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Behavioral characterization of co-exposure to cannabinoids and hormonal contraceptives in female rats

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Abstract:	Hormonal contraceptives are among the most widely used drugs by young healthy women to block ovulation and avoid pregnancy. They reduce the ovarian secretion of estradiol and progesterone, hormones that also modulate neuronal plasticity, cognitive functions, emotions and mood. Cannabis is the most commonly used illicit drug worldwide and its use is increasing among young women, many of which regularly take the “pill”. Despite evidence of a bidirectional interaction between the endocannabinoid system and gonadal hormones, only very few studies have examined the consequences of cannabis consumption in young females under hormonal contraceptives treatment. To fill this gap, this study evaluated the behavioral effects of co-exposure to chronic 1) hormonal contraceptives, i.e., ethinyl estradiol (EE) plus levonorgestrel (LNG), one of the synthetic estrogen-progestin combinations of hormonal contraceptives, and 2) a cannabinoid receptor agonist, i.e., WIN 55,212-2 (WIN), on motor activity, emotional states and cognitive functions in young adult female rats (8-11/experimental group). Hormonal and cannabinoid treatment started at post-natal day (PND) 52 and 56, respectively, while behavioral testing occurred between PND 84-95. The results show that chronic EE-LNG treatment, at doses (0.020 and 0.060 mg/rat, respectively) known to drastically reduce plasma progesterone levels, and the contextual exposure to WIN, at a dose (12.5 µg/kg/infusion) known to be rewarding in the rat, alters the hormonal milieu but does not modify spontaneous locomotor activity, anxiety-like state (as measured by the elevated plus maze and the marble burying tests) and cognitive abilities (as measured by the novel object recognition and the prepulse inhibition tests) in young adult female rats. Yet, co-exposure to EE-LNG and WIN tends to increase the duration of immobility and to reduce the time spent swimming in the forced swimming test, suggesting the development of a depressive-like state. These findings allow deepening the current knowledge on the interaction between cannabinoid agonists and hormonal contraceptives and suggest that low, rewarding doses of cannabinoids do not significantly alter the motor and cognitive skills and do not induce anxiety in females that use hormonal contraceptives, but may rather induce a depressive-like state.
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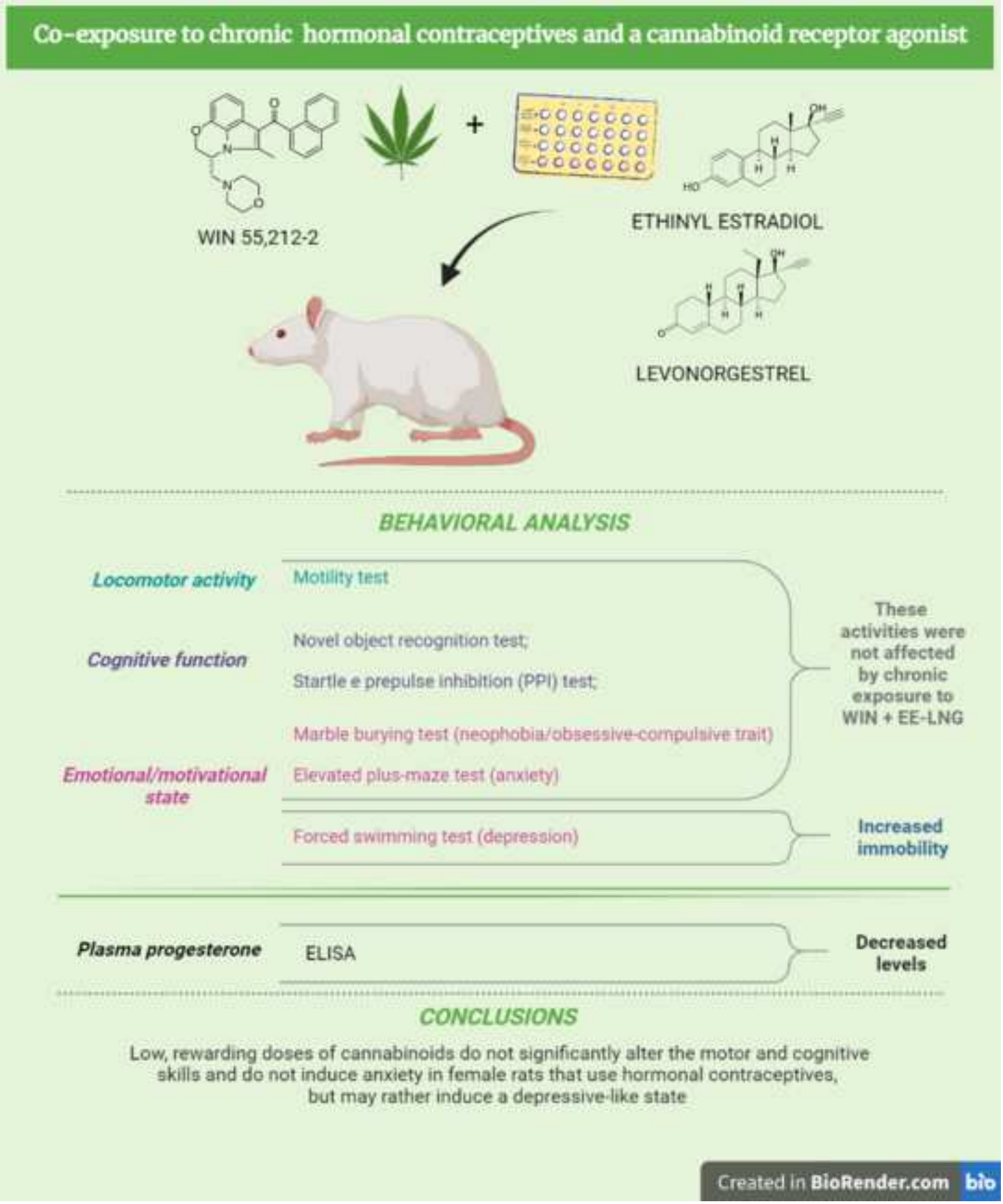
Highlights

- Hormonal contraceptives - cannabinoid agonist behavioral interactions in rats
- Hormonal contraceptives plus cannabinoids do not alter spontaneous locomotor activity
- Hormonal contraceptives plus cannabinoids do not alter anxiety-like state
- Hormonal contraceptives plus cannabinoids do not alter cognitive abilities
- Hormonal contraceptives plus cannabinoids may induce a depressive-like state



Behavioral characterization of co-exposure to cannabinoids and hormonal contraceptives in female rats

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Abstract

Hormonal contraceptives are among the most widely used drugs by young healthy women to block ovulation and avoid pregnancy. They reduce the ovarian secretion of estradiol and progesterone, hormones that also modulate neuronal plasticity, cognitive functions, emotions and mood. Cannabis is the most commonly used illicit drug worldwide and its use is increasing among young women, many of which regularly take the “pill”. Despite evidence of a bidirectional interaction between the endocannabinoid system and gonadal hormones, only very few studies have examined the consequences of cannabis consumption in young females under hormonal contraceptives treatment. To fill this gap, this study evaluated the behavioral effects of co-exposure to chronic 1) hormonal contraceptives, i.e., ethinyl estradiol (EE) plus levonorgestrel (LNG), one of the synthetic estrogen-progestin combinations of hormonal contraceptives, and 2) a cannabinoid receptor agonist, i.e., WIN 55,212-2 (WIN), on motor activity, emotional states and cognitive functions in young adult female rats (8-11/experimental group). Hormonal and cannabinoid treatment started at post-natal day (PND) 52 and 56, respectively, while behavioral testing occurred between PND 84-95. The results show that chronic EE-LNG treatment, at doses (0.020 and 0.060 mg/rat, respectively) known to drastically reduce plasma progesterone levels, and the contextual exposure to WIN, at a dose (12.5 µg/kg/infusion) known to be rewarding in the rat, alters the hormonal milieu but does not modify spontaneous locomotor activity, anxiety-like state (as measured by the elevated plus maze and the marble burying tests) and cognitive abilities (as measured by the novel object recognition and the prepulse inhibition tests) in young adult female rats. Yet, co-exposure to EE-LNG and WIN tends to increase the duration of immobility and to reduce the time spent swimming in the forced swimming test, suggesting the development of a depressive-like state. These findings allow deepening the current knowledge on the interaction between cannabinoid agonists and hormonal contraceptives and suggest that low, rewarding doses of cannabinoids do not significantly alter the motor and cognitive skills and do not induce anxiety in females that use hormonal contraceptives, but may rather induce a depressive-like state.

Keywords: hormonal contraceptives; cannabinoid agonists; ethinyl estradiol; levonorgestrel; emotional state; cognitive function

1. Introduction

Steroid hormones, synthesized both in the periphery and the central nervous system, influence key brain functions through rapid modulation of neuronal excitability via membrane receptors, or by promoting gene expression via intracellular steroid receptors. These so called neurosteroids regulate mood, emotional states, the stress response and cognition, and their levels are altered in neuropsychiatric disorders, including anxiety and depression, and following administration of psychoactive substances (Porcu et al., 2016). Chronic treatment with ethinyl estradiol (EE) and levonorgestrel (LNG), two of the synthetic estrogens and progestins used in the hormonal contraceptive (HC) pill, markedly decreased brain and plasma levels of progesterone and its neuroactive metabolite allopregnanolone in female rats (Concas et al., 2022). HCs are used from almost 60 years by more than 150 million women worldwide, mostly for contraceptive purposes, but also as a therapy for endometriosis, polycystic ovarian syndrome and menstrual cycle-related symptoms (Concas et al., 2022). Several studies have nowadays shown that HCs affect the central nervous system, altering the volume and structure of several brain regions with subsequent changes in brain function that may persist even after discontinuation (Petersen et al., 2023). This is particularly concerning for the increasing number of young women who start taking HCs early in adolescence, when the brain is still under development and influenced by steroid hormones.

Administration of several drugs of abuse, including alcohol, nicotine, morphine, and Δ9-tetrahydrocannabinol (THC), alters brain and plasma pregnenolone, progesterone and allopregnanolone levels (Concas et al., 2006; Grobin et al., 2005; Morrow et al., 2020; Vallée et al., 2014), and such alterations are thought to strengthen the rewarding properties of these drugs. Indeed, neurosteroids display rewarding properties in behavioral paradigms of drug addiction (Finn et al., 1997) and modulate dopamine release in the rat nucleus accumbens and prefrontal cortex (Motzo et al., 1996). Likewise, estradiol modulates motivation and dopamine release, enhancing the rewarding properties of alcohol (Hilderbrand and Lasek, 2018) and psychostimulants (Quigley et al., 2021), although it also

decreases risky choice and promotes risk aversion in both male and female rats (Orsini et al., 2021). Thus, chronic treatment with HCs might alter reward threshold and sensitivity to drugs of abuse, a notion strengthened by numerous evidence showing that females are more vulnerable to drug abuse than males, and that endogenous and exogenous steroid hormones are likely involved in this phenomenon (Quigley et al., 2021).

According to the United Nations Office on Drugs and Crime (UNODC)'s World Drug Report 2022, marijuana is still the most commonly abused drug worldwide, particularly among young adults, and cannabis legalization in several countries appears to have accelerated daily use and related health impacts. Cannabis consumption increased during the COVID-19 pandemic, and almost 6% of the global cannabis users estimated in 2020 (209 million people) were 15-16 years old (UNODC, 2022). Worryingly, early onset of cannabis use among adolescents has been shown to be associated with cognitive impairment (Pope et al., 2003), enhanced risk for depression (Hengartner et al., 2020), and transition from recreational use to cannabis use disorder (Behrendt et al., 2009). Adolescence is a critical period for brain development, marked by the increasing levels of gonadal hormones including estrogens and progesterone. These steroids interact with the endocannabinoid system in modulating, among others, the reproductive system, motivational processes and sensitivity to exogenous cannabinoids (Gorzalka and Dang, 2012; Struik et al., 2018). Bi-directional interactions between (endo)cannabinoids and gonadal hormones could account, at least in part, for the greater sensitivity of women to the subjective effects of cannabis, which may contribute to the faster progression from first use to abuse and the greater withdrawal symptoms of women relative to men (Cooper and Haney, 2014). In support to an enhanced vulnerability of the female sex to cannabis abuse, female rats were shown to be more sensitive to the reinforcing effects of cannabinoids (Fattore et al., 2007) and at higher risk to reinstate cannabinoid-seeking behavior after extinction (Fattore et al., 2010), with gonadal hormones significantly affecting cannabinoid CB_1 receptor density and function in selected brain areas (Castelli et al., 2014).

Since approximately 25% of women aged 15-44 who currently use contraception reported using the pill as their preferred method (Cooper, 2022) and the prevalence of cannabis use is increasing among the female population (UNODC, 2022), it can be easily assumed that a high number of girls take the pill and smoke cannabis. On the basis of the clinical and preclinical evidence available in the literature, concomitant use of exogenous steroid hormones and cannabinoids are very likely to interact and affect brain and behavior (Gorzalka and Dang, 2012). Yet, the effect of smoking marijuana in girls under HC treatment is still to be determined. Similarly, no preclinical study has been performed so far to test the effect of rewarding doses of cannabinoids in young females exposed to chronic HC treatment. In this study, we tested the hypothesis that co-exposure to EE-LNG, one of the estrogen-progestin combinations of the HC pills, and to WIN 55,212-2 (WIN), a synthetic cannabinoid agonist known to induce rewarding effects and sustain motivated behavior in rats, may affect motor, emotional and cognitive behavior, as well as plasma progesterone in female rats.

2. Materials and Methods

2.1 Animals

Female Sprague-Dawley rats (Charles River, Italy) aged 32-35 days were housed 4/cage and maintained on an artificial 12 h light/dark inverted cycle (light ON at 7 PM) at a constant temperature of 22±2°C, a relative humidity of 65% and with food and water available *ad libitum*. After arrival in the animal facility, rats were handled once daily for 5 minutes for 10 consecutive days before undergoing surgery for the implantation of an intravenous catheter, after which they were housed individually to avoid damage to the external part of the catheter.

All 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 (authorization n.

969/2019-PR).

2.2 Surgery for intravenous catheter implantation (PND 42-45)

Following 10 days of acclimation, starting on post-natal day (PND) 42, animals were deeply anaesthetized with isoflurane (3%) and a permanent intravenous (i.v.) catheter was surgically implanted into the external jugular vein and secured to the middle scapular region. During the 7 days following surgery, rats were allowed to recover individually in their own home cage and received a daily i.v. infusion of gentamicin (0.16 mg/kg) followed by 0.2 ml of a heparinized (1%) sterile saline solution to flush the antibiotic through the catheter. Antibiotics and anesthetics were purchased as sterile solutions from local distributors. As shown in Figure 1, at the end of the 10-day recovery, animals started the chronic treatment with HCs.

2.3 Hormonal contraceptives treatment (PND 52-97)

Daily HC treatment started from PND 52. Rats were injected subcutaneously (s.c.) at the nape of the neck with either a combination of EE (0.020 mg, Sigma-Aldrich E4876) and LNG (0.060 mg, Sigma-Aldrich N2260) (EE-LNG-treated group) or vehicle (VEH-treated group) once a day at 4 PM. The EE-LNG combination was dissolved in one drop of Tween 80 and distilled water and administered in a volume of 1 ml/rat (Porcu et al., 2012). Animals were subjected to behavioral tests, or were euthanized 20 to 24 hours after the last EE-LNG daily treatment (see timeline on Figure 1).

2.4 Cannabinoid treatment (PND 56-98)

Daily cannabinoid treatment started from PND 56. WIN (Tocris, UK) was dissolved in one drop of Tween 80 and then diluted in heparinized (1%) 0.9% sterile saline solution and i.v. infused at a unit dose of 12.5 µg/kg/0.1 ml until reaching a cumulative dose of 0.3 mg/kg (WIN-treated group). This WIN dosage regimen was selected based on previous studies showing that it increases dopamine release in the rat nucleus accumbens (Fadda et al.,

2006) and corresponds to the mean amount of cannabinoid agonist daily self-administered by trained rats (Fattore et al., 2007; Fattore et al., 2010), which suggests to induce rewarding effects. To closely mimic the self-administration pattern of cannabinoid intake observed in our previous studies (Fattore et al., 2007; Fattore et al., 2010), rats were daily placed in self-administration boxes for 2 hours and WIN was infused at the same rate of infusion (100 µl/5 s), at the same unit dose (12.5 µg/kg/infusion) and approximately at the same time interval until reaching a cumulative i.v. dose of 0.3 mg/kg. Specifically, rats received 12 infusions during the 1st half hour of the session, 7 infusions during the 2nd half hour, 3 infusions during the 60-90 min interval and 2 infusions during the last 30-min of the session. In parallel, control rats received i.v. infusion of sterile saline (SAL-treated group) under the same experimental conditions (i.e., with the only difference of receiving saline in the place of the cannabinoid). During each 2h-session, approximately 50% of EE-LNG- or VEH-treated animals were infused with WIN (WIN-treated group), while the remaining 50% with saline (SAL-treated group). Rats were euthanized on PND 98, 2 hours after the WIN/SAL administration session (see timeline on Figure 1). WIN treatment took place once daily, 7 days/week, during the dark phase of the cycle (between 9.30 AM and 13.30 PM) and was performed in operant chambers (29.5×32.5×23.5 cm), each encased in a sound- and light-attenuating cubicle equipped with a ventilation fan (Med Associates, USA). In each chamber, the two levers (used by rats in self-administration studies as *modus operandi* to obtain the drug) were kept retracted during the 2h-session, and WIN (or saline) was delivered through a swivel device connected to the implanted catheter by an infusion pump controlled remotely by the experimenter through a computer with Med Associates software. The WIN solution was freshly remixed every day before use.

2.5 Behavioral testing (PND 84-95)

Behavioral tests started at PND 84, i.e., after 33 days of EE-LNG/VEH treatment and 29 days of WIN/SAL infusion. Experiments were conducted during the dark phase (between 1 PM and 4 PM) in dedicated rooms under dim light (20 lux). Rats were left undisturbed in

each experimental room for 1 hour acclimatation period before starting behavioral testing. Each animal underwent the following sequence of behavioral tests at least 1 hour after the last WIN infusion: locomotor activity (LA), novel object recognition (NOR), forced swimming (FST), marble burying (MB), elevated plus maze (EPM), and prepulse inhibition (PPI), with at least 45 hours (i.e., every two days) or 69 hours (i.e., every three days) separating two consecutive test to prevent carryover effects (see timeline on Figure 1).

2.5.1 Locomotor activity

Rats were individually tested for LA under standardized environmental conditions using the Digiscan Animal Activity Analyser (Omnitech Electronics, USA) that assessed animals' activity for 60 minutes at 10-min intervals, beginning immediately after the animal was placed in the cage (Spano et al., 2013). Each operant cage (42×30×60 cm) was equipped with two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 4 cm above the cage floor, and a further set of 16 horizontal beams whose height could be adapted to the size of the animals. The following behavioral parameters were measured: 1) horizontal activity, i.e., the total number of beam interruptions that occurred in the horizontal sensors; 2) vertical activity, i.e., the total number of beam interruptions that occurred in the vertical sensors, i.e., the number of times the rat rose onto its hind legs (number of rearings); 3) total distance travelled (cm). The arena was wiped out with H_2O_2 before each trial to prevent olfactory cues.

2.5.2 Marble burying

The MB test, a cost-effective rodent model of compulsive-like activity with good face validity, was conducted in open transparent plastic boxes (54×34.5×20 cm) with the floor covered with 5 cm of fresh sawdust as previously described (Zanda et al., 2017). Twenty-four standard glass marbles (1.5 cm in diameter, arranged in six rows of four marbles each) were placed uniformly over the sawdust surface. On the test day, rats were individually placed in the cage in a marble-free area (34.5×15 cm) and their activity was videotaped for 30 min. At

the end of the session, animals were gently removed from the test cage and the number of marbles partially (≥67%) and totally (>95%) buried was counted by an experimenter blind to the pharmacological treatments. Bedding was replaced and marbles were thoroughly cleaned with soapy water, followed by H_2O_2, before each test to avoid the presence of olfactory cues.

2.5.3 Elevated plus maze

The EPM test, commonly used to assess anxiety-like behavior in rodents, was conducted as previously described (Porcu et al., 2012). The black polyvinyl chloride maze comprised two open and two closed arms (12×60 cm) that converged on a small central square (12×12 cm), which served as starting point, thus reproducing the shape of a plus sign; the apparatus was elevated 50 cm from the floor. The rat was placed in the center of the maze apparatus (i.e., at the start point) facing an open arm, and allowed to freely explore for 5 minutes. Rats' behavior was videotaped by a camera positioned above the maze. Three independent researchers blind to the treatment group measured the time spent by each rat into the open vs. the closed arms and counted the number of entries into open and closed arms, considering the animal inside a specific arm when it had all the four paws inside that arm. The EPM was thoroughly cleaned with H_2O_2 before each test to avoid olfactory cues.

2.5.4 Forced swimming test

The FST is commonly used in animal studies to evaluate the effect of environmental or pharmacological manipulations on mood and their ability to induce a depressive-like state in rodents that are forced to swim without possibility of escaping. The FST was conducted according to our previous studies (Zanda et al., 2017). Briefly, animals were individually placed in transparent Plexiglas cylinders (height: 40 cm; diameter: 20 cm) containing 20 cm of water at 25°C. After 5 minutes, they were removed from the water and dried in a heated environment (30°C) for 30 minutes. The sessions were videotaped and the videos analyzed by three independent researchers blind to the experimental condition. Animals' behaviors

were classified according to three possible and mutually exclusive categories: swimming, climbing (i.e., attempts to climb the wall of the cylinder), and immobility (i.e., floating in the water performing the minimum amount of movements with the anterior paws to maintain its head above the water surface). The rats' emotional state was determined by quantifying the time spent swimming or trying to escape in any direction (sign of reaction) or staying completely immobile (sign of behavioral hopelessness or passivity). An increase or decrease in immobility time is considered a sign of a depressant or antidepressant-like effect, respectively.

2.5.5 Novel object recognition

Ability to discriminate novel objects in the NOR test is a marker of non-spatial short-term memory. The NOR test was carried out in a black box (60×60 cm) according to our previous studies (Boi et al., 2022). Briefly, each rat was given a 10-min habituation session (habituation trial) and 3 min later placed in the box and allowed to explore two identical objects for 10 min (training session, T1). After a 1-h interval, the second trial (choice session, T2) started and lasted 3 min, with 2 objects placed in the same positions and the animal exposed to the same condition as in T1, except that one object was replaced with a new one (novel object). The objects to be discriminated were made of glass, plastic or metal and had different shapes and colors, devoid of any natural significance, and were thoroughly cleaned with H_2O_2 before each trial to avoid olfactory cues. Exploratory behavior was defined as the animal directing its nose toward the object at a distance ≤2 cm and/or sniffing, biting or touching the objects with the nose. Sitting on, climbing over and/or turning around the objects were not considered exploratory behaviors. Combination (novel/old) and position (right/left) of objects were counterbalanced to compensate for lateralization. Training and test sessions were recorded with a camera and the total time spent exploring the objects during T1 and T2 and the frequency of approaches, i.e., numbers of approaching the two objects, were measured by three independent researchers blind to the experimental condition. Recognition skills were calculated using the following formula: N/(N+F) × 100,

where N=time spent exploring (or frequency of contacts with) the novel object, and F=time spent exploring (or frequency of contacts with) the familiar one.

2.5.6 Prepulse inhibition

The apparatus for the detection of the startle reflex consisted of 4 standard cages, each placed inside a sound-attenuated and ventilated chamber (Med Associated, USA). Startle cages were non-restrictive Plexiglas cylinders (diameter 9 cm) mounted on a piezoelectric accelerometer platform connected to an analogue-digital converter. Background noise and acoustic bursts were conveyed through two speakers, placed in proximity to the startle cage, in order to produce a variation in sound intensity within 1 dB. Both startle cages and speakers were connected to a PC dedicated to the detection and analysis of all variables of the cage. Before each session, calibration of acoustic stimuli and mechanical responses was checked. On test day, rats were individually placed in the test cage for a 5-min acclimatization period with a 70 dB white noise background that continued for the remainder of the session. Animals were then tested on 3 consecutive blocks of trials. The 1st and 3rd blocks consisted of 5 pulse-alone trials of 40 ms at 115 dB, while the 2nd block (test block) was a pseudorandom sequence of 50 trials including 12 pulse-alone trials, 30 pulse trials preceded by 73, 76 or 82 dB prepulses of 20 milliseconds (10 for each level of prepulse loudness), and 8 no-stimulus trials where the only background noise was delivered. Inter-trial intervals were selected randomly between 10 and 15 s, while the inter-stimulus intervals were set at 100 milliseconds. The percent (%) PPI was calculated based on the values relative to the second block only and using the following formula: 100 - [(mean startle amplitude for prepulse+pulse trials/mean startle amplitude for pulse-alone trials) × 100], as previously described (Spano et al., 2010).

2.6 Progesterone assay

On PND 98, three days after the last behavioral test, rats were euthanized by decapitation; blood was collected from the trunk into EDTA-coated tubes and centrifuged at 900xg for 10

min at 4°C. The resulting plasma was frozen at -20°C until use. Progesterone levels were quantified by an enzyme-linked immunosorbent assay kit (DEV9988, Demeditec Diagnostics GmbH, Germany), according to manufacturer's instructions, using a 96-well plate pre-coated with progesterone antiserum. Each sample was run in duplicate. The lowest detectable level is 0.156 ng/ml; within- and between-assay coefficients of variation ranged 5-12% and 6-14%, respectively.

2.7 Statistical analysis

A sample size of 7.75 per experimental group was estimated using G*Power software (G*Power 3.1.9.2; based on α=0.05, power=0.80), a value consistent with the numbers used in the past for similar studies. Thus, we determined a sample size of ≥ 8 per experimental group throughout the experiment (specifically, 9 Vehicle + Saline, 11 Vehicle + WIN, 8 EE-LNG + Saline and 11 EE-LNG + WIN rats/group). Statistical analysis was performed using commercially available statistical programs (Statistica 8.0, StatSoft Inc., Tulsa, OK, USA; GraphPad Prism 8.0, GraphPad Software, San Diego, CA, USA). Normality was checked by the Kolmogorov-Smirnov test, which indicated that data met the requirement for parametric analysis. Data are presented as mean ± SEM and statistical comparisons were performed by two-way analysis of variance (ANOVA), with EE-LNG and WIN treatments as factors, except for locomotor activity whose data were analyzed by two-way repeated measures ANOVA, with EE-LNG and WIN treatments as factors and time as repeated measure. ANOVAs were followed by Tukey's multiple comparisons *post hoc* test. A p value <0.05 was considered statistically significant.

3. Results

3.1 Chronic co-exposure to EE-LNG and WIN does not alter spontaneous locomotor activity

Locomotor activity decreased over time to a similar extent in all experimental groups (Figure 2). A significant effect of time was observed for horizontal activity (Fig. 2A, $F_{5,175}$=99.96;

p<0.00001), vertical activity (Fig. 2B, $F_{5,175}$=41.90; p<0.00001), and total distance travelled (Fig. 2C, $F_{5,175}$=99.43; p<0.00001). For vertical activity, two-way ANOVA also revealed a significant effect of WIN treatment ($F_{1,35}$=6.56; p=0.015) and a time x WIN treatment interaction ($F_{5,175}$=3.53; p=0.005); likewise, for total distance travelled there was also a significant time x WIN treatment interaction ($F_{5,175}$=4.25; p=0.001) (see Supplementary Tables 1 and 2 for a summary of the ANOVA and *post hoc* results). Overall, chronic treatment with EE-LNG and co-exposure with WIN decreased locomotor activity with time, similar to control female rats.

3.2 Chronic co-exposure to EE-LNG and WIN does not induce neophobia / compulsive traits in the marble burying test

No differences among experimental groups were observed in the number of fully (>95%) buried marbles (Fig. 3A, EE-LNG treatment: $F_{1,35}$=0.001, p=0.974; WIN treatment: $F_{1,35}$=1.22, p=0.276; interaction $F_{1,35}$=0.04, p=0.848), partially (≥67%) buried marbles (Fig. 3B, EE-LNG treatment: $F_{1,35}$=2.30, p=0.138; WIN treatment: $F_{1,35}$=1.22, p=0.277; interaction $F_{1,35}$=0.46, p=0.502), or in the total number (fully+partially) of buried marbles (Fig. 3C, EE-LNG treatment: $F_{1,35}$=0.31, p=0.582; WIN treatment: $F_{1,35}$=1.74, p=0.195; interaction $F_{1,35}$=0.16, p=0.691). Overall, chronic treatment with EE-LNG and co-exposure with WIN did not affect burying behavior in female rats.

3.3 Chronic co-exposure to EE-LNG and WIN differentially affects emotional behavior in the elevated plus maze and forced swimming tests

Regarding anxiety-like behavior, no differences among experimental groups were observed in the number of open arm entries (Fig. 4A, EE-LNG treatment: $F_{1,35}$=0.67, p=0.419; WIN treatment: $F_{1,35}$=0.52, p=0.477; interaction $F_{1,35}$=0.45, p=0.507) or time spent in the open arms (Fig. 4B, EE-LNG treatment: $F_{1,35}$=0.009, p=0.923; WIN treatment: $F_{1,35}$=1.63, p=0.209; interaction $F_{1,35}$=0.52, p=0.477). Likewise, no significant differences were observed in the number of closed arm entries (EE-LNG treatment: $F_{1,35}$=1.25, p=0.271; WIN treatment:

$F_{1,35}$=3.62, p=0.065; interaction $F_{1,35}$=0.02, p=0.873) or time spent in the closed arms (EE-LNG treatment: $F_{1,35}$=0.74, p=0.394; WIN treatment: $F_{1,35}$=0.38, p=0.543; interaction $F_{1,35}$=0.60, p=0.443) (data not shown). Overall, chronic treatment with EE-LNG and co-exposure with WIN did not affect anxiety-like behavior in female rats.

By contrast, co-exposure to HCs and cannabinoids altered depressive-like behavior in the FST. Two-way ANOVA for immobility (Fig. 4C), revealed a significant effect of EE-LNG treatment ($F_{1,35}$=5.09, p=0.030) and WIN treatment ($F_{1,35}$=5.59, p=0.024), but no interaction ($F_{1,35}$=0.42, p=0.522). Increased immobility was found following both EE-LNG and WIN treatments, although the only significant change revealed by the *post hoc* test (+36%, p=0.011) was between VEH/SAL-treated rats and EE-LNG/WIN-treated rats (Fig. 4C). For swimming (Fig. 4D), we found a significant effect of WIN treatment ($F_{1,35}$=6.52, p=0.015), but no effect of EE-LNG treatment ($F_{1,35}$=3.16, p=0.084) or their interaction ($F_{1,35}$=1.31, p=0.260), although the only significant change revealed by the *post hoc* test (-22%, p=0.018) was between VEH/SAL-treated rats and EE-LNG/WIN-treated rats (Fig. 4D). Finally, no change in climbing behavior (Fig. 4E) was observed among groups (EE-LNG treatment: $F_{1,35}$=2.13, p=0.154; WIN treatment: $F_{1,35}$=0.31, p=0.580; interaction $F_{1,35}$=2.18, p=0.149). Overall, concurrent exposure to HC and cannabinoid agonists seems to induce depressive-like behavior in female rats.

3.4 Chronic co-exposure to EE-LNG and WIN does not alter cognitive performance in the novel object recognition and prepulse inhibition tests

All groups displayed similar exploration of the novel object, both for time (Fig. 5A, EE-LNG treatment: $F_{1,35}$=0.06, p=0.806; WIN treatment: $F_{1,35}$=2.46, p=0.126; interaction $F_{1,35}$=2.39, p=0.131) and frequency (Fig. 5B, EE-LNG treatment: $F_{1,35}$=1.55, p=0.221; WIN treatment: $F_{1,35}$=0.16, p=0.695; interaction $F_{1,35}$=0.01, p=0.919) of investigation, suggesting that chronic treatment with EE-LNG and co-exposure with WIN does not affect recognition memory in female rats.

Likewise, all groups displayed a similar percentage of PPI (Fig. 5C, EE-LNG treatment: $F_{1,35}$=0.04, p=0.841; WIN treatment: $F_{1,35}$=0.75, p=0.392; interaction $F_{1,35}$=0.16, p=0.692), suggesting that chronic treatment with EE-LNG and co-exposure with WIN does not affect sensory-motor gating abilities.

3.5 Chronic treatment with EE-LNG, alone and plus WIN, decreases plasma progesterone level

As illustrated in Figure 6, two-way ANOVA for plasma progesterone levels revealed a significant effect of EE-LNG treatment ($F_{1,35}$=96.95, p<0.0001), but no effect of WIN treatment ($F_{1,35}$=2.75, p=0.106) and no interaction ($F_{1,35}$=2.54, p=0.119). As expected (Concas et al., 2022), chronic EE-LNG treatment markedly decreased plasma progesterone concentrations in SAL-treated rats (-85%, p<0.0001). Interestingly, WIN treatment *per se* was ineffective, and it did not alter the extent of EE-LNG-induced decrease in plasma progesterone levels (-81%, p<0.0001).

4. Discussion

There is a complex interplay between steroid hormones and cannabinoids, with endocannabinoid signaling being able to affect ovarian function in both physiological and pathophysiological states. This study investigated for the first time whether prolonged exposure to a cannabinoid agonist, at a dose regimen known to be rewarding in rats, may interact with HCs to alter behavior in female rats. Chronic HC administration inhibited ovulation, as shown by the marked suppression in circulating progesterone levels, as expected (Concas et al., 2022). Surprisingly, WIN did not affect both basal and the HC-induced decrease in plasma levels of this hormone. To our knowledge, an effect of WIN administration on plasma progesterone levels has never been reported. However, while acute THC administration increased plasma progesterone levels in male rats (Grobin et al., 2005), chronic administration of cannabinoid agonists reduces gonadotropin-releasing hormone secretion, ultimately suppressing gonadal progesterone (Gorzalka and Dang,

2012). Thus, WIN, at the low dose used in our experiments, may exert a less potent effect on peripheral progesterone levels.

The effect of chronic EE-LNG treatment and WIN co-exposure was examined on different domains as the prolonged use of both HCs (Concas et al., 2022) and cannabinoid receptor agonists (Howlett and Abood, 2017) are known to affect motor, cognitive and emotional domains. Contrary to our expectations, findings showed that combination of HCs and low doses of the cannabinoid agonist does not induce significant motor or cognitive alterations. Yet, it increased immobility time (with a concomitant reduction in swimming time) in the FST, suggesting a depressive-like state, while not affecting anxiety-like behavior.

The finding that motor activity was not significantly different in animals exposed to EE-LNG or WIN is in line with previous evidence showing that both HCs (Simone et al., 2015) and this cannabinoid regimen of exposure (Spano et al., 2013) *per se* do not significantly alter motor parameters in rats. Here, we demonstrated for the first time that the combination of the two pharmacological treatments also does not alter spontaneous motor activity, despite estrogen, progesterone and cannabinoid CB_1 receptors are all widely distributed in rat brain regions regulating motor systems. Importantly, lack of motor effects of the HC and cannabinoid chronic treatment excludes the possibility that animals' performance in the other behavioral tests may be impaired by nonspecific motor effects.

This study is the first to evaluate the effect of chronic treatment with EE-LNG, low rewarding doses of WIN, or their combinations, on burying behavior in female rats, and findings of no significant changes suggest that a prolonged exposure to HCs and/or a mild chronic stimulation of CB_1 receptors does not trigger obsessive-compulsive behavior or neophobia in rats. Yet, since ovarian hormones are known to modulate compulsive behavior in the signal attenuation rat model of obsessive-compulsive disorder (OCD) (Flaisher-Grinberg et al., 2009) and to affect symptom severity in OCD patients (Vulink et al., 2006), we expected to observe a significant effect of EE-LNG in the MB test. In support to our original hypothesis, LNG was found to be effective in contrasting OCD symptoms in a young woman (Perciaccante and Perciaccante, 1993). However, low doses of EE were found to be

anxiolytic in the shock-probe defensive burying, while higher doses increased freezing behavior in rats (Simone et al., 2015); moreover, LNG also did not affect burying behavior in female rats (Picazo et al., 1998), which is in line with our finding using EE-LNG, suggesting inconsistent effects of a specific HC treatment on this parameter, likely depending in part on the test used to measure burying behavior. Conversely, lack of significant effects of WIN in the MB test was not totally unexpected, since adolescent exposure to a higher dose of WIN (1.2 mg/kg/day, PND 30-43) was reported not to affect food neophobia in adult male rats (Schoch et al., 2018), while in humans cannabis use seems to ameliorate, rather than trigger, OCD symptoms (Szejko et al., 2020).

In line with data from the MB test, we did not find significant effects of EE-LNG and/or WIN in the EPM test. With respect to HCs, this result was unexpected since we previously observed anxiety-like behavior in the EPM test following chronic EE-LNG treatment in female rats (Porcu et al., 2012). However, those rats were only subjected to the EPM test, thus avoiding repeated manipulations and testing. Further, the different HC dose regimen might also contribute to the discrepant results in the EPM test; while higher EE and LNG doses (0.030 and 0.125 mg, respectively) were anxiogenic (Porcu et al., 2012), intermediate doses (0.030 and 0.060 mg, respectively) did not alter behavior, and lower doses (0.010 and 0.020, respectively) induced an anxiolytic effect in the same rat strain (Simone et al., 2015), suggesting that EE and LNG may affect anxiety-like behavior in a dose-dependent manner. Regarding WIN's effect on anxiety-like behavior in rats, there is a degree of inconsistency in the literature. WIN elicited anxiety in the EPM test when acutely administered at doses higher than those used in the present study (Komaki et al., 2015), an effect that was found to be partially more pronounced in pubertal than adult rats (Klugmann et al., 2011). However, other studies reported no effect of WIN (2 mg/kg, i.p.) on anxiety-like behavior in the EPM (Cassar et al., 2022). Importantly, anxiolytic effects were detected in rats that underwent both WIN self-administration and WIN passive (experimenter-given) administration at comparable self-administered doses when tested in the locomotor activity cages, where they constantly spent less time on the box margins (and more at the center) than controls (Spano

et al., 2013), which is evocative of a reduced level of anxiety. It is therefore possible that, similarly to the above-mentioned study, WIN treatment in our study might induce anxiolytic effects, although too mild to be detected in the EPM test.

The only significant effects induced by chronic exposure to the EE-LNG/WIN combination were detected in the FST, where we observed an increase in immobility time and a concomitant reduction in the time spent swimming, thus revealing a depressive-like behavior in animals that received the concomitant HC and cannabinoid treatments with respect to VEH/SAL-treated animals. However, neither EE-LNG nor WIN *per se* affected depressive-like behavior in the FST. With respect to HCs, we previously reported that chronic EE-LNG administration (0.030-0.125 mg, respectively) reduced immobility in the FST on test day (i.e., 24 hours following a pre-test session) (Santoru et al., 2014). However, increased immobility may be indicative of a passive behavior necessary to cope with the acute swim stress, rather than being suggestive of an antidepressant effect of HCs (Concas et al., 2022). The current results of lack of changes in immobility induced by chronic HC treatment suggest that these rats are not depressed, in line with lack of changes in anxiety-like behavior. This interpretation also agrees with lack of changes in anhedonia in the sucrose preference test following chronic EE-LNG treatment (Santoru et al., 2014). In line with our results, female rats exposed during adolescence (PND 35-45) to THC displayed depressive-like behavior in the FST, contrary to male rats that in the same test did not show behavioral despair (Rubino et al., 2008). Yet, no effects of either adolescent cannabis smoke or THC exposure in the FST was also reported (Bruijnzeel et al., 2019), while adulthood treatment with a more potent cannabinoid agonist (HU-210, 0.1 mg/kg), induced an antidepressant-like response in the FST (Morrish et al., 2009).

Prolonged exposure to EE-LNG in combination with WIN did not significantly affect animals' performance in the NOR test. Likewise, treatment with EE-LNG alone (i.e., plus saline) also had no effect, in line with our previous observation that chronic EE-LNG treatment did not affect recognition memory in the NOR and novel place location tests (Boi et al., 2022). Effects of EE-LNG on recognition memory in rats might be dose-dependent as lower doses

(10-20 µg) were found to impair, while a higher EE dose (30 µg) improved performance in the NOR test (Simone et al., 2015). These findings are not surprising as HCs' effects on cognition vary in both women and animals depending on the cognitive domains examined, length of treatment, doses and type of HC administered (Concas et al., 2022). Similarly, chronic treatment with the cannabinoid agonist did not induce recognition memory deficits, in line with our previous observation (Spano et al., 2010) but in contrast with other studies that observed persistent object recognition deficits after chronic pubertal WIN treatment (Schneider et al., 2008). Yet, the different experimental design may explain such a discrepancy, including the higher WIN dose (1.2 mg/kg administered intraperitoneally once/daily vs. a cumulative dose of 0.3 mg/kg achieved after 24 intravenous infusions of WIN 12.5 µg/kg over a 2-h daily session), and the earlier age window for WIN treatment (PND 40-65 vs. PND 56-95). Actually, the age at which WIN exposure occurs appears particularly critical, given that when the same WIN schedule is administered to adult rats (PND>70), chronic cannabinoid exposure does not alter behavior (Schneider and Koch, 2003).

Finally, we showed for the first time that chronic EE-LNG treatment, both alone and in combination with chronic WIN, does not alter PPI in female rats, in line with previous studies showing no change in PPI in rats with different levels of ovarian hormones (i.e., intact, ovariectomized, and ovariectomized rats treated with 17β-estradiol) (Vaillancourt et al., 2002) or exposed to a similar cannabinoid chronic treatment (Spano et al., 2010). However, our finding is in contrast with previous studies showing that estrogenic compounds are able to affect baseline PPI in rats (Sbisa et al., 2018). Indeed, human studies reported discrepant findings on HCs' effects on the prepulse inhibition and startle responsiveness, with a recent study describing HC-related PPI differences (Naysmith et al., 2022), while Gogos (2013) did not observe significant PPI differences between HC and non-HC users. To complicate the issue, Beck et al. (2008) reported facilitated fine motor coordination and startle responsiveness in women taking HC, Holloway et al. (2011) found that PPI was lower in young HC users depending on stimulus intensity, and Borgström et al. (2008) reported lower

PPI in HC users with negative mood in comparison to users without negative mood, positing that PPI differences may result from HC-related negative affect, rather than HC use *per se*. The finding that WIN does not impair sensorimotor gating in rats is further supported by a study showing that acute, subchronic, and chronic treatment with WIN did not affect PPI in rats (Bortolato et al., 2005), although discrepant findings were also reported after prepubertal and pubertal prolonged exposure to higher WIN doses, i.e., 1.2 vs. 0.3 mg/kg (Schneider and Koch, 2003).

We are aware that the current study is not without limitations. Firstly, we cannot rule out the possibility that different estrogen-progestin combinations, doses and route of administration might elicit a different outcome. Likewise, we cannot rule out the possibility that HCs and rewarding doses of cannabinoids might alter behavior in other species, including humans or their effects might be age-dependent, being more pronounced in young adolescents, an age window that, due to its short duration, is difficult to capture in rats subjected to long-term chronic drug treatments.

5. Conclusions

We showed that co-exposure to HCs and low but rewarding doses of cannabinoids does not significantly alter the motor and cognitive skills and does not induce anxiety in female rats, but it may induce a depressive-like state. These findings allow deepening the current knowledge on the interaction between cannabinoid agonists and hormonal contraceptives. Future studies are needed to further investigate the mechanisms behind the depressive-like effect and its implications for women's health.

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Author Contributions Statement

LF, AC and PP: Conceptualization; LF, AP, LC, CC, CS, PP: Investigation; LF, AP, MGP, PP: Data curation and formal analysis; MS: Resources; LF, PP: Funding acquisition, Project administration, Supervision and Writing - original draft; LF, AP, LC, CC, CS, MGP, MS, AC, PP: Writing - review & editing.

Author Confirmation Statement

The manuscript has been read and approved by all authors.

Author Disclosure Statement

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Timeline of the experimental procedures. Upon arrival at postnatal day (PND) 32-35, rats were allowed to acclimate before undergoing surgery for catheter insertion into the jugular vein. Starting on PND 52 they received daily subcutaneous injections of the EE-LNG combination (0.020 and 0.060 mg, respectively). On PND 56 they started daily infusions of WIN 55,212-2 (12.5 µg/kg). Behavioral tests took place between PND 84 and PND 95 in the following order: locomotor activity (LA, PND 84), novel object recognition (NOR, PND 86), forced swimming (FST, PND 88), marble burying (MB, PND 91), elevated plus maze (EPM, PND 93), and prepulse inhibition (PPI, PND 95). Rats were sacrificed on PND 98 to collect plasma samples for determination of progesterone levels.

Figure 2. Effect of long-term EE-LNG treatment and co-exposure to WIN 55,212-2 on locomotor activity. Spontaneous locomotor activity was assessed on PND 84 and horizontal activity (A), vertical activity (B), and total distance travelled (C), were collected every 10 minutes over the 60 minutes test. Horizontal and vertical activities are expressed as mean counts of photobeam interruptions; distance travelled is in cm. Data represent the mean ± SEM of values from 9 (Vehicle + Saline), 11 (Vehicle + WIN), 8 (EE-LNG + Saline) or 11 (EE-LNG + WIN) rats/group. *p<0.05, **p<0.01, ***p<0.0001 vs. the respective 10 minutes value for every experimental group (repeated measures ANOVA followed by Tukey's *post hoc* test; exact p values are shown in Supplementary Table 2).

Figure 3. Effect of long-term EE-LNG treatment and co-exposure to WIN 55,212-2 in the marble burying test. Neophobia/compulsive behavior was assessed in the marble burying test on PND 91. Data are reported as number of marbles fully (>95%, A) and partially (>67%, B) buried. Panel C illustrates the total number of marbles that triggered burying behavior (fully + partially buried). Each bar represents the mean ± SEM of values

from 9 (Vehicle + Saline), 11 (Vehicle + WIN), 8 (EE-LNG + Saline) or 11 (EE-LNG + WIN) rats/group.

Figure 4. Effect of long-term EE-LNG treatment and co-exposure to WIN 55,212-2 on anxiety- and depressive-like behavior. Anxiety-like behavior was assessed in the elevated plus maze test on PND 93. Data are reported as number of entries (A) and time spent (B) in the open arms of the maze. Depressive-like behavior was assessed in the forced swimming test on PND 88. Data are reported as time (seconds) spent in immobility (C), swimming (D) and climbing (E) behavior. Each bar represents the mean ± SEM of values from 9 (Vehicle + Saline), 11 (Vehicle + WIN), 8 (EE-LNG + Saline) or 11 (EE-LNG + WIN) rats/group. *p<0.05, two-way ANOVA followed by Tukey's *post hoc* test.

Figure 5. Effect of long-term EE-LNG treatment and co-exposure to WIN 55,212-2 on cognitive performance in the novel object recognition and prepulse inhibition tests. Recognition memory was assessed in the novel object recognition (NOR) test on PND 86. Data are reported as percent time (A) and percent frequency (B) of exploration of the novel vs. familiar object. Prepulse inhibition (PPI) was assessed on PND 95 and data are reported as percent PPI (C). Each bar represents the mean ± SEM of values from 9 (Vehicle + Saline), 11 (Vehicle + WIN), 8 (EE-LNG + Saline) or 11 (EE-LNG + WIN) rats/group.

Figure 6. Effect of long-term EE-LNG treatment and co-exposure to WIN 55,212-2 on plasma progesterone levels. Progesterone levels were measured in plasma samples collected on PND 98 at the end of all behavioral tests. Data are expressed as ng/ml and each bar represents the mean ± SEM of values from 9 (Vehicle + Saline), 11 (Vehicle + WIN), 8 (EE-LNG + Saline) or 11 (EE-LNG + WIN) rats/group. *p<0.0001, two-way ANOVA followed by Tukey's *post hoc* test.

Figure 1

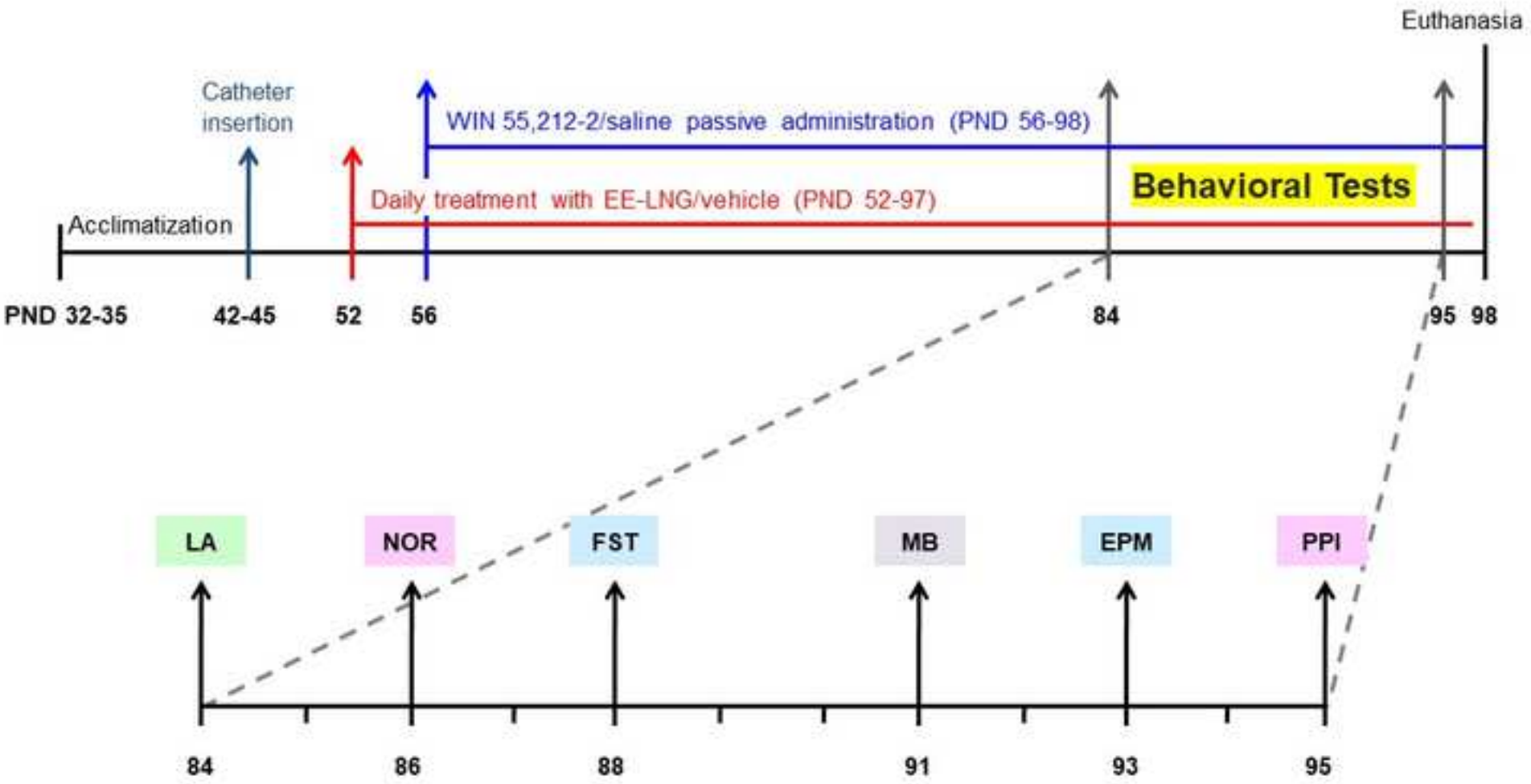


Figure 2

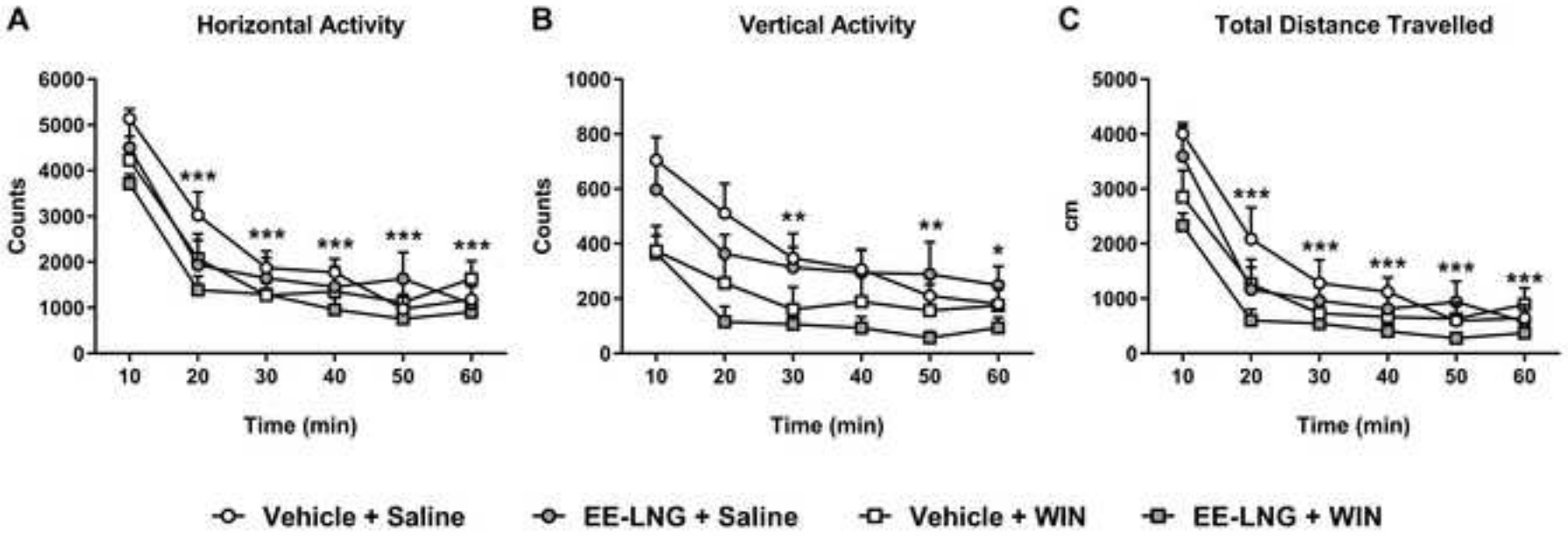
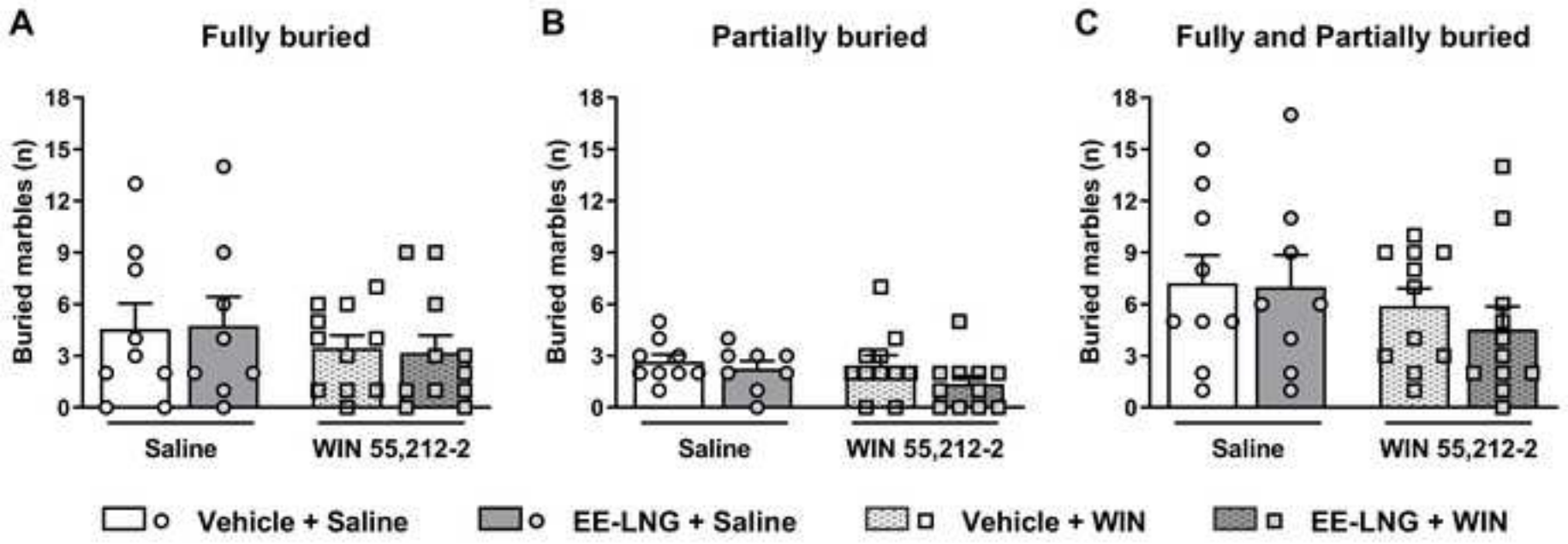


Figure 3



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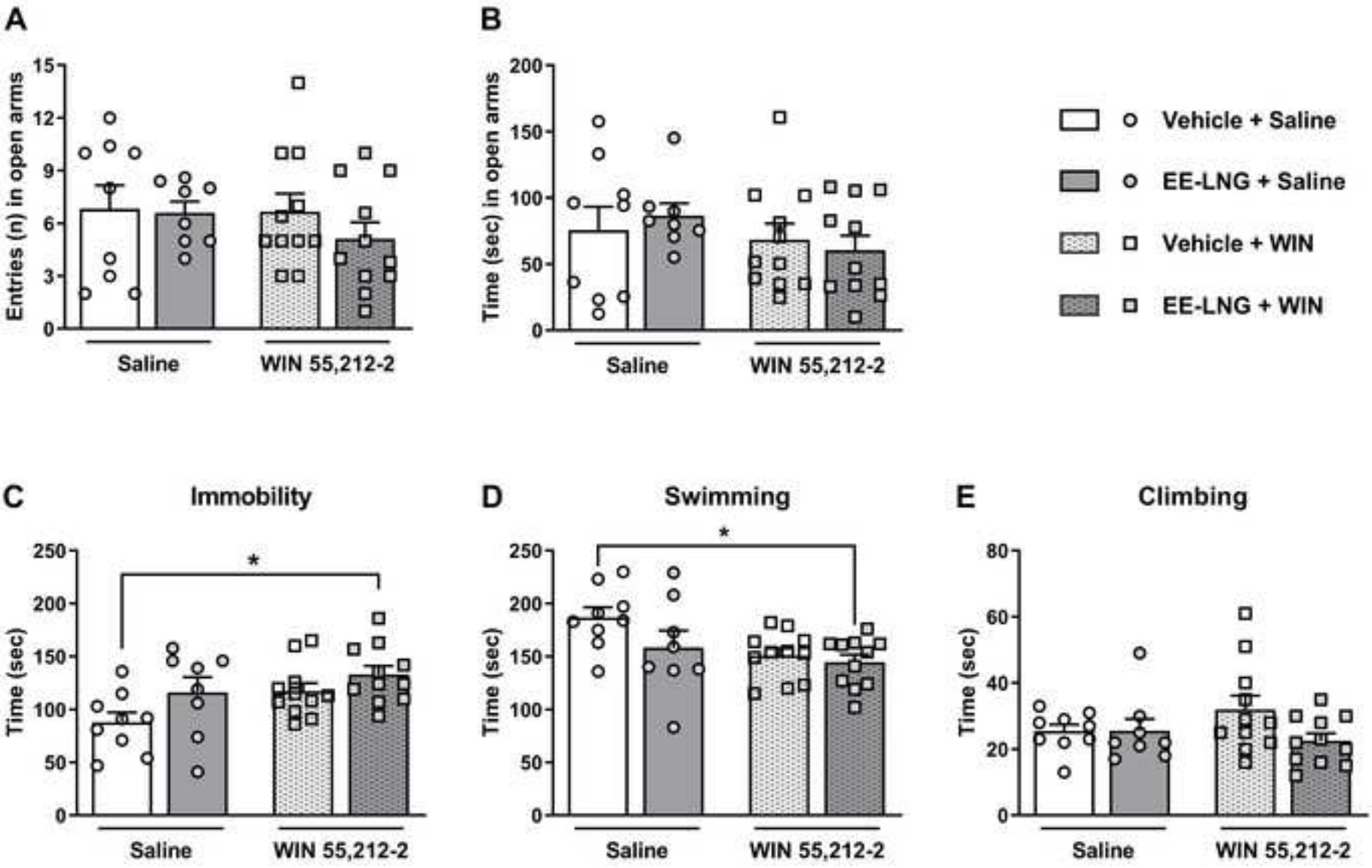


Figure 5

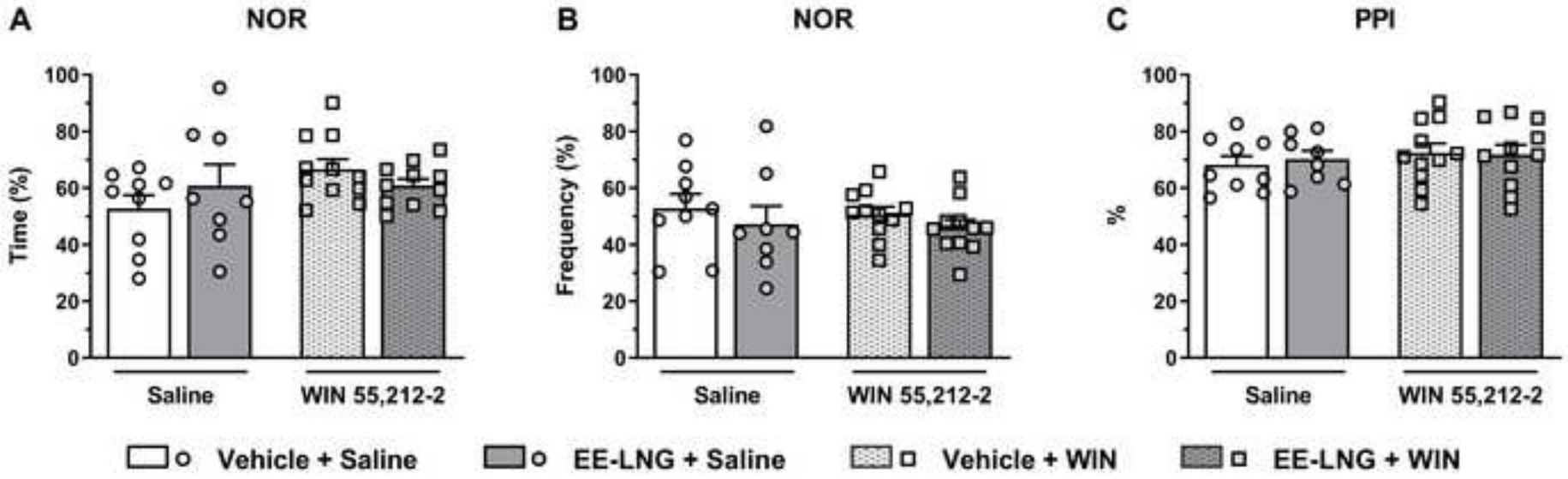
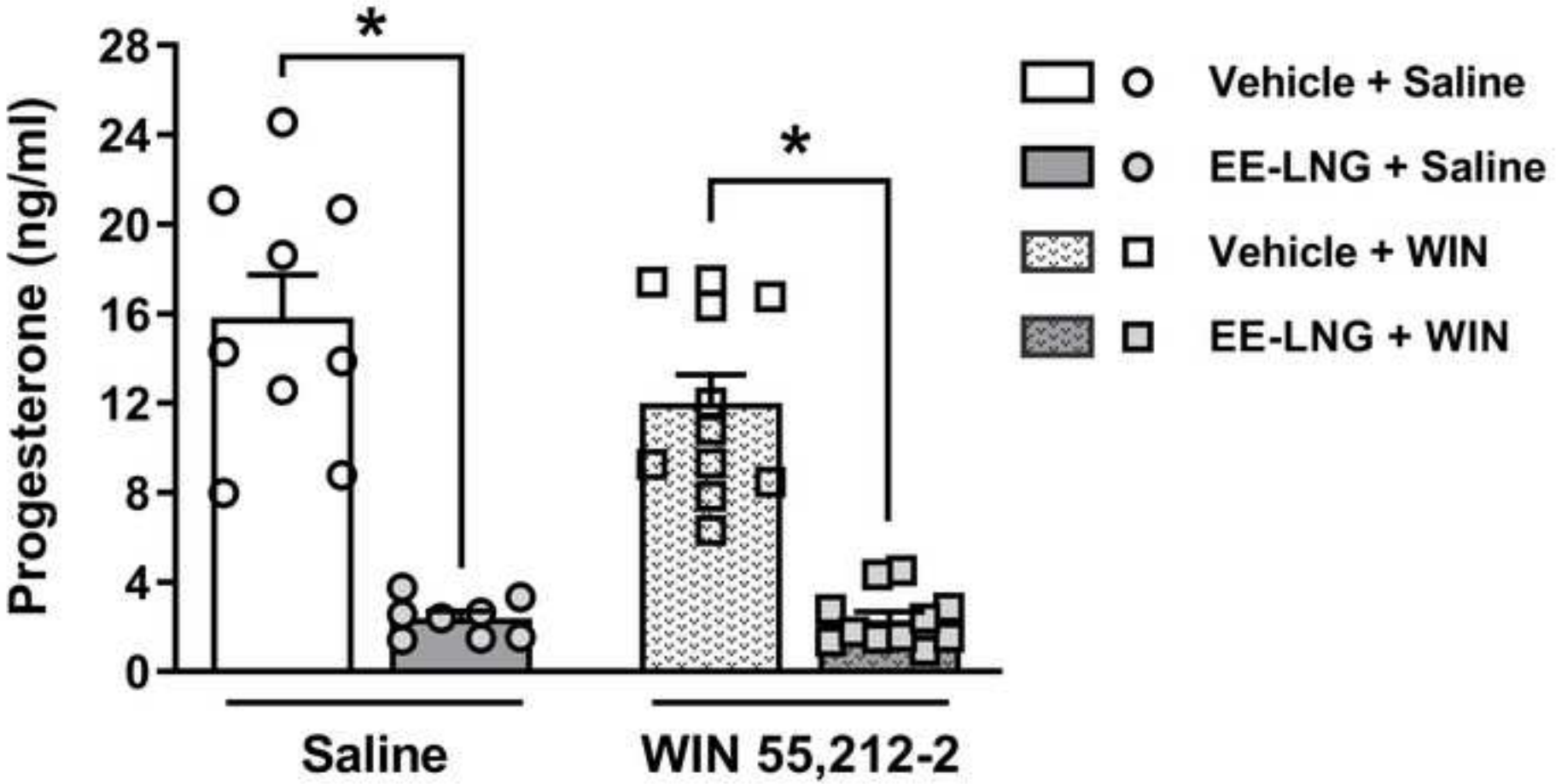


Figure 6



Author Disclosure Statement

The authors declare that they have no conflicts of interest.

Ethical Statement

The authors declare that the manuscript is based on an original research. All animal experiments comply with the ARRIVE guidelines and all experimental procedures were conducted according to the European Parliament and the Council Directive 2010/63/EU and the Italian Legislative decree n.26, 4 March 2014, and were approved by the Italian Ministry of Health.

Supplementary Table 1.

Detailed results for the two-way ANOVA repeated measures for each parameter assessed in the locomotor activity test.

Effect	Horizontal activity	Vertical activity	Total distance travelled
EE-LNG treatment	$F_{1,35}$=1.19; p=0.281	$F_{1,35}$=0.53; p=0.470	$F_{1,35}$=1.65; p=0.208
WIN treatment	$F_{1,35}$=1.92; p=0.174	**$F_{1,35}$=6.56; p=0.015**	$F_{1,35}$=3.62; p=0.065
EE-LNG x WIN	$F_{1,35}$=0.06; p=0.812	$F_{1,35}$=0.14; p=0.708	$F_{1,35}$=0.07; p=0.800
Time	**$F_{5,175}$=99.96; p<0.00001**	**$F_{5,175}$=41.90; p<0.00001**	**$F_{5,175}$=99.43; p<0.00001**
Time x EE-LNG	$F_{5,175}$=2.11; p=0.067	$F_{5,175}$=1.61; p=0.160	$F_{5,175}$=1.75; p=0.126
Time x WIN	$F_{5,175}$=1.92; p=0.093	**$F_{5,175}$=3.53; p=0.005**	**$F_{5,175}$=4.25; p=0.001**
Time x EE-LNG x WIN	$F_{5,175}$=1.24; p=0.292	$F_{5,175}$=1.74; p=0.127	$F_{5,175}$=0.84; p=0.524

Supplementary Table 2.

Exact p values for the Tukey *post hoc* analysis for each parameter assessed in the locomotor activity test.

	Vehicle+Saline	EE-LNG+Saline	Vehicle+WIN	EE-LNG+WIN
Horizontal activity				
20 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
30 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
40 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
50 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
60 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
Vertical activity				
20 min	*p=0.111*	p=0.024	*p=0.841*	p=0.0004
30 min	p=0.00002	p=0.0008	p=0.009	p=0.0002
40 min	p=0.00002	p=0.0002	*p=0.064*	p=0.00006
50 min	p=0.00002	p=0.0001	p=0.006	p=0.00002
60 min	p=0.00002	p=0.00002	p=0.025	p=0.00006
Total distance travelled				
20 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
30 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
40 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
50 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002
60 min	p=0.00002	p=0.00002	p=0.00002	p=0.00002