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Sex-specific behavioral deficits induced at early life by prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 depend on mGlu5 receptor signaling

Running title: Sex specific cannabinoid-induced behavioral deficits and mGlu5R

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Abstract

Background and Purpose: Cannabis sativa is the illicit drug most commonly used among pregnant and breastfeeding women. Different studies reported long-term adverse effects induced by in utero exposure to the main component of Cannabis, Δ^9-tetrahydrocannabinol (THC), both in rodents and humans. However, little is known about any potential sex-dependent effects of cannabis consumption during pregnancy on newborns at early developmental ages.

Experimental Approach: We studied the effects of prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 (WIN; 0.5 mg/kg from GD5 to GD20) on the emotional reactivity and cognitive performance of male and female rat offspring from infancy through adolescence and tested the role of mGlu5 receptor signaling in the observed effects.

Key Results: Prenatally WIN-exposed male infant pups emitted less isolation-induced ultrasonic vocalizations (USVs) compared with male control pups when separated from the dam and siblings and showed increased locomotor activity, while females were spared. These effects were normalized when male pups were treated with the positive allosteric modulator of mGlu5 receptor CDPPB. When tested at the prepubertal and pubertal periods, WIN-prenatally exposed rats of both sexes did not show any difference in social play behavior, anxiety and temporal order memory.

Conclusion and Implications: We reveal a previously undisclosed sexual divergence in the consequences of fetal cannabinoids on newborns at early developmental ages, which is dependent on mGlu5 receptor signaling. These results provide new impetus for the urgent

need to investigate the functional and behavioral substrates of prenatal cannabinoid exposure in both the male and female offspring.

Keywords: in utero cannabis, ultrasonic vocalizations, social play, elevated plus-maze, temporal order memory, mGlu5 receptors.

Abbreviations: WIN, the cannabinoid receptor agonist WIN 55,212-2; CTRL, control; GD, gestational day; PND postnatal day; USVs, Isolation-induced ultrasonic vocalizations; % TO, percentage of time spent in the open arms; % OE, percentage of open arm entries; SAP, number of stretched-attend postures; HDIPS, number of exploratory head dips; SEM, standard error of mean; VEH, vehicle; CDPPB, the positive allosteric modulator of mGlu5 receptors.

Bullet point summary:

What is already known?

- Cannabis sativa is the illicit drug most commonly used among pregnant and breastfeeding women and the long-term adverse effects induced by in utero cannabis have been described both in rodents and humans.
- However, the majority of these studies have been conducted exclusively in the male progeny

What this study adds?

- We demonstrate for the first time that fetal exposure to cannabinoids causes sex-specific, mGlu5-related behavioral alterations in the progeny at early developmental periods.
- Our results provide new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabinoid exposure in both the male and female offspring.

Clinical significance

- The dissemination of our results represents a means of preventative education for women using marijuana while pregnant.
- Our results provide new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabis exposure in both sexes.

Introduction

Cannabis sativa (marijuana) is the illicit drug most commonly used among pregnant and breastfeeding women (Brown, 2017; SAMHSA, 2013; Scheyer, 2019). The main active principle of cannabis, Δ^9-tetrahydrocannabinol (THC), enters maternal circulation and readily crosses the placenta (Hutchings *et al.*, 1989). Thus, prenatal cannabis exposure might exert deleterious effects on the fetus. Nowadays, the legalization of medical and recreational cannabis is increasing throughout the United States and many other Countries debate on its possible legalization. In this context, rigorous scientific research about the impact of cannabis use on health and well-being becomes more important than ever.

Human studies have provided invaluable information on the detrimental effects of prenatal cannabis exposure on the offspring from the neonatal period through early adulthood (Crume *et al.*, 2018; El Marroun *et al.*, 2018; Huizink, 2014; Ryan *et al.*, 2018), revealing increased tremors, startles and altered sleep patterns at birth (Calvigioni *et al.*, 2014; Volkow *et al.*, 2017) and significant impairment of higher cognitive functions beyond infancy (Fried, 2002; Fried *et al.*, 1998; Grant *et al.*, 2018; Huizink *et al.*, 2006; Leech *et al.*, 1999; Passey *et al.*, 2014; Smith *et al.*, 2006). However, one weakness of human studies is that they cannot control for environmental and genetic factors. Therefore, a wide array of animal studies has been performed to better evaluate the contribution of prenatal cannabis to adverse, even subtle neurodevelopmental consequences in the offspring (Grant *et al.*, 2018; Trezza *et al.*, 2012).

The endocannabinoid system plays a relevant role in a broad spectrum of neurodevelopmental processes: notably, CB1 cannabinoid receptors, already functional around gestational days (GD) 11-14 in rats, are involved in embryonal implantation, neural development and control of synaptic communication (Berghuis *et al.*, 2007; Harkany *et al.*, 2007). Pioneering animal studies have demonstrated specific deficits in prenatally cannabis-

exposed rodent offspring at different developmental periods (Grant *et al.*, 2018; Richardson *et al.*, 2016; Trezza *et al.*, 2012). Interestingly, some of the behavioral deficits displayed by rodents prenatally exposed to cannabinoids have been related to changes in brain glutamatergic neurotransmission (Antonelli *et al.*, 2004; Antonelli *et al.*, 2005; Castaldo *et al.*, 2007; Mereu *et al.*, 2003).

Noteworthy, the majority of these studies were conducted exclusively in the male progeny. Therefore, an urgent need exists to understand the effects of prenatal cannabis exposure also in female progeny. Pioneering preclinical and clinical studies reported sexually-dimorphic responses to cannabinoids when administered during the gestational and/or early postnatal periods (Navarro *et al.*, 1995; Vela *et al.*, 1998; Wang *et al.*, 2006; Wang *et al.*, 2004). Recently, our laboratories also revealed a previously undisclosed sexual divergence in the consequences of fetal cannabinoids at adulthood: prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 (WIN) reduced social interactions in adult male but not female rats and altered neuronal excitability and synaptic plasticity in the prefrontal cortex of male rats only. These deficits were paralleled by decreased levels of mGlu5R mRNA. Amplifying mGlu5R signaling with a positive allosteric modulator of mGlu5R normalized the social and synaptic deficits displayed by male WIN-exposed rats (Bara *et al.*, 2018).

Based on these findings, this study follows up our recent report showing sex-dependent effects of in utero cannabinoid exposure at adulthood and aimed to test the effects of prenatal exposure to WIN in the male and female infant and prepubertal and pubertal rat offspring, to evaluate possible sex-dependent effects induced by in utero WIN exposure on emotional reactivity and cognitive performance at developmental ages earlier than adulthood. The interaction between cannabinoid and mGlu5R has been extensively explored by using pharmacological, electrophysiological and anatomical approaches (Araque *et al.*, 2017; Jung *et al.*, 2012; Katona *et al.*, 2008; Lafourcade *et al.*, 2007; Liang *et al.*, 2014; Won *et al.*, 2012). Importantly, mGlu5R partecipate in the developmental regulation of the endocannabinoid system: indeed, the developmentally dependent increase in endocannabinoid mobilization (that occurs between the neonatal and juvenile stages) correlates with increases in the levels of protein expression of mGlu5R (Liang *et al.*, 2014). Based on this evidence and our recent findings on the interaction between cannabinoid and mGlu5R in modulating behavioral and synaptic states in the context of nutrition (Manduca *et al.*, 2017) and in utero cannabinoid exposure (Bara *et al.*, 2018), we here investigated whether the positive allosteric modulator of mGlu5R CDPPB normalized the behavioral deficits induced by in utero cannabinoid exposure.

Methods

Animals

Wistar (RGD_13508588) female rats (Charles River, Italy), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day 0 (GD0). Pregnant rats were singly housed in Macrolon cages (40 × 26 × 20 cm), under controlled conditions (temperature 20-21 °C, 55-65% relative humidity and 12/12 h light cycle with lights on at 07:00 a.m.). Food and water were available ad libitum. The synthetic cannabinoid receptor agonist WIN55,212-2 (WIN, 0.5 mg/kg) was administered to the dams subcutaneously (s.c.) daily from GD 5 to GD 20 (WIN group, n=27). Control dams (CTRL group, n=26) received a similar volume injection of the vehicle solution. Newborn litters found up to 05:00 p.m. were considered to be born on that day (postnatal day (PND) 0). On Postnatal day (PND) 1, litters were culled to four males and four females. On PND 21, pups were weaned and housed in groups of three. The experiments were carried out on the male and female offspring at three different developmental ages: 1. infancy (PNDs 10 and 13); 2. prepubertal period (PND 28-35 for males and PND 22-28 for females) and 3. puberty (PND 50-60 for males and PND 30-40 for females offspring). Puberty corresponds to vaginal opening and first ovulation (i.e. around 5 weeks) for female and preputial separation for male rats (Beckman *et al.*, 2003; Korenbrot *et al.*, 1977; Schneider, 2013).

To avoid the so called "litter effects", one pup of both sexes per litter from different litters per treatment group was randomly used in each experiment (CTRL= 118 males and 89 females or WIN= 123 males and 87 females) as described in the "Handbook of Behavioral Teratology (CV Vorhees); Developmental and Reproductive Toxicology: A Practical Approach (RD Hood". For power analysis, sample size (n) was based on our previous experiments and power analysis with the software GPower. Potential outliers within each data set were calculated using the GraphPad software. Sample size is indicated in the figure legends and represented in the figures as scatter dot plot. All behavioral tests were assessed by a trained observer who was unaware of treatment condition to reduce performance bias. Reproduction data including body weights of the dams (calculated from GD 1 to GD 21 and expressed as body weight gain in percentage) and the length of pregnancy, the litter size, weight gains of pups and postnatal viability (calculated as the number of live animals of both sexes at PND 21 (i.e. weaning) / the number of live animals of both sexes at PND 1 in percentage) were also measured.

The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) (Kilkenny *et al.*, 2010) guidelines, with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

Drug treatment

WIN 55,212-2 mesylate ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone) (WIN, Sigma, Italy and National Institute of Mental Health, USA) was suspended in 5% polyethylene glycol, 5% Tween 80, and 90% saline and given subcutaneously (s.c.) at a volume of 1 ml/kg to the gestating dams. The dose of WIN used in this study (0.5 mg/kg) has been estimated to correspond to a moderate, or even to a low, exposure to cannabis in humans (Antonelli *et al.*, 2004; Compton *et al.*, 1992; French *et al.*, 1997), and it does not induce any sign of toxicity and/or gross malformations in the rat offspring (Mereu *et al.*, 2003). The positive allosteric modulator of mGlu5 receptors CDPPB (3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl) benzamide) (National Institute of Mental Health, USA) was dissolved in 5% Tween 80/5% polyethylene glycol/saline and given intraperitoneally (i.p.) at the dose of 1.5 mg/kg 30 min before testing to offspring. Drug doses and pre-treatment intervals were based on previous work and pilot experiments. Solutions were freshly prepared on the day of the experiment and were administered in a volume of 2.5 ml/kg to offspring.

Behavioral tests

Isolation-induced ultrasonic vocalizations (USVs)

USVs are emitted by rodent pups when removed from the nest and play an important communicative role in mother-offspring interactions (Manduca *et al.*, 2012). On PND 10, the isolation-induced USVs emitted by CTRL- and WIN-exposed pups were recorded as previously described (Antonelli *et al.*, 2005; Melancia *et al.*, 2018). Briefly, pups were individually removed from the nest and placed into a black Plexiglas arena (30 cm × 30 cm), located inside a sound-attenuating and temperature-controlled chamber. Pup USVs were detected for 15 sec by an ultrasound microphone (Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies between 10 and 200 kHz and fixed at 15 cm above the arena and analyzed quantitatively (numbers of calls/15 sec).

Homing behavior

The homing behavior test exploits the tendency of immature rodent pups to maintain body contact with the dam and siblings and to discriminate their own home cage odor from a neutral odor, which is an early indicator of social discrimination (Bignami, 1996). The homing behavior test was performed as previously described (Servadio *et al.*, 2018). Briefly, on PND 13 the litter was separated from the dam and kept for 30 min in a temperature-controlled holding cage. Then, each pup was placed into a Plexiglas box whose floor was covered for 1/3 with bedding from the pup's home cage, and for 2/3 with clean bedding. The pup was located at the side of the box covered by clean bedding, and its behavior was video recorded for 4 min for subsequent analysis. The following parameters were scored by an observer, unaware of animal treatment, using the Observer 3.0 software (Noldus, The Netherlands): latency (sec) to reach the home-cage bedding area; total time (sec) spent by the pup in the nest bedding area; total number of entries into the nest bedding area; locomotor activity, expressed as the total number of crossings in the test box.

Social play behavior

Social play behavior is one of the earliest forms of non-mother-directed social behavior very abundant during the juvenile phases of life in mammalian species, including rats (Vanderschuren *et al.*, 2016). The test was performed as previously described (Manduca *et al.*, 2016). Prepubertal and pubertal rats were individually habituated to the test cage for 10 min on each of the 2 days before testing. The test was performed between 9 a.m. and 2 p.m. under low light condition and consisted of placing the animal together with a similarly treated partner into the test cage for 15 min. Behavior was assessed per pair of animals and analyzed by a trained observer who was unaware of the treatment condition to reduce performance bias, using the Observer 3.0 software (Noldus Information Technology, The Netherlands). Both animals in a test pair had received the same treatment during gestation (CTRL- or WIN- in utero). Animals in a test pair did not differ by >10 g in body weight and had no previous common social experience (i.e., they were not cage mates).

In rats, a bout of social play behavior starts with one rat soliciting ('pouncing') another animal, by attempting to nose or rub the nape of its neck. If the animal that is pounced upon fully rotates to its dorsal surface, 'pinning' is the result (one animal lying with its dorsal surface on the floor with the other animal standing over it), which is considered the most characteristic posture of social play behavior in rats (Pellis *et al.*, 2009).

We determined: 1. frequency of pinning; 2. frequency of pouncing; 3. time spent in social exploration (i.e. the total amount of time spent in non-playful forms of social interaction, like sniffing any part of the body of the test partner, including the anogenital area, or grooming any part of the partner body).

Elevated plus-maze test

The elevated plus-maze apparatus comprised two open (50 × 10 × 40 cm3; l × w × h) and two closed arms (50 × 10 × 40 cm3; l × w × h) that extended from a common central platform (10 × 10 cm2). The test was performed as previously described (Manduca *et al.*, 2015; Trezza *et al.*, 2008). Rats were individually placed on the central platform of the maze for 5 min. Each 5-min session was recorded with a camera positioned above the apparatus for subsequent behavioral analysis carried out an observer, unaware of animal treatment, to reduce performance bias, using the Observer 3.0 software (Noldus, The Netherlands).The following parameters were analyzed:

• % time spent in the open arms (% TO): (seconds spent on the open arms of the maze/seconds spent on the open + closed arms) × 100. Time on the open quadrants was timed from the moment all four paws of the rat were placed on an open section and ended when all four paws re-entered a closed quadrant;

• % open arm entries (% OE): (the number of entries into the open arms of the maze/number of entries into open + closed arms) × 100;

• Number of closed arm entries;

• Number of stretched-attend postures (SAP) made from the exit of a "closed" quadrant towards an "open" quadrant. This exploratory posture can be described as a forward elongation of the body, with static hind-quarters, followed by a retraction to the original position.

• Number of exploratory head dips (HDIPS) made over the edge of the platform, either from the exit of the "closed" quadrant, or whilst on the "open" quadrant;

Temporal order memory test

Animals were habituated to the experimental arena (40 × 40 cm) without objects for 10 min daily for 2 d before testing. This task consisted of two sample phases and one test trial (Barker *et al.*, 2007; Manduca *et al.*, 2017). In each sample phase, rats were allowed to explore two copies of an identical object for a total of 4 min. Different objects were used for sample Phases 1 and 2, with a delay between the sample phases of 1 h. After 3 h from sample

Phase 2, rats performed the test trial (4 min duration) where a third copy of the objects from sample Phase 1 and a third copy of the objects from sample Phase 2 were used. The positions of the objects in the test and the objects used in sample Phase 1 and sample Phase 2 were counterbalanced between the rats. An intact temporal order memory requested the subjects to spend more time exploring the object from Sample 1 (i.e., the object presented less recently) compared with the object from Sample 2 (i.e., the "new" object). The discrimination ratio was calculated as the difference in time spent by each animal exploring the object from sample Phase 1 compared with the object from sample Phase 2 divided by the total time spent exploring both objects in the test phase. Negative discrimination means that animals investigated more the object in Phase 2 than the object in Phase 1. Each 4-min session was recorded with a camera positioned above the apparatus for subsequent behavioral analysis carried out an observer, unaware of animal treatment, to reduce performance bias, using the Observer 3.0 software (Noldus, The Netherlands).

Statistical analysis

Data are expressed as mean ± SEM and adhere to BJP guidelines. To assess the effects of the prenatal treatments (WIN or CTRL) in the male and female offspring, the behavioral data were analyzed by Two-way ANOVA, with treatment and sex as factors. Two-way ANOVA was also used to assess the effects of prenatal (WIN or CTRL) and postnatal (CDPPB or vehicle) treatments. Three-way ANOVA was also used to assess the effects of prenatal (WIN or CTRL) treatments in both male and female offspring depending on the different developmental ages (prepubertal and pubertal periods). To assess the effects on reproduction data, the data were analyzed by using Student's t-test (WIN or CTRL). Statistical significance was set at $p < 0.05$ with no further distinction made for $p < 0.01$ and $p< 0.001$. If main or interaction effects were significant, the Student–Newman–Keuls post hoc test was used for individual group comparisons. The software Sigma Plot (RRID:SCR_003210) and GraphPad Prism (RRID:SCR_002798) were used for statistical analysis of the data. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Random allocation of animals to treatment groups and to behavioral tasks and blinding of investigators assessing outcomes were adopted to reduce selection and detection bias in our trials.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Reproduction data

No differences in body weight gains were observed between WIN- and CTRL-treated dams. Prenatal WIN exposure did not affect pregnancy length, litter size at birth, postnatal viability and pup weight gain at different developmental ages (Table 1).

Prenatal exposure to the cannabinoid receptor agonist WIN caused sex-dependent deficits in social communication and locomotion in the infant rat offspring

Prenatally WIN-exposed male pups emitted less USVs at infancy (PND 10) when separated from the dam and siblings compared with male CTRL-pups (Figure 1: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$<0.05]). Interestingly, the deleterious effects of in utero WIN exposure on USVs were specific to the male offspring. Post hoc analysis revealed that prenatally-WIN exposed female pups showed no difference in the rate of USVs at PND 10 when compared to age-matched females from CTRL-group (Figure 1) suggesting that prenatal exposure to the cannabinoid WIN causes sex-dependent deficits in early social communication of the offspring.

When tested in the homing behavior test at PND13, male and female pups prenatally exposed to WIN did not differ from control animals in the latency to reach the nest arena (Figure 2A: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s]), in the total time spent in the nest zone (Figure 2B: [$p_{(sex)}$=n.s; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and in the number of entries in the nest zone (Figure 2C: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]). However, the frequency of crossing in the test arena was increased specifically in the male WIN-exposed offspring, while WIN-exposed females were spared (Figure 2D: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$<0.05]), suggesting a sex-dependent detrimental effects induced by prenatal cannabinoid exposure on early life locomotion.

Prenatal exposure to the cannabinoid receptor agonist WIN had no effect on social play, anxiety-like behaviors and temporal order memory in the prepubertal progeny

When tested at prepubertal period, WIN- and CTRL-prenatally exposed rats did not show any difference in social play behavior (Figure 3A-B). A detailed analysis of the various social play parameters revealed that pinning (Figure 3A: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and pouncing (Figure 3B: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) frequencies were similar between WIN- and CTRL- animals of both sexes. Moreover, the time spent in general social exploration (including non-playful forms of social interaction, like sniffing) was unchanged during the 15-min session ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not showed).

To test whether prenatal cannabinoid exposure induced deficits in emotional control and cognitive abilities, we tested WIN- and CTRL offspring of both sexes for their anxiety-like behavior and temporal order memory. No differences between WIN- and CTRL prenatally exposed prepubertal rats were found in the elevated plus-maze. Specifically, there was no change in the percentage of time spent in the open arms of the maze (Figure 3C: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s.]), in the percentage of open arm entries (Figure 3D: $p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s.]) and in the number of closed-arm entries (considered as a measure of locomotion in the maze) ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown). Also the SAP ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown), and the HDIPS ([$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s.], data not shown) were not influenced by in utero WIN exposure in neither the male nor the female offspring.

Regarding their cognitive abilities, prepubertal CTRL and WIN-exposed animals of both sexes displayed identical discrimination index (Figure 3E: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and exploration time (Figure 3F: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) in the temporal order memory task. Thus, prenatal WIN exposure did not induce deficits in social play, anxiety-like behaviors and temporal order memory in the progeny of both sexes at the prepubertal period.

Prenatal exposure to the cannabinoid receptor agonist WIN did not induce deficits in social play, anxiety-like behaviors and changes in temporal order memory in the pubertal progeny

When tested in the pubertal period, no differences in social play behavior were found between WIN- and CTRL prenatally exposed rats. Thus, pinning (Figure 4A: [$p_{(sex)}$<0.05; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and pouncing (Figure 4B: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) frequencies were similar in WIN- and CTRL- animals in both sexes, suggesting no main

effects of in utero cannabinoid exposure on social play behavior at pubescence. Moreover, the time spent in general social exploration (including non-playful forms of social interaction, like sniffing) was unchanged during the 15-min session ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not showed).

Further, no differences between WIN- and CTRL prenatally exposed rats were found in the elevated plus-maze. Specifically, there was no change in the percentage of time spent in the open arms of the maze (Figure 4C: [$p_{(sex)}$<0.05; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]), in the percentage of open arm entries (Figure 4D: [$p_{(sex)}$<0.05; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]), in the number of closed-arm entries ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown), in SAP ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown), and HDIPS ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown). When tested in the temporal order memory task, pubertal animals displayed identical discrimination index (Figure 4E: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and exploration time (Figure 4F: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) suggesting an intact temporal order memory. Collectively, these results show that prenatal WIN exposure did not induce deficits in social play, anxiety-like behaviors and temporal order memory in the progeny at pubescence.

Positive allosteric modulation of mGlu5 receptors corrected the behavioral deficits induced by prenatal exposure to the cannabinoid receptor agonist WIN in the male offspring at infancy

Our previous works demonstrated the ability of mGlu5 positive allosteric modulation to correct synaptic and behavioral deficits induced by prenatal WIN exposure at adulthood (Bara *et al.*, 2018). Along this line, we found that systemic treatment with the positive allosteric modulator of mGlu5 receptors CDPPB (1.5 mg; i.p.) at PND 10 normalized the altered USV profile displayed by WIN-exposed pups but remained without effect in the CTRL group, indicating selectivity of the drug effects to the disease-state (Figure 5A: [$p_{(WIN\ in\ utero)}$=n.s.; $p_{(CDPPB)}$=n.s.; $p_{(WIN\ in\ utero\ x\ CDPPB}$<0.05]). Specifically, post hoc analysis revealed that CDPPB rescued the decrease of USVs induced by in utero WIN treatment at PND10 in the male offspring. Furthermore, we found that potentiating mGlu5 signaling by CDPPB administration normalized the hyper-locomotion induced by prenatal exposure to WIN in the male offspring tested in the homing behavior test at PND 13 (Figure 5B: [$p_{(WIN\ in\ utero)}$<0.05; $p_{(CDPPB)}$=n.s.; $p_{(WIN\ in\ utero\ x\ CDPPB)}$<0.05) without affecting the number of crossing in the CTRL-group. Overall, these data show that potentiating mGlu5 signaling normalized the behavioral deficits induced by prenatal exposure to cannabinoids in the infant offspring.

Discussion

In the present study, we demonstrated for the first time that fetal exposure to cannabinoids causes sex-specific, mGlu5-related behavioral alterations in the progeny at early developmental periods. Specifically, we found that prenatal exposure to the synthetic cannabinoid WIN altered isolation-induced USVs and locomotor activity in the male but not female infant rat offspring. Conversely, the social, emotional and cognitive profile was spared in the offspring of both sexes tested at the prepubertal and pubertal periods. Interestingly, potentiating mGlu5R signaling reverted the early behavioral deficits displayed by WIN-exposed infant male rats. Infant rodents produce USVs in response to separation from the mother and the nest and USVs are a potent tool to detect subtle effects of adverse events during development (Branchi *et al.*, 2006; Branchi *et al.*, 2001; Cuomo *et al.*, 1987; Insel *et al.*, 1986). It has previously been shown that cannabinoid exposure during pregnancy and/or lactation alters isolation-induced USVs (Antonelli *et al.*, 2005; Trezza *et al.*, 2008). These early studies, however, were only performed in the male offspring, while the consequences induced by developmental cannabinoid exposure in the female offspring were not investigated. Here, we report that male but not female WIN-exposed pups display a decreased rate of isolation-induced USVs compared to control rats. Whether the decreased USV emission displayed by WIN-exposed male pups could be the consequence of an altered maternal responsiveness, which is one of the factors tuning the rate of USV emission of the offspring (D'Amato *et al.*, 2005), is an interesting issue that deserves further investigation. Related to this, previous studies reported disrupted maternal behavior in lactating rats exposed to very high doses of THC (Bromley *et al.*, 1978; Navarro *et al.*, 1995). Conversely, other authors failed to detect changes in maternal care in rhesus monkeys exposed to low doses of THC during pregnancy and lactation (Golub *et al.*, 1981). Recently, it has been shown that THC administered to pregnant mice (GD 5.5 – GD 17.5) at a "non-intoxicating" daily dose (3mg/kg, intraperitoneal) did not alter maternal behavior or physical measures (Tortoriello *et al.*, 2014) suggesting that moderate doses of cannabinoid should not alter maternal behavior and in turn influence mum-pup interaction. By this evidence, we cannot certainly exclude that prenatal exposure to low doses of WIN (0.5 mg/kg, subcutaneously) may induce any alteration in maternal behavior which in turn may contribute to the altered pattern of emotionality displayed by WIN-exposed male pups. Therefore, this issue still remains absolutely intriguing.

The synthetic cannabinoid receptor agonist used in this study (i.e. WIN) has effects that are highly comparable to those of the main active principle of Cannabis THC regardless they

differ in affinity at CB1 receptors and profile of action (Compton *et al.*, 1992; Wiley *et al.*, 2002). Therefore, we hypothesize that the sex-specific behavioral deficits we here observed at early life stages after prenatal WIN exposure could be similar to those obtained by administering THC during the prenatal period. In support of this hypothesis, we recently demonstrated that prenatal THC administration (from GD 5–GD 20) induced similar sex-specific synaptic deficits in the prefrontal cortex of adult rats without any sign of toxicity and/or gross malformations in the rat offspring as WIN did (Bara *et al.*, 2018). However, in a recent inhalation mouse study, a dose of ~ 0.5 mg/kg/day THC smoke from GD 5.5–17.5 produced deficits in fetal growth and reduced birth weights in cannabis-exposed male offspring suggesting that low-dose exposure to THC via inhalation can compromise fetal development (Benevenuto *et al.*, 2017). This highlights that differences in the treatment schedule, routes of administration, doses and animal strain may account for different results following in utero cannabinoid exposure.

During the early phases of postnatal life, olfaction, and in particular the learned association between maternal odors and maternal stimulation, is crucial for the development of social behavior and social recognition (Terry *et al.*, 1996). Therefore, we tested the infant offspring in the homing behavior test, which requires intact sensory, olfactory and motor capabilities that allow the pup to recognize the mother's odor among others (Bignami, 1996). Both WIN-exposed male and female pups were able to use olfactory cues to discriminate between a neutral odor and their own home cage odor. Interestingly, however, locomotor activity in the test arena was increased specifically in prenatally WIN-exposed male rats, while females were spared, suggesting a sex-dependent detrimental effects of prenatal WIN exposure on early life locomotor activity. Maternal exposure to cannabinoid drugs during pregnancy and/or lactation might particularly affect the ontogeny of motor behaviors: an age-dependent hyperlocomotion has been reported in the lactating offspring of mothers receiving THC (during GD 6–12) (Borgen *et al.*, 1973). Other studies demonstrated that rats pre- and/or postnatally exposed to cannabinoid displayed motor hyperactivity at infancy and adolescence, but not at adulthood (Bara *et al.*, 2018; Mereu *et al.*, 2003; Navarro *et al.*, 1995; Silva *et al.*, 2012). These preclinical studies are in line with human data showing that children of both sexes prenatally exposed to cannabis are hyperactive and impulsive starting around age 6 (Fried *et al.*, 2001; Goldschmidt *et al.*, 2004; Sharapova *et al.*, 2018). Altogether, the abnormal USV profile and locomotor activity displayed by WIN-exposed male pups indicate the presence of sex-specific deficits in social communication and locomotion at early life stages. Previous evidence suggested that prenatal exposure to WIN permanently alters GABA

and glutamate circuits in the prefrontal cortex and hippocampus of the offspring (Antonelli *et al.*, 2004; Antonelli *et al.*, 2005; Mereu *et al.*, 2003; Saez *et al.*, 2014). Notably, a reduction in cortical glutamatergic neurotransmission and NMDA receptor activity has been reported (Antonelli *et al.*, 2005; Mereu *et al.*, 2003). These alterations might result in an inappropriate assembly of neuronal network that could represent a substrate for the observed emotional and locomotor dysfunctions displayed by the WIN-exposed male offspring. Based on this experimental evidence and the prominent role of mGlu5R in synaptic endocannabinoid-mediated signaling (Araque *et al.*, 2017), we tested the ability of CDPPB, a well-described positive allosteric modulator of mGlu5Rs, to rescue the behavioral deficits displayed by WIN-exposed male pups. We found that systemic administration of CDPPB normalized the altered USVs profile and the increased locomotion induced in male pups by prenatal WIN exposure. This finding extends our previous data demonstrating the ability of mGlu5 positive allosteric modulation to correct synaptic and behavioral deficits induced by prenatal cannabinoid exposure at adulthood (Bara *et al.*, 2018). Female did not show the behavioral deficits displayed by the male offspring at infancy; however we cannot exclude that the administration of CDPPB *per se* could affect their USVs and homing performances since CDPPB is known to affect cognitive and operant responding tasks in rodents (Cleva *et al.*, 2012; Fowler *et al.*, 2013; Lee *et al.*, 2018). In the present study we used a dose of CDPPB (1.5 mg/kg) that did not affect early life behavioral parameters (i.e. USVs and homing behavior) in the CTRL male progeny, therefore we hypothesize that the same dose would not have an effect *per se* in the female progeny. Related to this, it should be considered that prenatal exposure to WIN induced sex-related differences in the postsynaptic mGluR proteins at adulthood (Bara *et al.*, 2018) and that mGlu5R modulate spine plasticity in the nucleus accumbens of female mice depending on estrogen receptors (Peterson *et al.*, 2015) suggesting the importance of sex-dependent specificity of the mGluR signaling in the brain.

Moreover, it remains to clarify how prenatal WIN exposure induces sex-specific detrimental behavioral effects at early life stages. Different studies have focused on the sexual dimorphism of the endocannabinoid system, which could explain at least in part the sex dissimilarities in the consequences induced by in utero cannabinoid exposure. Beside molecular and structural differences (Castelli *et al.*, 2014; Garrett *et al.*, 2009; Rodriguez de Fonseca *et al.*, 1994), prenatal exposure to cannabinoids throughout gestation induces sex-specific effects on dopaminergic neurotransmission in the limbic forebrain (Alpar *et al.*, 2016; Navarro *et al.*, 1995; Rodriguez de Fonseca *et al.*, 1991) and also changes in the ontogenetic expression of tyrosine hydroxylase gene (Navarro *et al.*, 1995). Moreover, sex-

differences in mRNA expression levels for mGlu1R have been reported in the prefrontal cortex of adult rats prenatally exposed to WIN, with an increase in mGlu1R mRNA levels exclusively in the male progeny (Bara *et al.*, 2018). In humans, impaired dopamine D2 receptor expression in amygdala is most evident in males in association with prenatal cannabis exposure suggesting a potential pathway for altered emotional regulation (Wang *et al.*, 2004). Interestingly, 10-year-old boys prenatally exposed to marijuana are more susceptible to behavioral problems than girls (Goldschmidt *et al.*, 2004). However, the neurobiological mechanisms underlying maternal exposure to cannabinoids still remain complex. Changes in the epigenetic role of steroid hormones (both sex-steroids and glucocorticoids) on brain development induced by prenatal cannabinoid exposure could be responsible for some specific behavioral effects that we here found at early life stages. Indeed, it has been proposed that the epigenetic effects of abused drugs including marijuana on brain development might be the result of both drug mimicking or modification of the action of natural hormones which play a very important role in neuronal phase during early stages of brain development and cortical organization during perinatal ages in rodents (Navarro *et al.*, 1995). Moreover, marijuana exposure in early fetal life also decreases the expression of genes (through histone lysine methylation) for dopamine D2 receptors in brain areas mediating rewarding processes (i.e. nucleus accumbens) which may explain higher rates of drug addiction in adults exposed prenatally to marijuana (DiNieri *et al.*, 2011). Prenatal exposure to THC also causes substantial changes in gene expression levels of several other significant systems in the brain that are linked to the endocannabinoid signalosome such as the opioid, glutamate, and GABA systems, which may persist well into adulthood (Jutras-Aswad *et al.*, 2009; Navarro *et al.*, 1995) and sex-dependent affect behavioral outcomes since early life stages.

Profound changes in behavioral repertoire and physiological status occur between weaning and puberty; it is during this stage that mammals progressively achieve sexual maturation and establish a sense of independence from their primary caregivers (Spear, 2000; Vanderschuren *et al.*, 2016). This process of development involves several behavioral processes influenced by endocannabinoid signaling (Hill *et al.*, 2012; Solinas *et al.*, 2008; Zanettini *et al.*, 2011). We here showed that prenatally WIN-exposed animals of both sexes did not exhibit deficits in social play, neither anxious-like behaviors in the elevated plus-maze test nor cognitive deficits in temporal order memory at the prepubertal and pubertal periods. The endocannabinoid system has a strong interaction with different neurotransmitters present from early stages of brain development (Alpar *et al.*, 2016). It could be that in utero WIN

administration induced mGluR sex-mediated deficits at early life stages (as we found in the present study) and then the reorganization of this system occurs and different targets (such as dopamine or opioids) become predominant in mediating specific motivational, rewarding and emotional processes that we here did not explore. For instance, prenatal THC-induced reorganization of the dopamine system occurs within this sensitive period and might disrupt reward circuits by genetic and epigenetic modifications (DiNieri *et al.*, 2011; Spano *et al.*, 2007).

Previous findings from our group demonstrated that perinatal exposure to THC (GD 15 to PND 9) altered social play and induced anxiety-like behaviors in the male rat offspring (Trezza *et al.*, 2008). Moreover, it has been recently shown that the postnatal exposure to the cannabinoid receptor agonist CP 55,940 from PND 4 to PND 10, a period of brain development equivalent to the third trimester in human, increased the time spent in the open arms of the elevated-plus maze in offspring of both sexes at pre-pubertal period (Breit *et al.*, 2019). We here showed that prenatally WIN-exposed animals of both sexes did not exhibit anxious-like behaviors in the elevated plus-maze. The discrepancy with our present findings may depend on the different cannabinoid agonists used (THC or CP 55,940 vs WIN) and the treatment schedule (perinatal or postnatal vs prenatal exposure). Moreover, it is possible that a longer activation of endocannabinoid neurotransmission that extends beyond birth till after the early postnatal period may be required to disrupt social play behavior and to induce an anxious-like phenotype in the elevated plus-maze.

Furthermore, social play behavior (van Kerkhof *et al.*, 2013) and temporal order memory (Barker *et al.*, 2007) are mediated by functional activity in the prefrontal cortex and certain levels of regional frontal specificity to the effects of prenatal cannabinoid exposure have been demonstrated (Bara *et al.*, 2018). The fact that the prefrontal cortex develops late in to postnatal life (i.e. late adolescence/early adulthood) (Arain *et al.*, 2013; Kolb *et al.*, 2012) and that temporal order memory requires cortical more than hippocampal integrity (Barker *et al.*, 2017) may explain the normal behavior of (pre)pubertal WIN-exposed rats in these tasks compared to their impaired performance when tested for other forms of memory (Antonelli *et al.*, 2005; Castaldo *et al.*, 2007; Drazanova *et al.*, 2019; Ferraro *et al.*, 2009; Mereu *et al.*, 2003). Further, it can be hypothesized that perturbations of the fetal endocannabinoid system induced by in utero exposure to WIN predisposed the offspring to abnormalities in memory and altered emotionality later in life (Richardson *et al.*, 2016): thus, WIN in utero induced an imbalanced brain circuit at sub-threshold levels (non-manifested during pre-pubescent and pubescent period) that can precipitate neurodevelopmental disease by otherwise sub-

threshold stimuli later in life (Bara *et al.*, 2018; Campolongo *et al.*, 2007; Mereu *et al.*, 2003; Tortoriello *et al.*, 2014; Trezza *et al.*, 2008; Vargish *et al.*, 2017).

Overall our results clearly show previously undisclosed sexual divergence in the consequences of fetal cannabinoids at early stages providing new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabinoid exposure in both male and female offspring.

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Table 1. Reproduction data following in utero exposure to WIN. Dam weight gain was calculated from GD 1 to GD 21 for n=10 dams per group (WIN vs CTRL). Pup weight at different developmental ages was calculated for n=9 CTRL and n=10 WIN-exposed male and n=8 CTRL- and WIN-exposed female pups from different litters. Data represent mean values ± SEM.

Treatment Group	Dam weight gain %	Pregnancy length (days)	Litter size	Postnatal viability (%)	Pup weight (g)							
					PND10		PND13		PND25		PND45	
					♂	♀	♂	♀	♂	♀	♂	♀
CTRL	34.3 ± 1.91	22.6 ± 0.29	12.9 ± 0.78	87.9 ± 1.048	24.6 ± 0.65	23.9 ± 0.67	30.3 ± 0.82	29.0 ± 1.02	71.7 ± 1.83	59.6 ± 1.55	162.8 ± 1.99	142.1 ± 3.10
WIN	33.1 ± 1.42	22.6 ± 0.17	12.7 ± 0.67	88.4 ± 1.352	23.2 ± 0.70	23.5 ± 0.46	30.5 ± 0.75	28.5 ± 0.87	68.7 ± 2.23	58.9 ± 1.33	158.7 ± 2.90	143.9 ± 3.71

Table 1: Reproduction data following in utero exposure to the cannabinoid WIN

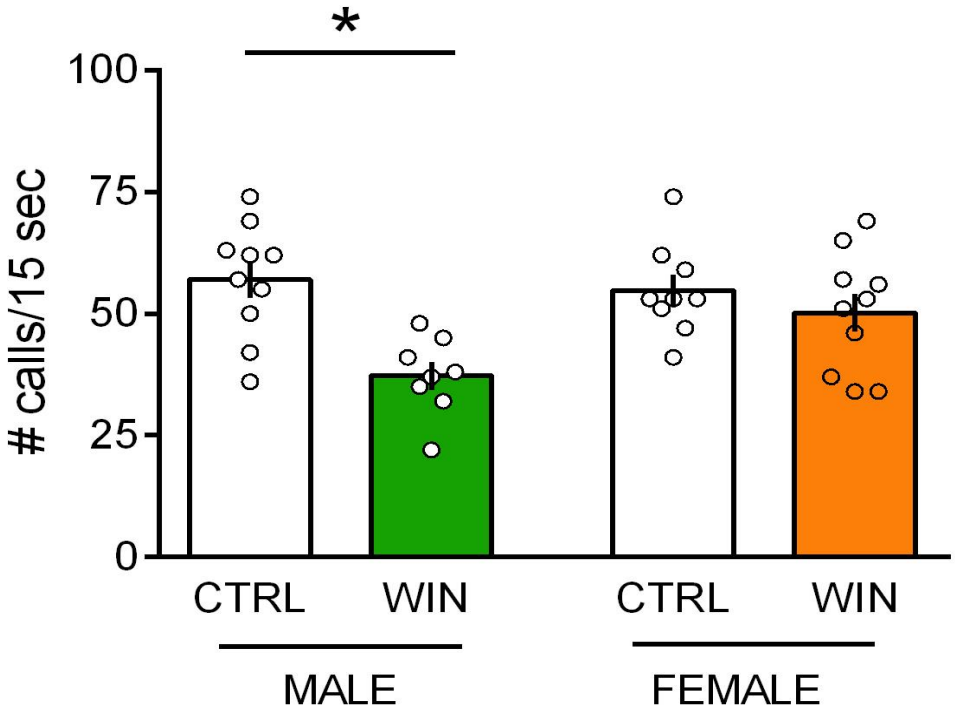

Figure 1. Prenatal WIN exposure induces sex-specific communicative deficits in the infant rat offspring. At infancy (PND 10) male progeny from dams exposed during gestation to WIN vocalized significantly less compared to CTRL-pups when separated from the dam and siblings. In contrast, the communicative profile of female littermates was normal (males: CTRL n=10, WIN n=8; females: CTRL n=9, WIN n=10). Specifically, prenatally-WIN exposed male but not female showed a decrease in the rate of USVs/15 sec compared to their age-matched male progeny from CTRL-group. Scatter dot plot represents each animal. Error bars indicate SEM. *p<0.05. Student–Newman–Keuls test.

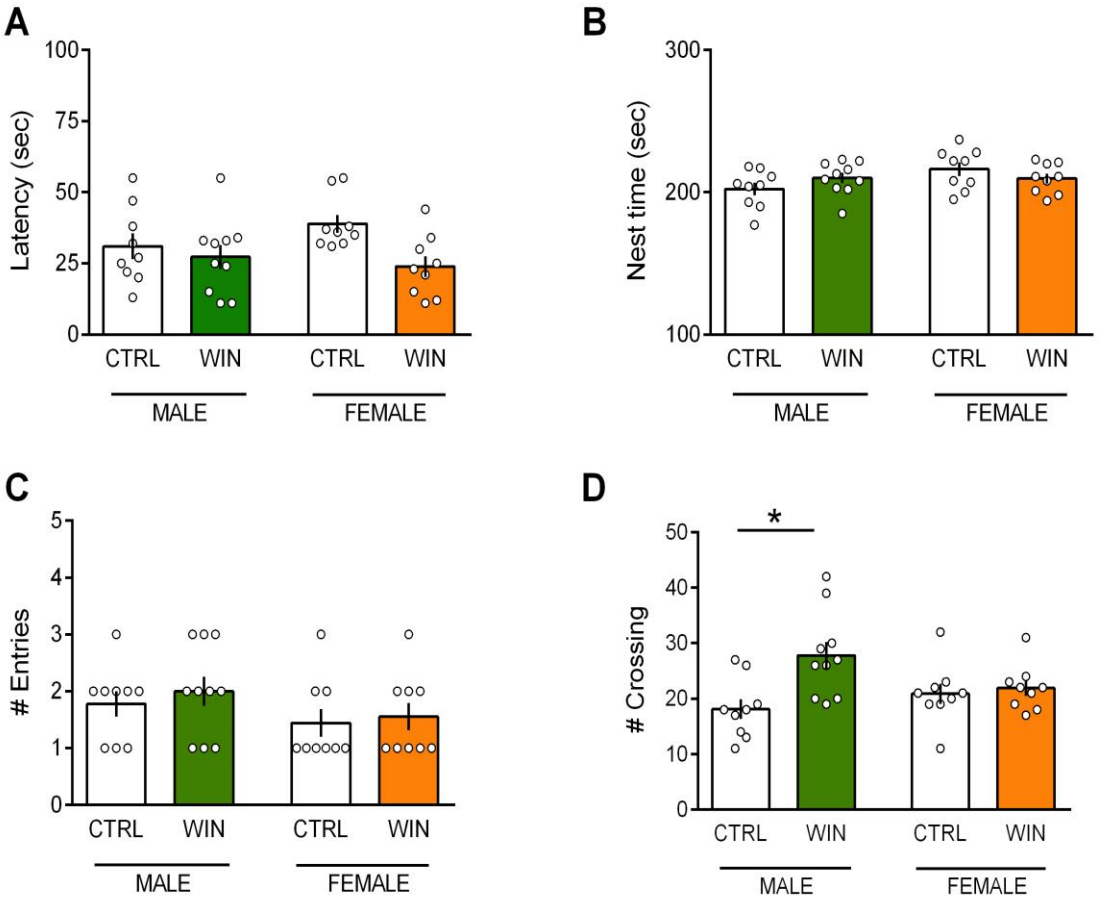

Figure 2. Prenatal WIN exposure induces sex-specific deficits in locomotor activity in the infant rat offspring. A-D, In male and female progeny at PND13 prenatal WIN exposure did not alter the latency to reach the nest arena (**A**), the total time spent in the nest zone (**B**)and the number of entries in the nest zone (**C**) in the homing test. Conversely, the frequency of crossing in the test arena was increased only in male offspring exposed in utero to WIN, while female were spared (males: CTRL n=9, WIN n=10; females: CTRL n=9, WIN n=9) (**D**). Scatter dot plot represents each animal. Error bars indicate SEM. *p<0.05. Student–Newman–Keuls test.

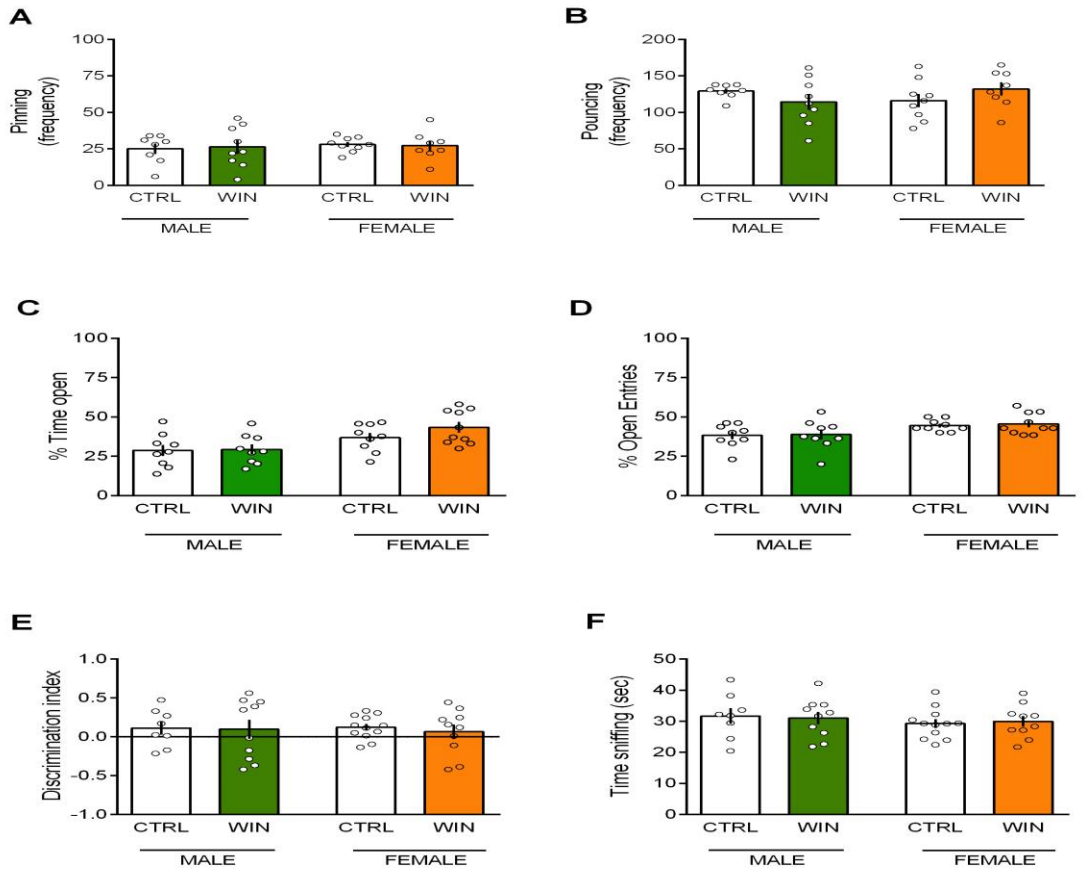

Figure 3. Prenatal WIN exposure does not affect social, anxious and cognitive behaviors in prepubescent male and female offspring.A-B, No differences between WIN-exposed and CTRL animals in both sexes were found in the social play behavior in the prepubertal period as expressed in the frequency of pinning (**A**) and pouncing (males: CTRL n=8, WIN n=9; females: CTRL n=9, WIN n=8) (**B**). In the elevated plus-maze test, prenatal cannabinoid exposure did not modify the percentage of time spent in the open arms (**C**) and the percentage of open arm entries (males: CTRL n=9, WIN n=9; females: CTRL n=9, WIN n=10) (**D**). **E-F**, In the temporal order memory task, male and female prepubertal rats exposed in utero to the cannabinoid WIN showed no differences in their discrimination index (**E**) and in the total time exploring the objects during the test phase (males: CTRL n=8, WIN n=10; females: CTRL n=12, WIN n=10) (**F**). Scatter dot plot represents a pair of animals for social behavior (A-B) and each animal for the elevated plus-maze (C-D) and the temporal order task (E-F). Error bars indicate SEM.

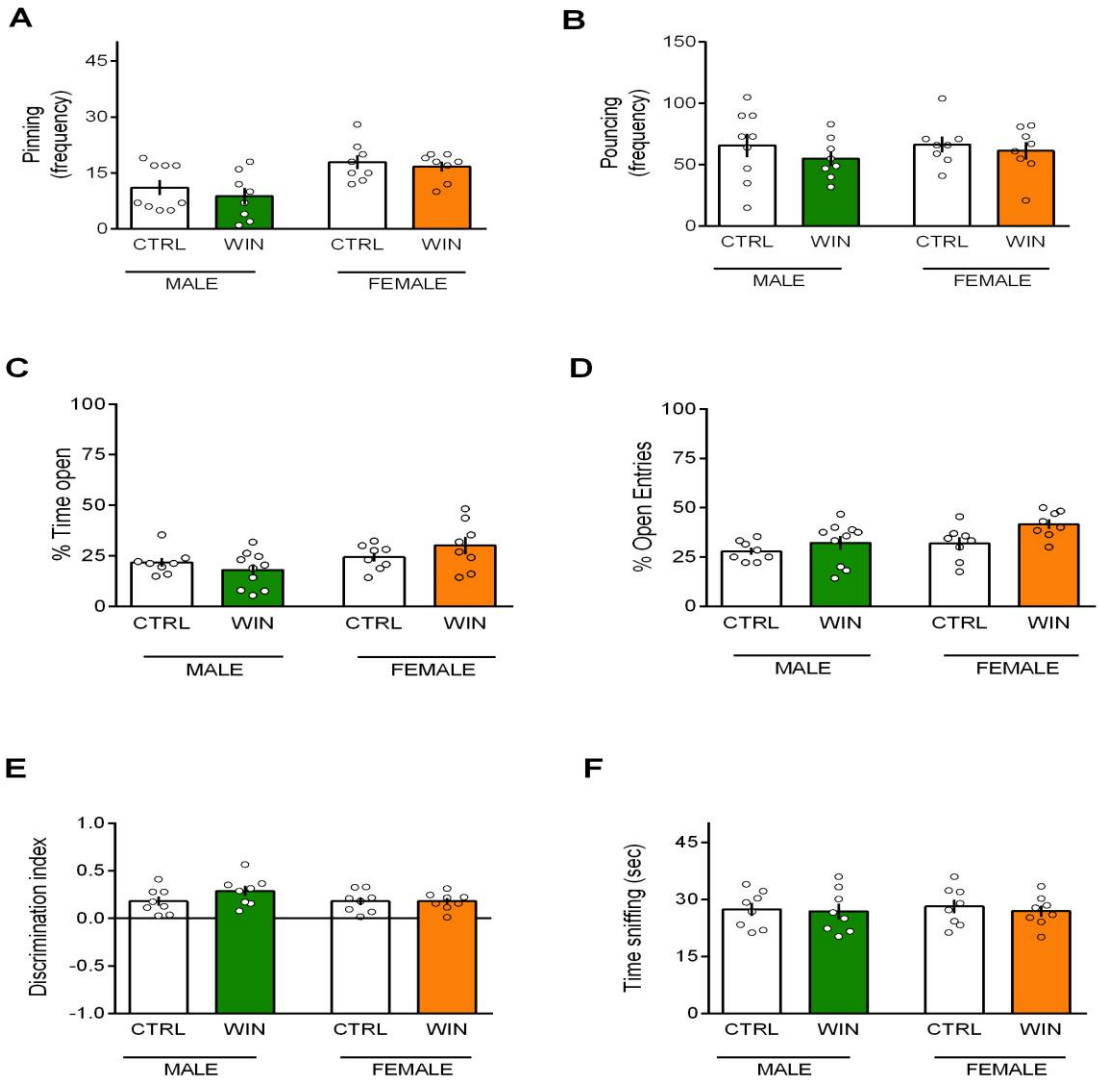

Figure 4. Prenatal WIN exposure does not affect social, cognitive and anxious behaviors in pubescent offspring of both sexes. A-B, Prenatal cannabinoid exposure did not alter social behavior at puberty in WIN-exposed and CTRL animals as expressed in the frequency of pinning (**A**) and pouncing (**B**) (males: CTRL n=9, WIN n=8; females: CTRL n=8, WIN n=8). In the elevated plus-maze test, prenatal cannabinoid exposure did not modify the percentage of time spent in the open arms (**C**) and the percentage of open arm entries (males: CTRL n=8, WIN n=10; females: CTRL n=8, WIN n=8) (**D**). **E-F**, In the temporal order memory task, male and female pubertal rats exposed to the cannabinoid WIN in utero showed no differences in their discrimination index (**E**) and in the total time exploring the objects during the test phase (males: CTRL n=8, WIN n=8; females: CTRL n=8, WIN n=8) (**F**). Scatter dot plot represents a pair of animals for social behavior (A-B) and each animal for the elevated plus-maze (C-D) and the temporal order task (E-F). Error bars indicate SEM.

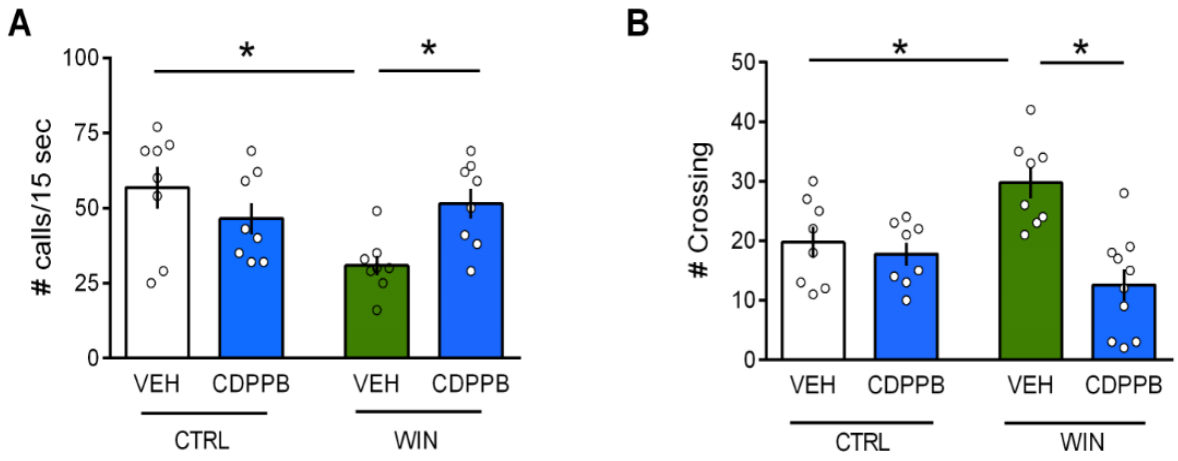

Figure 5. Positive allosteric modulation of $mGlu_5$ receptors normalizes the communicative and locomotor deficits displayed by male pups prenatally exposed to WIN.A, Systemic administration of CDPPB (1.5 mg/Kg, i.p.) at PND 10 rescued the decrease in the rate of USVs in male rats prenatally exposed to WIN without affecting the USV frequency in the CTRL-group (males CTRL: VEH n=8, CDPPB n=8; males WIN: VEH n=8, CDPPB n=8). **B,** In the homing behavior, treatment with CDPPB (1.5 mg/Kg, i.p.) at PND 13 corrects the increase in the frequency of crossing in male rats prenatally exposed to WIN without affecting the number of crossing in the CTRL-group (males CTRL: VEH n=8, CDPPB n=8; males WIN: VEH n=8, CDPPB n=10). Scatter dot plot represents each animal. Error bars indicate SEM. *p<0.05. Student–Newman–Keuls test.