone H3 PTMs in hippocampal nuclear extracts. As shown in Figure 4, long-term EE-LNG treatment altered histone H3 methylation ($F_{3,20}$=5.18, p=0.008, η^2 =0.437) and acetylation ($F_{3,28}$=7.18, p=0.001, η^2 =0.435). Specifically, a significant increase in histone H3 methylation on lysine 9 was observed in EE-LNG-treated rats 14 days after drug discontinuation compared to proestrus (+48%, p<0.05), and diestrus 1 (+56%, p<0.01) controls. This effect was accompanied by a parallel reduction in histone H3 acetylation on lysines 9 and 14, observed in both groups of EE-LNG-treated rats (EE-LNG 24h: -57%, p<0.01; EE-LNG 14d: -66%, p<0.001).

3.4. Effect of EE-LNG treatment on long-term plasticity at hippocampal CA1-CA3 excitatory synapses

To evaluate the effect of long-term EE-LNG treatment on hippocampal long-term plasticity of glutamatergic CA3-CA1 synapses, we first recorded dendritic fEPSPs in the CA1 field to examine the input-output (I–O) relationship obtained by stimulating the Schaffer's collateral afferents with increasing current intensity (from 0 to 1.0 mA). One-way ANOVA revealed a significant change in the stimulation intensity able to evoke half of the maximal response among groups ($F_{3,107}$=4.28, p=0.007, η^2 =0.107). The post-hoc test showed a difference between vehicle-treated rats in diestrus 1 compared with those in proestrus (proestrus: 0.56 ± 0.02 mA; diestrus 1: 0.43 ± 0.02 mA, p=0.003), while no difference was found in EE-LNG-treated rats tested 24 hours or 14 days after the last administration (EE-LNG 24h: 0.51 ± 0.03 mA, p=0.62; EE-LNG 14d: 0.50 ± 0.03 mA, p=0.38), compared to vehicle-treated rats in proestrus or diestrus 1.

Subsequently, LTP was induced in the CA1 region by HFS that was delivered to the Schaffer's collateral afferents 10 min after stable baseline was established (Figure 5). ANOVA revealed significant changes in the magnitude of the resulting LTP ($F_{3,82}$=2.79, p=0.045, η^2 =0.093); while no differences were observed between vehicle-treated rats in proestrus and diestrus 1, and among vehicle- and EE-LNG-treated rats, the post-hoc analysis highlighted a significant difference in the magnitude of long term plasticity between the two groups of EE-LNG-treated rats, with those tested 14 days after drug discontinuation showing a greater LTP compared to those tested 24 hours after drug discontinuation (p=0.026, Figure 5C).

3.5. Behavioral evaluation of learning and memory following EE-LNG treatment

We next evaluated whether changes in BDNF, histone H3 PTMs, and synaptic plasticity induced by long-term EE-LNG treatment and its discontinuation altered cognitive performance in behavioral tests of learning and memory.

3.5.1. Recognition memory in the NOR and NPL tests

In the NOR test all groups displayed similar exploration of the novel object, both for time ($F_{3,44}$=1.64, p=0.195, η^2 =0.1003, Figure 6A) and frequency ($F_{3,44}$=1.34, p=0.273, η^2 =0.084, Figure 6B) of investigation. Likewise, in the NPL test, all groups showed comparable time ($F_{3,44}$=0.39, p=0.763, η^2 =0.026, Figure 6C) and frequency ($F_{3,44}$=0.45, p=0.719, η^2 =0.030, Figure 6D) of exploration of the object moved to a new location, suggesting the long-term EE-LNG treatment and its discontinuation do not affect recognition memory in female rats.

3.5.2. Spatial learning, memory and behavioral flexibility in the Morris water maze test

We also evaluated spatial learning, memory and behavioral flexibility in the Morris water maze test. Spatial learning was assessed over four days of training in which all rats appeared to swim normally and had no difficulty in locating the platform. Overall, no effect of EE-LNG treatment was observed in learning performance. As training progressed, latency to reach the platform decreased for all experimental groups (Figure 7A). Repeated measures ANOVA revealed a significant effect of time ($F_{3,531}$=65.74, p<0.0001, η^2 =0.182), no effect of treatment ($F_{2,177}$=3.02, p=0.052, η^2 =0.011), and no interaction ($F_{6,531}$=0.60, p=0.730, η^2 =0.003), indicative of a decrease in latency over the 4-day training period for all groups tested. Given that platform search strategy might have been influenced by motor abilities (i.e. changes in swim speed), training trials were also analyzed using the proximity measure to calculate the cumulative search error (CSE) to account for putative changes in swim speed (Gallagher et al., 1993). Repeated measures ANOVA revealed a significant effect of time ($F_{3,531}$=72.48, p<0.0001, η^2 =0.195), no effect of treatment ($F_{2,177}$=2.94, p=0.056, η^2 =0.010), and no interaction ($F_{6,531}$=1.05, p=0.392, η^2 =0.006); similar to latency, CSE also decreased in all groups over the 4-day training period (Figure 7B), suggesting that all groups tested acquired spatial learning in the Morris water maze task. Finally, we observed a decrease in total distance travelled over the 4-day training period; repeated measures ANOVA revealed a significant effect of time ($F_{3,531}$=94.79, p<0.0001, η^2 =0.257), but no effect of treatment ($F_{2,177}$=2.52, p=0.083, η^2 =0.007), and no interaction ($F_{6,531}$=0.85, p=0.533, η^2 =0.005) (Figure 7C).

Memory performance was evaluated during the probe trial and results are reported in Table 2. No changes among experimental groups were observed in latency to reach the zone where the platform

was located ($F_{2,33}$=0.79, p=0.458, η^2 =0.036), and in swim speed ($F_{2,33}$=0.02, p=0.977, η^2 =0.001). A trend for an effect of treatment was observed for average proximity to platform vs. opposite quadrant (treatment: $F_{2,33}$=2.93, p=0.064, η^2 =0.037; quadrant: $F_{1,33}$=0.15, p=0.701, η^2 =0.002; interaction $F_{2,33}$=0.47, p=0.630, η^2 =0.015). Frequency in quadrant east was greater than average frequency in the other quadrants (quadrant: $F_{1,33}$=17.41, p=0.0001, η^2 =0.092; treatment: $F_{2,33}$=0.48, p=0.621, η^2 =0.015; interaction $F_{2,33}$=1.13, p=0.333, η^2 =0.012), with the EE-LNG 14d group showing a greater frequency in quadrant east than average frequency in the other quadrants (p<0.005). However, time spent in quadrant east was not greater than average time spent in the other quadrants for all the groups tested (quadrant: $F_{1,33}$=2.36, p=0.132, η^2 =0.042; treatment: $F_{2,33}$=0.86, p=0.430, η^2 =0.007; interaction $F_{1,33}$=0.86, p=0.429, η^2 =0.031).

We next evaluated behavioral flexibility by performing a reversal learning experiment in the Morris water maze test. Similar to learning performance, EE-LNG treatment did not affect behavioral flexibility. All rats rapidly learned the new platform location as latency to reach the platform was already low on day 1 of reversal learning, compared to day 1 of learning; as training progressed, latency to reach the platform further decreased for all experimental groups (Figure 7D). Repeated measures ANOVA revealed a significant effect of time ($F_{3,531}$=52.95, p<0.0001, η^2 =0.145), but no effect of treatment ($F_{2,177}$=0.17, p=0.841, η^2 =0.001), and no interaction ($F_{6,531}$=0.45, p=0.844, η^2 =0.002). CSE also decreased over time in all groups; repeated measures ANOVA revealed a significant effect of time ($F_{3,531}$=49.25, p<0.0001, η^2 =0.132), with no effect of treatment ($F_{2,177}$=0.12, p=0.885, η^2 =0.001), and no interaction ($F_{6,531}$=0.49, p=0.819, η^2 =0.003), further suggesting that reversal learning was not affected by long-term EE-LNG treatment (Figure 7E). Finally, total distance travelled over the 4-day reversal learning training period also decreased over time; repeated measures ANOVA revealed a significant effect of time ($F_{3,531}$=49.56, p<0.0001, η^2 =0.147), but no effect of treatment ($F_{2,177}$=1.58, p=0.208, η^2 =0.006), and no interaction ($F_{6,531}$=0.86, p=0.526, η^2 =0.005) (Figure 7F).

Lastly, we evaluated behavioral flexibility during the reversal learning probe trial and results are reported in Table 3. All rats equally remembered the platform location in the reversal learning phase and no changes among experimental groups were observed in latency to reach the zone where the platform was located ($F_{2,33}$=0.88, p=0.421, η^2 =0.040), and in swim speed ($F_{2,33}$=1.97, p=0.152, η^2 =0.085). A significant effect of quadrant was observed for average proximity to platform vs. opposite quadrant (quadrant: $F_{1,33}$=91.07, p<0.0001, η^2 =0.572; treatment: $F_{2,33}$=0.08, p=0.922, η^2 =0.001; interaction $F_{2,33}$=1.18, p=0.317, η^2 =0.015), suggesting that all rats remembered platform location.

Likewise, both frequency and time spent in quadrant west were greater than average time spent in the other quadrants for all groups tested (Frequency, quadrant: $F_{1,33}$=92.63, p<0.0001, η^2 =0.408; treatment: $F_{2,33}$=0.28, p=0.755, η^2 =0.005; interaction $F_{2,33}$=0.34, p=0.712, η^2 =0.003. Time, quadrant: $F_{1,33}$=89.49, p<0.0001, η^2 =0.619; treatment: $F_{2,33}$=1.18, p=0.316, η^2 =0.004; interaction $F_{2,33}$=1.19, p=0.312, η^2 =0.017).

4. Discussion

We report that long-term EE-LNG treatment alters BDNF content and induces epigenetic modifications in female rat hippocampus. BDNF regulates neuronal survival and differentiation during development and the structure and functions of different neuronal circuits throughout life, such as activity-dependent synaptic plasticity (Bekinschtein et al., 2014). It also plays a crucial role in learning, both in the formation of different types of memories and in maintaining long-lasting storage of information especially in the hippocampus (Pang et al., 2004; Tyler et al., 2002). Its alterations have often been associated with loss of cognitive functions, and with the pathogenesis of neurodegenerative diseases such as Alzheimer's (Song et al., 2015), but also with psychiatric diseases, including mood disorders and depression (Autry and Monteggia, 2012). It is well-known that neurosteroids regulate BDNF expression (Naert et al., 2007; Sohrabji et al., 1995), and estradiol directly promotes its expression through an estrogen response element in the BDNF gene (Sohrabji et al., 1995), but also through its signaling cascade involving ERK-mediated phosphorylation of CREB and epigenetic modifications such as histone acetylation, which both increase BDNF gene expression (Taxier et al., 2020). Hormonal contraceptives, by inhibiting the hypothalamic-pituitary-ovarian axis, decrease estradiol and progesterone concentrations from the ovaries (Levin et al., 2018), and prevent the increase in allopregnanolone levels during the luteal phase of the menstrual cycle (Follesa et al., 2002); we thus hypothesized that long-term treatment with hormonal contraceptives might alter hippocampal BDNF expression in female rats. Compared to vehicle-treated rats in proestrus, BDNF mRNA and protein were reduced in vehicle-treated rats in diestrus 1, and in EE-LNG-treated rats; however, such decrease was not statistically significant, except for protein content in EE-LNG-treated rats tested 14 days after drug discontinuation. Blunted circulating BDNF levels were reported in hormonal contraceptives' users during the luteal phase of the menstrual cycle (Pluchino et al., 2009), and hippocampal BDNF protein, but not mRNA immunoreactivity, was reduced in female rats treated with EE-LNG, 30-60 μg (Simone et al.,

2015), thus partially agreeing with our finding, probably because of methodological differences (species, tissue, techniques to assess BDNF).

While endogenous estradiol is involved in controlling BDNF levels, we cannot rule out the possibility that the synthetic EE in the hormonal contraceptive formulation, also able to bind estrogen receptors, might play a role in BDNF synthesis, partially compensating for the lack of endogenous estrogens. This hypothesis could explain why we and others (Simone et al., 2015) fail to observe reductions in BDNF expression following EE-LNG treatment, similar to the ones observed in ovariectomized rats. In fact, ovariectomy, which also disrupts the hypothalamic-pituitary-ovarian axis, decreased hippocampal BDNF mRNA expression in female rats, an effect prevented by hormone replacement (Berchtold et al., 2001; Gibbs, 1999; Singh et al., 1995). However, ovariectomy did not affect BDNF protein (Gibbs, 1999), leading to the hypothesis that different mechanisms might regulate BDNF mRNA and protein, including an uncoupling of transcriptional and translational mechanisms, or an increase in protein degradation from the hippocampus (Gibbs, 1998).

Transcriptional (Nibuya et al., 1996) and epigenetic mechanisms (Tsankova et al., 2006) contribute most to regulate BDNF expression. While phospho-CREB expression did not change, an altered expression pattern of the histone PTMs was found in EE-LNG-treated rats compared to vehicle-treated rats in proestrus and diestrus 1. The increase in histone H3 methylation on lysine 9 and the parallel reduction of its acetylation on lysines 9 and 14 suggest that hormonal contraceptives' treatment might be involved in the reduction of hippocampal BDNF through chromatin compaction. Interestingly, significant changes in BDNF protein and histone PTMs are observed 14 days after discontinuation from long-term EE-LNG treatment, although histone acetylation is also reduced in EE-LNG-treated rats tested 24 hours after the last treatment. The functional significance of this observation is unknown, since no changes in learning and memory or in LTP were observed in this group. In addition to cognitive function and synaptic plasticity, BDNF is also involved in mood disorders and depression (Autry and Monteggia, 2012), and depressed patients have lower serum BDNF (Shimizu et al., 2003). Likewise, histone methylation has been related to chronic stress (Tsankova et al., 2006), while histone acetylation has been associated to antidepressant effects (Covington et al., 2009), suggesting that the effects of EE-LNG on BDNF and chromatin remodeling in the hippocampus might be relevant for the manifestation of depressive symptoms. Indeed, long-term EE-LNG treatment induced anxiety-like behavior in female rats (Follesa et al., 2002; Porcu et al., 2012), and mood changes and depression have been reported in approximately 4-10% of hormonal contraceptives' users (Lundin et al., 2017; Poromaa and Segebladh, 2012; Segebladh et al., 2009), being a common reason for their discontinuation (Kulkarni, 2007).

Interestinlgy, use of hormonal contraceptives was associated with a greater risk for first diagnosis of depression and first prescription of antidepressants, especially among adolescents (Skovlund et al., 2016), who also reported higher depressive symptoms, compared to same age non-hormonal contraceptives' users (de Wit et al., 2020). However, the molecular mechanisms responsible for such effects are still unknown; thus, changes in BDNF and histone PTMs in rat hippocampus, persisting at 14 days after EE-LNG discontinuation, might be relevant for the occurrence of depressive symptoms, particularly in vulnerable women, and deserve further investigation.

Hippocampal BDNF mRNA expression was found to fluctuate across the rat estrus cycle, although opposite results have been reported, with both a peak BDNF concentration occurring in diestrus 2 (Gibbs, 1998), as well as a greatest mRNA immunoreactivity reported in proestrus, compared to diestrus 1 and 2, and to ovariectomized rats (Scharfman et al., 2003). Our findings somehow parallel this latest report, although the higher BDNF mRNA and protein observed in proestrus rats was not statistically significant compared to the other groups. Methodological differences in mRNA quantification might account for the discrepancy.

BDNF is involved in synaptic plasticity (Bekinschtein et al., 2014) and it is necessary for LTP expression in the hippocampus (Pang et al., 2004). Furthermore, neurosteroids (including estradiol) have also been shown to facilitate LTP (Taxier et al., 2020). Indeed, neuronal excitability increases with high endogenous estradiol levels, and adult female Long-Evans and Lister hooded rats show increased LTP in proestrus, compared to diestrus and estrus, although this effect was observed only in the afternoon, when estradiol levels were estimated to be at their peak (Good et al., 1999; Warren et al., 1995). However, we did not observe changes in LTP across the estrus cycle, likely because of strain differences or time; in fact, our rats were euthanized in the morning, and we might have missed the afternoon peak estradiol level necessary to increase LTP in proestrus rats. Following long-term EE-LNG treatment we found only a trend for a decrease in hippocampal LTP in rats tested 24 hours after the last drug administration, while no change, compared to vehicle-treated rats in proestrus and diestrus 1, was observed in EE-LNG-treated rats tested 14 days after drug discontinuation. Nevertheless, LTP in rats tested 24 hours after the last drug administration was significantly lower than that measured 14 days after drug discontinuation. Given that neurosteroid levels are decreased by long-term EE-LNG treatment and normalized two weeks after drug discontinuation (Follesa et al., 2002), the slight reduction in LTP in EE-LNG-treated rats tested 24 hours after the last administration (albeit not significant compared to vehicle-treated rats), might be related to the low endogenous concentrations of neurosteroids, rather

than to changes in BDNF expression. This hypothesis is further substantiated by evidence that hippocampal estrogen is necessary for memory related LTP in females but not males (Vierk et al., 2012).

Several preclinical and clinical studies suggest that steroid hormones are involved in cognitive function. However, studies on hormonal contraceptives' effects on cognition have yielded mixed results in both women and animals. Likely explanations include different cognitive domains examined (verbal and spatial abilities, executive function, attention, working memory, etc.), different doses and type of progestin administered, and/or duration of treatment (Gogos et al., 2014; Pletzer and Kerschbaum, 2014; Warren et al., 2014). Moreover, genotype might be another important variable to consider when examining cognitive function in hormonal contraceptives' users (Gravelsins et al., 2021). Likewise, we cannot rule out the possibility that different doses or type of synthetic steroids used in hormonal contraceptives might alter cognitive abilities in rats; moreover, strain and/or species differences might also contribute to the actions of EE and LNG on cognition. The present finding that long-term EE-LNG treatment does not alter spatial learning and memory in the Morris water maze test agrees with our previous report (Santoru et al., 2014), and further extends the results to lack of changes in behavioral flexibility in the reversal learning test. Likewise, EE-LNG-treated rats tested 14 days after drug discontinuation, showed no changes in spatial learning, memory and behavioral flexibility in the Morris water maze test, consistent with lack of changes in hippocampal LTP, despite reduced BDNF levels. Similarly, EE-LNG treatment does not alter recognition memory in the NOR and NPL tests (which also involves visuospatial memory), at either 24 hours or 14 days after drug discontinuation. These results agree with previous studies showing that EE-LNG treatment at doses (30-60 µg) similar to the ones used in our experiments (20-60 µg), does not alter recognition memory in the novel object and novel context recognition tests. By contrast, lower EE and LNG doses (10 and 20 µg, respectively), which are not sufficient to reduce serum estradiol levels, impair recognition memory in the NOR test, suggesting that the effects of EE and LNG on recognition memory might be independent from circulating endogenous estradiol. Indeed, only the 30 µg EE dose improved performance in the NOR test, an effect related to the ability of EE to directly bind estrogen receptor alpha, thus promoting synaptogenesis in hippocampal neurons (Simone et al., 2015).

Hormonal fluctuations across the estrus cycle influence spatial learning and memory, with better spatial memory observed when estrogen levels are low (Duarte-Guterman et al., 2015; Warren and Juraska, 1997), although the opposite has also been reported (Berry et al., 1997; Healy et al., 1999). Testing in the Morris water maze task occurred over several consecutive days, thus we did not control for estrus cycle stage in vehicle-treated rats to avoid using a large number of animals. Furthermore,

testing occurred in the morning, thus minimizing the chance to catch the peak estradiol level in the afternoon of proestrus, a condition that might have contributed to lack of effects of EE-LNG treatment on this behavioral paradigm since all females were likely in a condition of low endogenous estradiol levels. Recognition memory in the NOR and NPL tests was not altered in vehicle-treated rats in proestrus and diestrus 1. Very few studies have examined the effects of natural estrus cycle on performance in the NOR test. Increased exploration of the novel object was reported in proestrus Wistar rats tested during the dark phase of the light-dark cycle (Walf et al., 2006). However, studies in female C57BL/6 mice tested during the light phase have yielded mixed results, with increased exploration of the new object in proestrus/estrus mice or no change across the estrus cycle (Chari et al., 2020; Cordeira et al., 2018).

5. Conclusions

We showed that long-term treatment with hormonal contraceptives decreases BDNF levels and induces epigenetic modifications in female rat hippocampus that persist up to 14 days after drug discontinuation. Notwithstanding, these effects are not accompanied by alterations in synaptic plasticity, recognition memory, or spatial learning, memory, and behavioral flexibility. These findings provide the first evidence of hippocampal epigenetic changes induced by hormonal contraceptives and complement previous studies on the neurobiological actions of this drug. The decrease in BDNF content, coupled with changes in histone PTMs, might be relevant for the occurrence of certain psychiatric symptoms, reported by some women using hormonal contraceptives. The finding that effects of EE-LNG treatment on BDNF and histone PTMs persist up to 14 days after drug discontinuation warrants future investigation to better understand the implications of such long-term consequences for women's health.

Figure Legends

Figure 1. **Long-term EE-LNG treatment alters the estrus cycle in female rats.** Rats were orally treated with the combination of EE (0.020 mg) and LNG (0.060 mg) or with vehicle once daily for four weeks. EE-LNG-treated rats were tested 24 hours and 14 days after the last drug treatment. Stage of the estrus cycle was determined by daily vaginal smears taken between 10:00 and 11:00 hours (after drug treatment) starting from the third week of treatment and up to sacrifice or behavioral tests. Data are expressed as a percentage of occurrence of each estrus cycle phase assessed in female rats that underwent biochemical, electrophysiological and behavioral (NOR/NPL test) experiments. Vehicle-

treated animals had a regular estrus cycle with all the phases (diestrus 1, diestrus 2, proestrus, estrus) alternating over 4 to 5 days. The EE-LNG 24 hours group had an 80% occurrence of a diestrus phase (sometimes resembling a diestrus 1, sometimes a diestrus 2), with occasional estrus and rare proestrus, while the EE-LNG 14 days group had a 63% occurrence of a diestrus phase, which might indicate a slow return to normal cycling.

Figure 2. Reduced BDNF expression in long-term EE-LNG-treated rats 14 days after drug discontinuation. Western Blot and relative densitometry of BDNF expression in cytosolic extracts from hippocampus of vehicle-treated female rats in Proestrus and Diestrus 1, EE-LNG-treated rats at 24 hours (EE-LNG 24h) and 14 days (EE-LNG 14d) after the last drug treatment. Data are shown as the ratio between BDNF and GAPDH as reference loading control. Each bar represents the mean ± S.E.M. of 6 samples from different animals. *$p<0.05$ compared to Proestrus; one-way ANOVA followed by Bonferroni's post-hoc test.

Figure 3. Long-term EE-LNG treatment does not alter phospho-CREB expression. Western Blot and relative densitometry of phospho-CREB expression in nuclear protein extracts from hippocampus of vehicle-treated female rats in Proestrus and Diestrus 1, EE-LNG-treated rats at 24 hours (EE-LNG 24h) and 14 days (EE-LNG 14d) after the last drug treatment. Data are shown as the ratio between CREB phosphorylation (on serine 133) and total CREB. Each bar represents the mean ± S.E.M. of 6 samples from different animals.

Figure 4. Long-term EE-LNG treatment alters histone H3 post-translational modifications. Western Blot and relative densitometries of Met H3 and Ac H3 expression in nuclear protein extracts from hippocampus of vehicle-treated female rats in Proestrus and Diestrus 1, EE-LNG-treated rats at 24 hours (EE-LNG 24h) and 14 days (EE-LNG 14d) after the last drug treatment. Data are shown as the ratio between histone H3 methylation (on lysine 9) or histone H3 acetylation (on lysines 9/14) and the total histone H3. Each bar represents the mean ± S.E.M. of 6 samples for Met H3 and 8 samples for Ac H3 from different animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to Proestrus; $^{\#\#}p<0.01$ compared to Diestrus 1; one-way ANOVA followed by Bonferroni's post-hoc test.

Figure 5. Difference in LTP magnitude recorded in vehicle-treated female rats in Proestrus and Diestrus 1, and following long-term EE-LNG treatment and its discontinuation. (A) Representative traces of fEPSPs before and 1 h after HFS application (red trace) are shown (scale bars: 1 mV, 5 ms) relative to all experimental groups tested. (B) Scatter plots representing the change in fEPSP slope values induced by HFS (indicated with a black arrow) in vehicle-treated rats during Proestrus and Diestrus 1, and in EE-LNG-treated rats tested 24 hours (EE-LNG 24h) and 14 days (EE-LNG 14d) after the last treatment. Data are expressed as percent of baseline values. The gray bar indicates the time window where the extent of LTP was calculated. (C) Bar graph of the averaged fEPSP slope values obtained during the last 10 min of LTP recording, compared with the relative baseline (number of samples are indicated as N/n where N = number of animals and n = number of recordings; Proestrus = 5/20, Diestrus 1 = 5/24, EE-LNG 24h = 5/17, EE-LNG 14d = 5/25). *$p<0.05$; one-way ANOVA followed by Bonferroni post-hoc test.

Figure 6. Long-term EE-LNG treatment does not alter investigation in the NOR and NPL tests. Female rats were orally treated with the combination of EE (0.020 mg) and LNG (0.060 mg) or with vehicle once a day for four weeks. Vehicle-treated rats were tested in the Proestrus and Diestrus 1 phases of the estrus cycle; EE-LNG-treated rats were tested 24 hours (EE-LNG 24h) and 14 days (EE-LNG 14d) after the last drug treatment. Data are reported as percent time (A) and frequency (B) of the novel object investigation in the NOR test, and percent time (C) and frequency (D) of object investigation in the novel location for the NPL test. Each bar represents the mean ± S.E.M. of values from 12 rats for each experimental group.

Figure 7. Long-term EE-LNG treatment does not alter spatial learning and behavioral flexibility in the Morris water maze test. Female rats were orally treated with the combination of EE (0.020 mg) and LNG (0.060 mg) or with vehicle once a day for four weeks. EE-LNG-treated rats were tested 24 hours (EE-LNG 24h) after the last drug treatment, thus receiving the drug each day, approximately 1 hour after the end of the trial. A second group of EE-LNG-treated rats began Day 1 of the Morris water maze test 14 days after the last drug treatment (EE-LNG 14d). Latency to reach the platform (A, D), cumulative search error (B, E), and total distance travelled (C, F) during the four days of training trials in the learning (A, B, C) and reversal learning (D, E, F) trials in the Morris water maze test. Data represent the mean ± S.E.M. of values from 12 rats for each experimental group. Symbols show significant changes vs. the respective

day 1 value for each group as follows: *vehicle-treated females, #EE-LNG 24h, §EE-LNG 14d; one symbol $p<0.05$, two symbols $p<0.01$, three symbols $p<0.001$, four symbols $p<0.0001$. Repeated measures ANOVA followed by Bonferroni post hoc test.

Table 1. Real-Time analysis of BDNF mRNA expression in vehicle- and EE-LNG-treated female rats.

SAMPLE	Ct BDNF	Ct Actin	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
Proestrus	24.49 ± 0.08	18.27 ± 0.03	6.22 ± 0.05	0.00	1.00
Diestrus 1	24.26 ± 0.27	17.83 ± 0.23	6.43 ± 0.05	0.21 ± 0.01	0.87
EE-LNG 24h	24.29 ± 0.39	18.03 ± 0.28	6.46 ± 0.11	0.23 ± 0.06	0.85
EE-LNG 14d	24.12 ± 0.55	17.47 ± 0.39	6.65 ± 0.15	0.43 ± 0.11	0.74

Female rats were orally treated with the combination of EE (0.020 mg) and LNG (0.060 mg) or with vehicle once daily for four weeks. Real-Time analysis of BDNF mRNA expression in hippocampus of vehicle-treated female rats in Proestrus and Diestrus 1, and EE-LNG-treated rats at 24 hours (EE-LNG 24h) and 14 days (EE-LNG 14d) after the last drug treatment. Data are mean ± S.E.M. of 4 samples from different animals.

Table 2. Long-term EE-LNG treatment does not alter memory in the Morris water maze test.

PROBE TRIAL	Vehicle	EE-LNG 24h	EE-LNG 14d
Latency to platform (s)	32.3 ± 5.1	33.7 ± 6.1	23.8 ± 5.2
Swim speed (cm/s)	18.5 ± 0.8	18.7 ± 0.9	18.4 ± 1.5
Average proximity platform (cm)	49.7 ± 1.7	45.6 ± 2.1	44.8 ± 3.3
Average proximity opposite (cm)	48.5 ± 1.5	45.5 ± 2.6	48.5 ± 2.1
Frequency in quadrant East (n)	4.8 ± 0.5	5.1 ± 0.6	5.8 ± 0.9
Average frequency in quadrants North + West + South (n)	3.8 ± 0.2	4.2 ± 0.3	3.9 ± 0.4[a]
Time in quadrant East (s)	15.1 ± 1.1	15.8 ± 2.0	18.3 ± 2.7
Average time in quadrants North + West + South (s)	14.8 ± 0.4	14.3 ± 0.7	13.6 ± 0.9

Female rats were orally treated with the combination of EE (0.020 mg) and LNG (0.060 mg) or with vehicle once a day for four weeks. They were subjected to the probe trial on day 5, following 4 days of training trials in the Morris water maze test. EE-LNG-treated rats were tested 24 hours (EE-LNG 24h) after the last drug treatment, thus they received the drug each day, approximately 1 hour after the end of the trial. A second group of EE-LNG-treated rats began Day 1 of the Morris water maze test 14 days after the last drug treatment (EE-LNG 14d). Data represent the mean ± S.E.M. of values from 12 rats for each experimental group. Latency and swim speed were analyzed by one-way ANOVA; average proximity, and time and frequency in quadrants were analyzed by two-way ANOVA (factors: quadrant and treatment). [a]$p<0.005$ vs. the respective frequency value in quadrant east (two-way ANOVA followed by Bonferroni post hoc test).

Table 3. Long-term EE-LNG treatment does not alter memory in the reversal learning probe trial in the Morris water maze test.

PROBE TRIAL	Vehicle	EE-LNG 24h	EE-LNG 14d
Latency to platform (s)	14.9 ± 2.2	12.8 ± 2.8	18.9 ± 4.4
Swim speed (cm/s)	21.6 ± 0.9	18.7 ± 0.9	20.6 ± 1.3
Average proximity platform (cm)	40.9 ± 1.3	38.1 ± 1.4	41.0 ± 2.4
Average proximity opposite (cm)	53.0 ± 1.2[b]	55.0 ± 1.3[b]	53.2 ± 1.3[a]
Frequency in quadrant West (n)	7.2 ± 0.4	7.2 ± 0.7	7.3 ± 0.8
Average frequency in quadrants North + East + South (n)	4.2 ± 0.2[c]	3.6 ± 0.2[c]	4.4 ± 0.5[c]
Time in quadrant West (s)	21.4 ± 1.2	24.4 ± 1.6	23.4 ± 1.8
Average time in quadrants North + East + South (s)	12.4 ± 0.4[c]	11.3 ± 0.5[c]	11.8 ± 0.6[c]

Female rats were orally treated with the combination of EE (0.020 mg) and LNG (0.060 mg) or with vehicle once a day for four weeks. They were subjected to the probe trial on day 12, following 4 days of reversal learning training trials in the Morris water maze test. EE-LNG-treated rats were tested 24 hours (EE-LNG 24h) after the last drug treatment, thus they received the drug each day, approximately 1 hour after the end of the trial. A second group of EE-LNG-treated rats began Day 1 of the Morris water maze test 14 days after the last drug treatment (EE-LNG 14d). Data represent the mean ± S.E.M. of values from 12 rats for each experimental group. Latency and swim speed were analyzed by one-way ANOVA; average proximity, and time and frequency in quadrants were analyzed by two-way ANOVA (factors: quadrant and treatment). [a]$p<0.001$ and [b]$p<0.0001$ vs. the respective average proximity platform value; [c]$p<0.0001$ vs. the respective value for quadrant west (two-way ANOVA followed by Bonferroni post hoc test).

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by the Banco di Sardegna Foundation, Italy (Grant ID: 2012.0255), and the University of Cagliari, Italy (Grant ID: Premialità 2012) to AC, and by Fondazione di Sardegna, Italy (Grant ID: 2019.1440) to PP.

References

Autry, A.E., Monteggia, L.M., 2012. Brain-derived neurotrophic factor and neuropsychiatric disorders. Pharmacol. Rev. 64, 238-258. https://doi.org/10.1124/pr.111.005108.

Bekinschtein, P., Cammarota, M., Medina, J.H., 2014. BDNF and memory processing. Neuropharmacology 76 Pt C, 677-683. https://doi.org/10.1016/j.neuropharm.2013.04.024.

Beltz, A.M., Hampson, E., Berenbaum, S.A., 2015. Oral contraceptives and cognition: A role for ethinyl estradiol. Horm. Behav. 74, 209-217. https://doi.org/10.1016/j.yhbeh.2015.06.012.

Berchtold, N.C., Kesslak, J.P., Pike, C.J., Adlard, P.A., Cotman, C.W., 2001. Estrogen and exercise interact to regulate brain-derived neurotrophic factor mRNA and protein expression in the hippocampus. Eur. J. Neurosci. 14, 1992-2002. https://doi.org/10.1046/j.0953-816x.2001.01825.x.

Berry, B., McMahan, R., Gallagher, M., 1997. Spatial learning and memory at defined points of the estrous cycle: effects on performance of a hippocampal-dependent task. Behav. Neurosci. 111, 267-274. https://doi.org/10.1037/0735-7044.111.2.267.

Caruccio, L., Banerjee, R., 1999. An efficient method for simultaneous isolation of biologically active transcription factors and DNA. J. Immunol. Methods 230, 1-10. https://doi.org/10.1016/S0022-1759(99)00100-3.

Chari, T., Griswold, S., Andrews, N.A., Fagiolini, M., 2020. The stage of the estrus cycle is critical for interpretation of female mouse social interaction behavior. Front. Behav. Neurosci. 14, 113. https://doi.org/10.3389/fnbeh.2020.00113.

Cordeira, J., Kolluru, S.S., Rosenblatt, H., Kry, J., Strecker, R.E., McCarley, R.W., 2018. Learning and memory are impaired in the object recognition task during metestrus/diestrus and after sleep deprivation. Behav. Brain Res. 339, 124-129. https://doi.org/10.1016/j.bbr.2017.11.033.

Covington, H.E., Maze, I., LaPlant, Q.C., Vialou, V.F., Ohnishi, Y.N., Berton, O., Fass, D.M., Renthal, W., Rush, A.J., Wu, E.Y., Ghose, S., Krishnan, V., Russo, S.J., Tamminga, C., Haggarty, S.J., Nestler, E.J., 2009. Antidepressant actions of histone deacetylase inhibitors. J. Neurosci. 29, 11451-11460. https://doi.org/10.1523/JNEUROSCI.1758-09.2009.

de Wit, A.E., Booij, S.H., Giltay, E.J., Joffe, H., Schoevers, R.A., Oldehinkel, A.J., 2020. Association of use of oral contraceptives with depressive symptoms among adolescents and young women. JAMA Psychiatry 77, 52-59. https://doi.org/10.1001/jamapsychiatry.2019.2838.

Duarte-Guterman, P., Yagi, S., Chow, C., Galea, L.A., 2015. Hippocampal learning, memory, and neurogenesis: Effects of sex and estrogens across the lifespan in adults. Horm. Behav. 74, 37-52. https://doi.org/10.1016/j.yhbeh.2015.05.024.

Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav. Brain. Res. 31, 47-59. https://doi.org/10.1016/0166-4328(88)90157-X.

Ennaceur, A., Michalikova, S., Bradford, A., Ahmed, S., 2005. Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks. Behav. Brain Res. 159, 247-266. https://doi.org/10.1016/j.bbr.2004.11.006.

Follesa, P., Porcu, P., Sogliano, C., Cinus, M., Biggio, F., Mancuso, L., Mostallino, M.C., Paoletti, A.M., Purdy, R.H., Biggio, G., Concas, A., 2002. Changes in GABA(A) receptor gamma 2 subunit gene expression induced by long-term administration of oral contraceptives in rats. Neuropharmacology 42, 325-336. https://doi.org/10.1016/S0028-3908(01)00187-3.

Gallagher, M., Burwell, R., Burchinal, M., 1993. Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. Behav. Neurosci. 107, 618-626. https://doi.org/10.1037/0735-7044.107.4.618.

Gibbs, R.B., 1998. Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. Brain Res. 787, 259-268. https://doi.org/10.1016/S0006-8993(97)01511-4.

Gibbs, R.B., 1999. Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult rat brain. Brain Res. 844, 20-27. https://doi.org/10.1016/S0006-8993(99)01880-6.

Gogos, A., 2013. Natural and synthetic sex hormones: effects on higher-order cognitive function and prepulse inhibition. Biol. Psychol. 93, 17-23. https://doi.org/10.1016/j.biopsycho.2013.02.001.

Gogos, A., Wu, Y.C., Williams, A.S., Byrne, L.K., 2014. The effects of ethinylestradiol and progestins ("the pill") on cognitive function in pre-menopausal women. Neurochem. Res. 39, 2288-2300. https://doi.org/10.1007/s11064-014-1444-6.

Good, M., Day, M., Muir, J.L., 1999. Cyclical changes in endogenous levels of oestrogen modulate the induction of LTD and LTP in the hippocampal CA1 region. Eur. J. Neurosci. 11, 4476-4480. https://doi.org/10.1046/j.1460-9568.1999.00920.x.

Gravelsins, L., Duncan, K., Einstein, G., 2021. Do oral contraceptives affect young women's memory? Dopamine-dependent working memory is influenced by COMT genotype, but not time of pill ingestion. PLoS One 16, e0252807. https://doi.org/10.1371/journal.pone.0252807.

Griksiene, R., Monciunskaite, R., Arnatkeviciute, A., Ruksenas, O., 2018. Does the use of hormonal contraceptives affect the mental rotation performance? Horm. Behav. 100, 29-38. https://doi.org/10.1016/j.yhbeh.2018.03.004.

Griksiene, R., Ruksenas, O., 2011. Effects of hormonal contraceptives on mental rotation and verbal fluency. Psychoneuroendocrinology 36, 1239-1248. https://doi.org/10.1016/j.psyneuen.2011.03.001.

Gurvich, C., Warren, A.M., Worsley, R., Hudaib, A.R., Thomas, N., Kulkarni, J., 2020. Effects of oral contraceptive androgenicity on visuospatial and social-emotional cognition: A prospective observational trial. Brain Sci. 10(4):194. https://doi.org/10.3390/brainsci10040194.

Healy, S.D., Braham, S.R., Braithwaite, V.A., 1999. Spatial working memory in rats: no differences between the sexes. Proc. Biol. Sci. 266, 2303-2308. https://doi.org/10.1098/rspb.1999.0923.

Islam, F., Sparkes, C., Roodenrys, S., Astheimer, L., 2008. Short-term changes in endogenous estrogen levels and consumption of soy isoflavones affect working and verbal memory in young adult females. Nutr. Neurosci. 11, 251-262. https://doi.org/10.1179/147683008X301612.

Johansson, I.M., Birzniece, V., Lindblad, C., Olsson, T., Bäckström, T., 2002. Allopregnanolone inhibits learning in the Morris water maze. Brain Res. 934, 125-131. https://doi.org/10.1016/S0006-8993(02)02414-9.

Kask, K., Bäckström, T., Nilsson, L.G., Sundström-Poromaa, I., 2008. Allopregnanolone impairs episodic memory in healthy women. Psychopharmacology (Berl) 199, 161-168. https://doi.org/10.1007/s00213-008-1150-7.

Kulkarni, J., 2007. Depression as a side effect of the contraceptive pill. Expert Opin. Drug Saf. 6, 371-374. https://doi.org/10.1517/14740338.6.4.371.

Levin, E.R., Vitek, W.S., Hammes, S.R., 2018. Estrogens, progestins, and the female reproductive tract. In: Brunton, L.L., Hilal-Dandan, R., Knollmann, B.C., (Eds), Goodman & Gilman's The Pharmacological Basis of Therapeutics, 13th edition. McGraw Hill, pp. 803-831.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275. https://doi.org/10.1016/S0021-9258(19)52451-6.

Luine, V.N., 2014. Estradiol and cognitive function: past, present and future. Horm. Behav. 66, 602-618. https://doi.org/10.1016/j.yhbeh.2014.08.011.

Lundin, C., Danielsson, K.G., Bixo, M., Moby, L., Bengtsdotter, H., Jawad, I., Marions, L., Brynhildsen, J., Malmborg, A., Lindh, I., Sundström Poromaa, I., 2017. Combined oral contraceptive use is associated with both improvement and worsening of mood in the different phases of the treatment cycle-A double-blind, placebo-controlled randomized trial. Psychoneuroendocrinology 76, 135-143. https://doi.org/10.1016/j.psyneuen.2016.11.033.

Mayo, W., Dellu, F., Robel, P., Cherkaoui, J., Le Moal, M., Baulieu, E.E., Simon, H., 1993. Infusion of neurosteroids into the nucleus basalis magnocellularis affects cognitive processes in the rat. Brain Res. 607, 324-328. https://doi.org/10.1016/0006-8993(93)91524-V.

Mihalik, J.P., Ondrak, K.S., Guskiewicz, K.M., McMurray, R.G., 2009. The effects of menstrual cycle phase on clinical measures of concussion in healthy college-aged females. J. Sci. Med. Sport. 12, 383-387. https://doi.org/10.1016/j.jsams.2008.05.003.

Mordecai, K.L., Rubin, L.H., Maki, P.M., 2008. Effects of menstrual cycle phase and oral contraceptive use on verbal memory. Horm. Behav. 54, 286-293. https://doi.org/10.1016/j.yhbeh.2008.03.006.

Naert, G., Maurice, T., Tapia-Arancibia, L., Givalois, L., 2007. Neuroactive steroids modulate HPA axis activity and cerebral brain-derived neurotrophic factor (BDNF) protein levels in adult male rats. Psychoneuroendocrinology 32, 1062-1078. https://doi.org/10.1016/j.psyneuen.2007.09.002.

Nibuya, M., Nestler, E.J., Duman, R.S., 1996. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. J. Neurosci. 16, 2365-2372. https://doi.org/10.1523/JNEUROSCI.16-07-02365.1996.

Pang, P.T., Teng, H.K., Zaitsev, E., Woo, N.T., Sakata, K., Zhen, S., Teng, K.K., Yung, W.H., Hempstead, B.L., Lu, B., 2004. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306, 487-491. https://doi.org/10.1126/science.1100135.

Paoletti, A.M., Lello, S., Fratta, S., Orrù, M., Ranuzzi, F., Sogliano, C., Concas, A., Biggio, G., Melis, G.B., 2004. Psychological effect of the oral contraceptive formulation containing 3 mg of drospirenone plus 30 microg of ethinyl estradiol. Fertil. Steril. 81, 645-651. https://doi.org/10.1016/j.fertnstert.2003.08.030.

Pisu, M.G., Boero, G., Garau, A., Casula, C., Cisci, S., Biggio, F., Concas, A., Follesa, P., Maciocco, E., Porcu, P., Serra, M., 2019. Are preconceptional stressful experiences crucial elements for the aetiology of autism spectrum disorder? Insights from an animal model. Neuropharmacology 157, 107686. https://doi.org/10.1016/j.neuropharm.2019.107686.

Plamberger, C.P., Van Wijk, H.E., Kerschbaum, H., Pletzer, B.A., Gruber, G., Oberascher, K., Dresler, M., Hahn, M.A., Hoedlmoser, K., 2021. Impact of menstrual cycle phase and oral contraceptives on sleep and overnight memory consolidation. J. Sleep Res. 30, e13239. https://doi.org/10.1111/jsr.13239.

Pletzer, B., Kronbichler, M., Aichhorn, M., Bergmann, J., Ladurner, G., Kerschbaum, H.H., 2010. Menstrual cycle and hormonal contraceptive use modulate human brain structure. Brain Res. 1348, 55-62. https://doi.org/10.1016/j.brainres.2010.06.019.

Pletzer, B.A., Kerschbaum, H.H., 2014. 50 years of hormonal contraception - time to find out, what it does to our brain. Front. Neurosci. 8, 256. https://doi.org/10.3389/fnins.2014.00256.

Pluchino, N., Cubeddu, A., Begliuomini, S., Merlini, S., Giannini, A., Bucci, F., Casarosa, E., Luisi, M., Cela, V., Genazzani, A.R., 2009. Daily variation of brain-derived neurotrophic factor and cortisol in women

with normal menstrual cycles, undergoing oral contraception and in postmenopause. Hum. Reprod. 24, 2303-2309. https://doi.org/10.1093/humrep/dep119.

Porcu, P., Barron, A.M., Frye, C.A., Walf, A.A., Yang, S.Y., He, X.Y., Morrow, A.L., Panzica, G.C., Melcangi, R.C., 2016. Neurosteroidogenesis today: Novel targets for neuroactive steroid synthesis and action and their relevance for translational research. J. Neuroendocrinol. 28, 19. https://doi.org/10.1111/jne.12351.

Porcu, P., Mostallino, M.C., Sogliano, C., Santoru, F., Berretti, R., Concas, A., 2012. Long-term administration with levonorgestrel decreases allopregnanolone levels and alters GABA(A) receptor subunit expression and anxiety-like behavior. Pharmacol. Biochem. Behav. 102, 366-372. https://doi.org/10.1016/j.pbb.2012.05.011.

Porcu, P., Serra, M., Concas, A., 2019. The brain as a target of hormonal contraceptives: Evidence from animal studies. Front. Neuroendocrinol. 55, 100799. https://doi.org/10.1016/j.yfrne.2019.100799.

Poromaa, I.S., Segebladh, B., 2012. Adverse mood symptoms with oral contraceptives. Acta Obstet. Gynecol. Scand. 91, 420-427. https://doi.org/10.1111/j.1600-0412.2011.01333.x.

Rabinowitz, A., Cohen, S.J., Finn, D.A., Stackman, R.W., 2014. The neurosteroid allopregnanolone impairs object memory and contextual fear memory in male C57BL/6J mice. Horm. Behav. 66, 238-246. https://doi.org/10.1016/j.yhbeh.2014.05.005.

Rapkin, A.J., Morgan, M., Sogliano, C., Biggio, G., Concas, A., 2006. Decreased neuroactive steroids induced by combined oral contraceptive pills are not associated with mood changes. Fertil. Steril. 85, 1371-1378. https://doi.org/10.1016/j.fertnstert.2005.10.031.

Rosenberg, L., Park, S., 2002. Verbal and spatial functions across the menstrual cycle in healthy young women. Psychoneuroendocrinology 27, 835-841. https://doi.org/10.1016/S0306-4530(01)00083-X.

Santoru, F., Berretti, R., Locci, A., Porcu, P., Concas, A., 2014. Decreased allopregnanolone induced by hormonal contraceptives is associated with a reduction in social behavior and sexual motivation in female rats. Psychopharmacology 231, 3351-3364. https://doi.org/10.1007/s00213-014-3539-9.

Sassoe-Pognetto, M., Follesa, P., Panzanelli, P., Perazzini, A.-Z., Porcu, P., Sogliano, C., Cherchi, C., Concas, A., 2007. Fluctuations in brain concentrations of neurosteroids are not associated to changes in gephyrin levels. Brain Res. 1169, 1-8. https://doi.org/10.1016/j.brainres.2007.06.057.

Scharfman, H.E., Mercurio, T.C., Goodman, J.H., Wilson, M.A., MacLusky, N.J., 2003. Hippocampal excitability increases during the estrous cycle in the rat: a potential role for brain-derived neurotrophic factor. J. Neurosci. 23, 11641-11652. https://doi.org/10.1523/JNEUROSCI.23-37-11641.2003.

Segebladh, B., Borgström, A., Odlind, V., Bixo, M., Sundström-Poromaa, I., 2009. Prevalence of psychiatric disorders and premenstrual dysphoric symptoms in patients with experience of adverse mood during treatment with combined oral contraceptives. Contraception 79, 50-55. https://doi.org/10.1016/j.contraception.2008.08.001.

Shimizu, E., Hashimoto, K., Okamura, N., Koike, K., Komatsu, N., Kumakiri, C., Nakazato, M., Watanabe, H., Shinoda, N., Okada, S., Iyo, M., 2003. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. Biol. Psychiatry 54, 70-75. https://doi.org/10.1016/S0006-3223(03)00181-1.

Simone, J., Bogue, E.A., Bhatti, D.L., Day, L.E., Farr, N.A., Grossman, A.M., Holmes, P.V., 2015. Ethinyl estradiol and levonorgestrel alter cognition and anxiety in rats concurrent with a decrease in tyrosine hydroxylase expression in the locus coeruleus and brain-derived neurotrophic factor expression in the hippocampus. Psychoneuroendocrinology 62, 265-278. https://doi.org/10.1016/j.psyneuen.2015.08.015.

Singh, M., Meyer, E.M., Simpkins, J.W., 1995. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. Endocrinology 136, 2320-2324. https://doi.org/10.1210/endo.136.5.7720680.

Skovlund, C.W., Mørch, L.S., Kessing, L.V., Lidegaard, Ø., 2016. Association of hormonal contraception with depression. JAMA Psychiatry 73, 1154-1162. https://doi.org/10.1001/jamapsychiatry.2016.2387.

Sohrabji, F., Miranda, R.C., Toran-Allerand, C.D., 1995. Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. Proc. Natl. Acad. Sci. U.S.A. 92, 11110-11114. https://doi.org/10.1073/pnas.92.24.11110.

Song, J.H., Yu, J.T., Tan, L., 2015. Brain-derived neurotrophic factor in Alzheimer's disease: Risk, mechanisms, and therapy. Mol. Neurobiol. 52, 1477-1493. https://doi.org/10.1007/s12035-014-8958-4.

Sundström-Poromaa, I., Comasco, E., Sumner, R., Luders, E., 2020. Progesterone - Friend or foe? Front. Neuroendocrinol. 59, 100856. https://doi.org/10.1016/j.yfrne.2020.100856.

Talani, G., Biggio, G., Sanna, E., 2011. Enhanced sensitivity to ethanol-induced inhibition of LTP in CA1 pyramidal neurons of socially isolated C57BL/6J mice: Role of neurosteroids. Front. Endocrinol. (Lausanne) 2, 56. https://doi.org/10.3389/fendo.2011.00056.

Talani, G., Licheri, V., Biggio, F., Locci, V., Mostallino, M.C., Secci, P.P., Melis, V., Dazzi, L., Carta, G., Banni, S., Biggio, G., Sanna, E., 2016. Enhanced glutamatergic synaptic plasticity in the hippocampal CA1 field of food-restricted rats: Involvement of CB1 receptors. Neuropsychopharmacology 41, 1308-1318. https://doi.org/10.1038/npp.2015.280.

Taxier, L.R., Gross, K.S., Frick, K.M., 2020. Oestradiol as a neuromodulator of learning and memory. Nat. Rev. Neurosci. 21, 535-550. https://doi.org/10.1038/s41583-020-0362-7.

Tsankova, N.M., Berton, O., Renthal, W., Kumar, A., Neve, R.L., Nestler, E.J., 2006. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat. Neurosci. 9, 519-525. https://doi.org/10.1038/nn1659.

Tyler, W.J., Alonso, M., Bramham, C.R., Pozzo-Miller, L.D., 2002. From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn. Mem. 9, 224-237. https://doi.org/10.1101/lm.51202.

Vierk, R., Glassmeier, G., Zhou, L., Brandt, N., Fester, L., Dudzinski, D., Wilkars, W., Bender, R.A., Lewerenz, M., Gloger, S., Graser, L., Schwarz, J., Rune, G.M., 2012. Aromatase inhibition abolishes LTP generation in female but not in male mice. J. Neurosci. 32, 8116-8126. https://doi.org/10.1523/JNEUROSCI.5319-11.2012.

Walf, A.A., Rhodes, M.E., Frye, C.A., 2006. Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. Neurobiol. Learn. Mem. 86, 35-46. https://doi.org/10.1016/j.nlm.2006.01.004.

Warren, A.M., Gurvich, C., Worsley, R., Kulkarni, J., 2014. A systematic review of the impact of oral contraceptives on cognition. Contraception 90, 111-116. https://doi.org/10.1016/j.contraception.2014.03.015.

Warren, S.G., Humphreys, A.G., Juraska, J.M., Greenough, W.T., 1995. LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. Brain Res. 703, 26-30. https://doi.org/10.1016/0006-8993(95)01059-9.

Warren, S.G., Juraska, J.M., 1997. Spatial and nonspatial learning across the rat estrous cycle. Behav. Neurosci. 111, 259-266. https://doi.org/10.1037/0735-7044.111.2.259.

Wharton, W., Hirshman, E., Merritt, P., Doyle, L., Paris, S., Gleason, C., 2008. Oral contraceptives and androgenicity: influences on visuospatial task performance in younger individuals. Exp. Clin. Psychopharmacol. 16, 156-164. https://doi.org/10.1037/1064-1297.16.2.156.

Figure 1

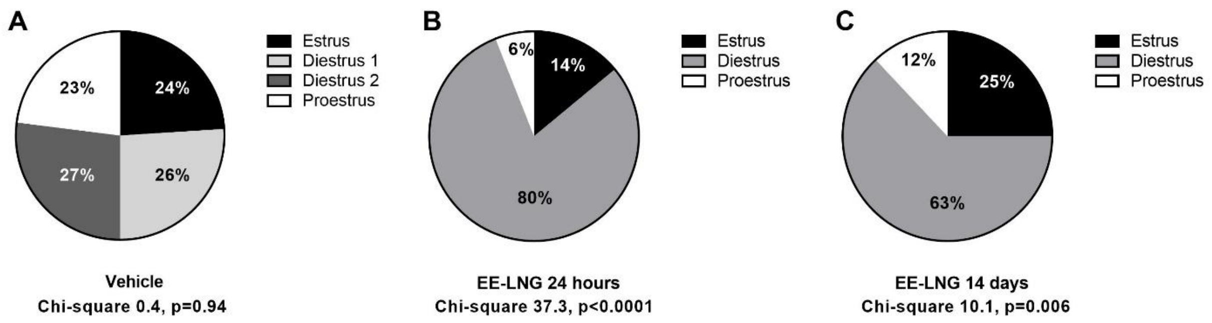

Figure 2

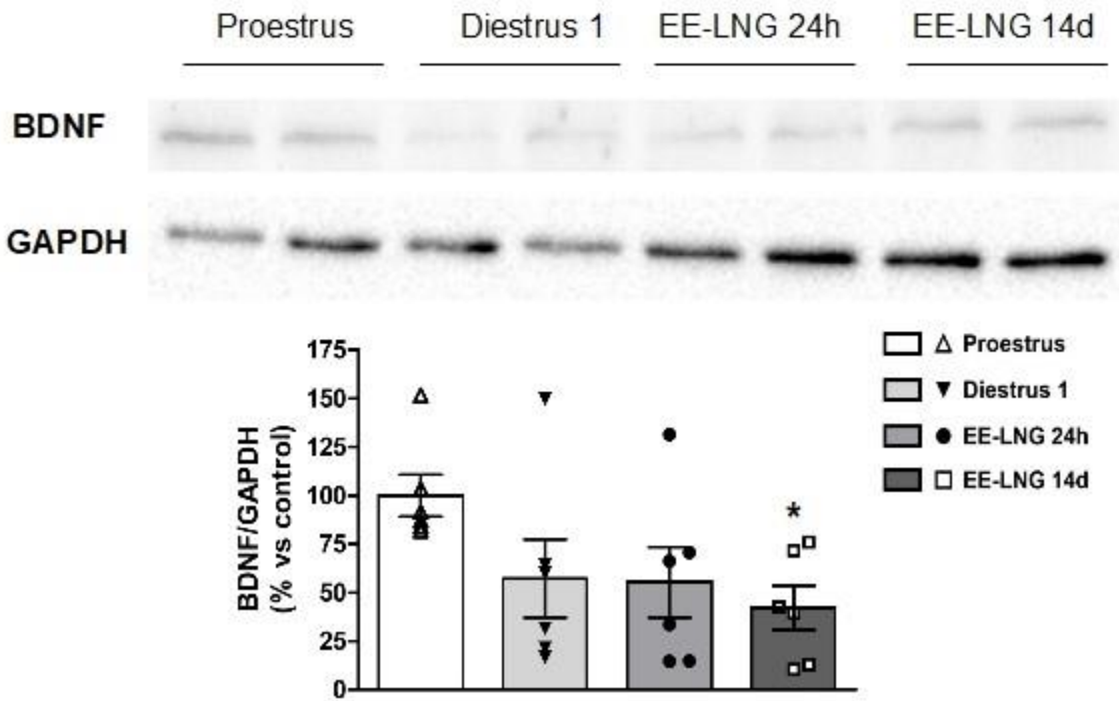

Figure 3

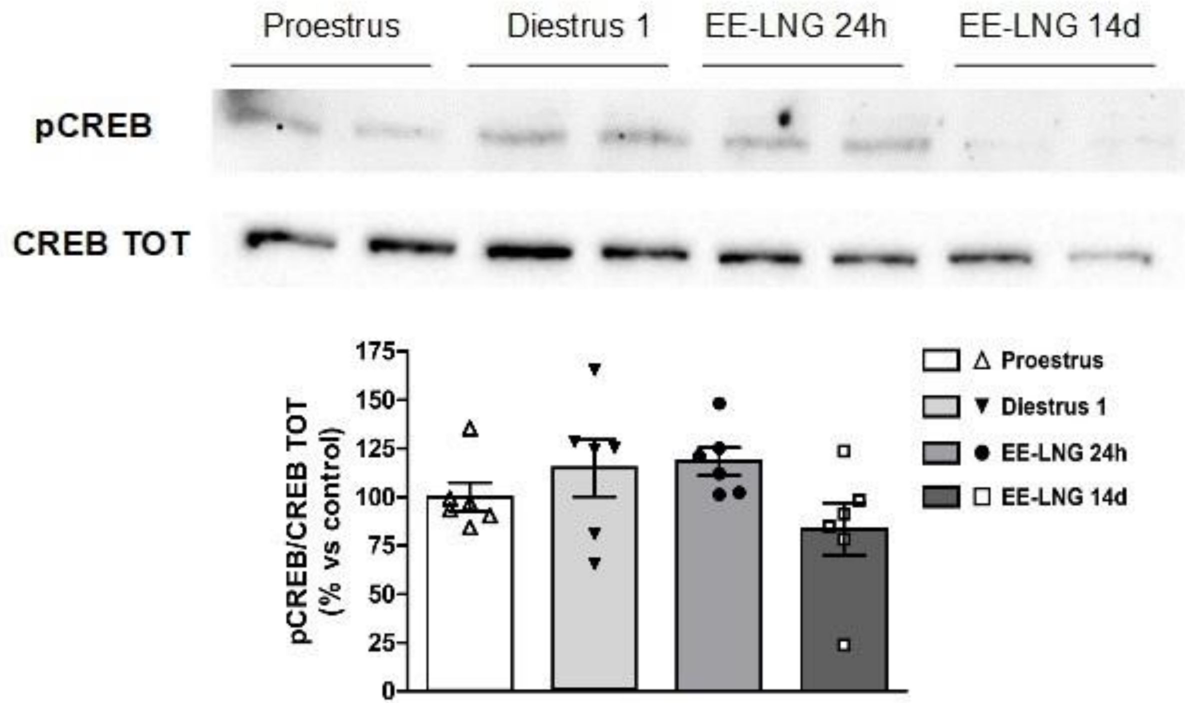

Figure 4

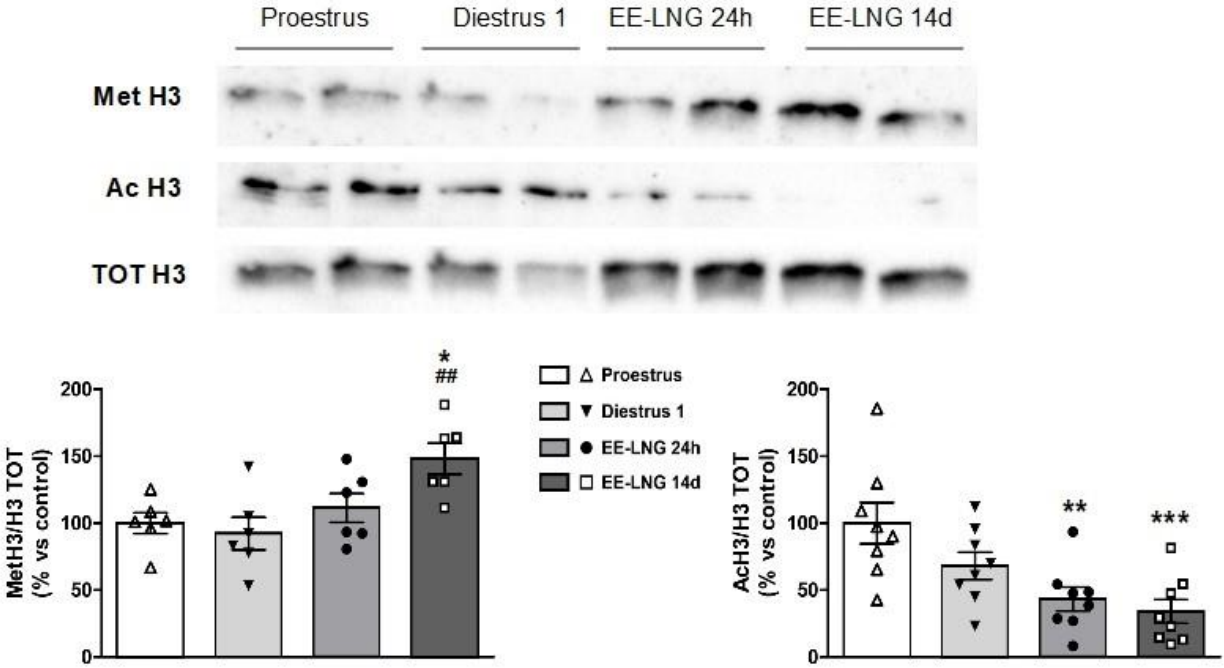

Figure 5

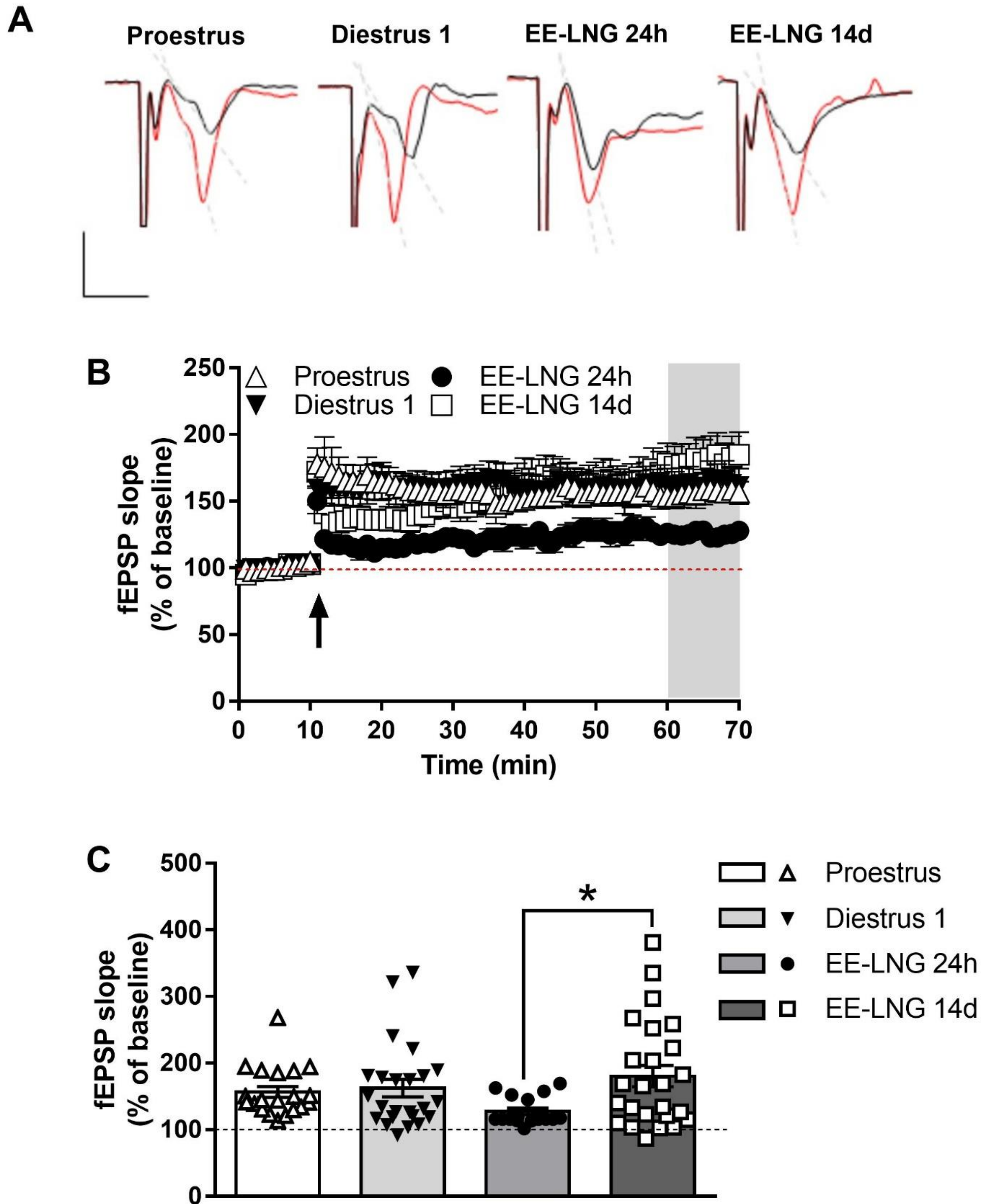

Figure 6

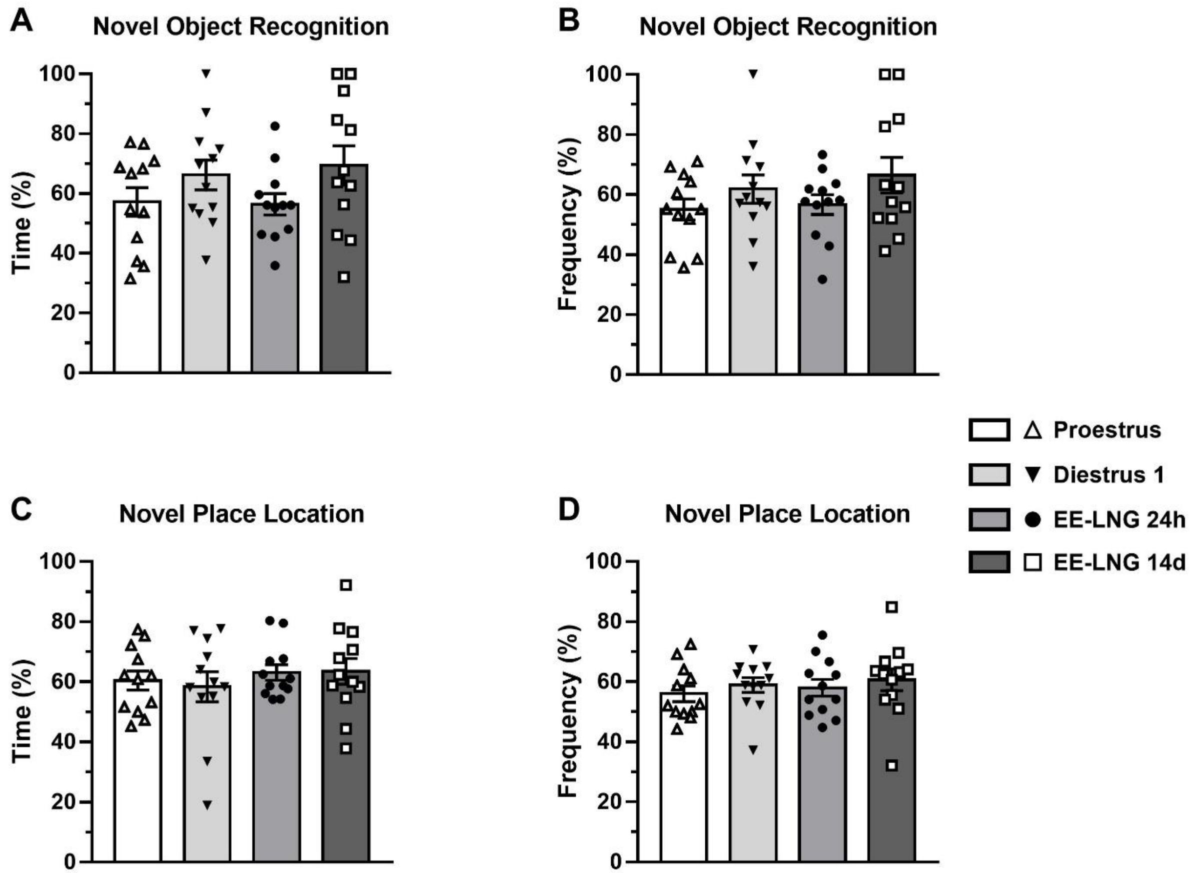

Figure 7