

Title: Nandrolone alters the behavioral response to cocaine as well as striatal and cortical dopamine receptors of prepubertal male rats.

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ABSTRACT:

Nandrolone is the anabolic androgenic steroid (AAS) most used by athletes and adolescents. The use of supraphysiologic doses has been associated with dysfunction in brain areas that regulate anxiety, motivation, and reward. This study investigated if exposure to nandrolone before puberty altered anxiety- and addictive-like behaviors. Changes in dopamine type 2 receptors (D2DR) in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) were also examined. Male rats received 10 daily injections of nandrolone decanoate (20 mg/kg) starting on day 28. Afterwards, they were tested in the elevated plus maze (EPM) and open field (OF). Their locomotor response (sensitization) and preference (conditioned place preference (CPP)) to cocaine (15 mg/kg) was also assessed. Nandrolone reduced anxiety and ambulation. Nandrolone-treated males also displayed sensitization to cocaine at an earlier age (day 44) than oil-treated males (day 52) and showed a 27% reduction in CPP to cocaine. Expression of D2DR in the NAc, and in the PFC of males tested for CPP was increased by nandrolone, whereas treatment with cocaine reduced accumbal D2DR. We hypothesize that nandrolone accelerates the development of the neural circuitry that regulates behavioral sensitization and reduces the rewarding property of cocaine, as manifested in the reduction of CPP. It is possible that the observed increase in accumbal D2DR may partially mediate the reduced anxiety and ambulation as well accelerate the maturation of the neural circuitry responsible for the sensitized response to cocaine.

Keywords: Sensitization, cocaine, adolescents, nandrolone, AAS, D2DR

Introduction

Anabolic-androgenic steroids (AAS) are derivatives of the main male gonadal hormone testosterone (T) developed in the 1950s to treat men with hypogonadism, delayed puberty (Basaria and Dobs, 2001) and to promote growth. The term anabolic comes from their effects of promoting metabolic processes, such as protein synthesis in muscle tissue and erythropoiesis (Kochakian and Welder, 1993). Androgenic comes from their masculinizing properties. Their anabolic properties make these substances appealing to athletes. AAS provides athletes with an edge in training and in competition, they increase muscle strength and endurance and promote competitive and aggressive behaviors (Bahrke et al., 1996).

Before the '90s, the use of AAS was circumscribed mainly to athletes during training and before competitions (Sjöqvist et al., 2008). However, during the last two decades, young men have been using AAS all year round to improve their physical appearance (Denham, 2006; Kindlundh et al., 1999). Use of AAS as self-medication is also on the rise among the female-to-male transgender population (Metastasio et al., 2018). In the USA, about 3-4 million people have used AAS, of those approximately 1 million have developed dependency (Pope et al., 2014a). Worldwide prevalence of AAS use is 3.3%, being higher for males (6.4%) than for females (1.6%) (Sagoe et al., 2014). Users of AAS report administering dosages that can be higher than 100X the physiological dose (Brower et al., 1990). This is of significant concern because AAS have deleterious side effects, particularly on the cardiovascular system. AAS also cause hepatic toxicity, decrease fertility and alter secondary sexual characteristics (Parkinson and Evans, 2006; van Amsterdam et al., 2010). Many studies find that AAS increase

psychiatric disorders (Pope et al 2014b; Oberlander and Henderson, 2012; Onakomaiya and Henderson, 2016). Some of the symptoms are associated with the exposure to AAS (mania, aggression, risk-taking behaviors, irritability) whereas others can result from AAS withdrawal (depression, loss of libido, suicidal thoughts, hypersomnia) (Pagonis et al., 2006; Pope and Katz, 1988). There is considerable variability in the display and range of these symptoms, in very few individuals these symptoms are disabling (Kaufman et al., 2015).

Androgens, including AAS, have rewarding properties (Johnson and Wood, 2001; Wood, 2004, 2002) and may contribute to the development of substance abuse and dependency to other drugs of abuse (Brower et al., 1989). Approximately 30-32% of subjects using AAS will develop a dependency to AAS (Pope et al., 2014a), this risk is higher for women and adolescents of both sexes (Kanayama et al., 2008; Penatti et al., 2011). AAS also promote dependence on other drugs such as opioids and cocaine (Pope et al., 2014a). Several studies report that abuse of drugs, particularly of cocaine, by AAS users is higher than in the general population (DuRant et al., 1995; A. M S Kindlundh et al., 2001). Because of their widespread use and adverse side effects, AAS were classified as a schedule III drug by the Drug Enforcement Agency (DEA).

Among the AAS, nandrolone (19-nortestosterone), in its long-lasting ester form of nandrolone decanoate (ND), is the AAS most widely used worldwide (Lood et al., 2012). The androgenic activity of this compound is lower than that of dihydrotestosterone (DHT); in contrast, its anabolic properties are higher than those of T (Saartok et al 1984) making it attractive for abuse by male and female athletes. Nandrolone has a higher affinity for the androgen receptor (AR) than T and is less susceptible to degradation by

the enzyme 17 beta-hydroxysteroid dehydrogenase, which may explain its enhanced anabolic properties. Moreover, although nandrolone and T can be reduced to DHT in target tissue that contains the enzyme 5 alpha-reductase, enzyme binding to nandrolone is weaker than to T (Bergink et al., 1985; Kicman, 2008; van der Vies et al., 1993). Also, binding of nandrolone to the AR is weaker than that of DHT. Taken together, this explains the stronger effects of nandrolone compared to T, on target tissues with no 5 alpha-reductase activity, and the weaker effect on tissues with a high 5 alpha-reductase activity, resulting in greater anabolic/androgenic properties (Tenniswood et al., 1982; van der Vies et al., 1985).

Adolescents, compared to children and adults, show the highest incidence of risk-taking behavior and of experimentation with drugs of abuse (SAMHSA, 2019). AAS are among the drugs that are most used by adolescents, particularly males. This is a significant concern since AAS can cross-sensitize with other drugs of abuse. Cocaine, one of the primary drugs used by people that abuse AAS, shares many of the harmful side effects on the cardiovascular system and also promotes risk-taking behavior, aggravating the detrimental health consequences when using both drugs.

Drugs of abuse exert their behavioral and addictive effects by acting on certain areas of the brain associated with decision making, motivation and reward, such as the prefrontal cortex (PFC) and nucleus accumbens (NAc) (Baker et al. 2003). Dopaminergic receptors in these brain areas participate in regulating addictive behaviors (Beyer and Steketee, 2002; Steketee and Walsh, 2005). In particular, decreased D2-like dopamine receptors (D2DR) expression in these brain areas are associated with increased novelty seeking and risk-taking, behaviors associated with

addiction (Goldstein and Volkow, 2002; Koob and Volkow, 2010). Manipulating striatal D2DR can alter the response to drugs of abuse, such as cocaine. Mice that lack striatal presynaptic D2DR show increased sensitivity to the locomotor activating effects of cocaine (Bello et al, 2011), whereas rats with increased D2DR sensitivity show enhanced cocaine self-administration (Marinelli and White, 2000).

This study investigated if exposure to nandrolone prior to puberty affected anxiety-like behaviors as well as the behavioral response to cocaine. The nucleus accumbens and prefrontal cortex were also examined to determine if nandrolone induces changes in the D2DR population of these brain substrates. To accomplish these goals, rats were injected with nandrolone decanoate (20 mg/kg) during postnatal days (PN) 28-37 and their behaviors assessed from days 38 to 62. Animals were sacrificed at days 54 or 63 and brain tissue removed for further analysis.

2. Materials and Methods

2.1 Animals

Pregnant Sprague Dawley rats were purchased from Charles Rivers Laboratories (Ballardvale St, MA, USA). Dams were housed in pairs, with water and Purina® rat chow provided ad libitum. They were maintained in a temperature and humidity-controlled room, under a (12L:12D) light-dark cycle with lights off at 5 PM. After parturition, pups were cross fostered, and each dam was housed separately with their litter. The litter was culled to 10 pups per dam, 5 males and 5 females. Pups remained with the mother until weaning (day 23). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico,

Medical Sciences Campus (Protocol 1140215) and adhere to USDA, NIH and AAALAC guidelines.

2.2 Drugs and chemicals

The AAS used in this study was 4-estren-17beta-ol-3-one decanoate (nandrolone decanoate) (Steraloids, Inc., Newport, RI, USA), dissolved in sesame oil. Nandrolone was administered subcutaneously (s.c.) at a dose of 20 mg/kg. The nandrolone dose of 20 mg/kg was selected since it is similar to the supraphysiological doses used by AAS users (Clark and Henderson, 2003). Cocaine-HCl (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sterile saline and administered intraperitoneally (i.p.) at a dose of 15 mg/kg. The dose of 15 mg/kg of cocaine has been used extensively in our laboratory and has been proven effective in inducing behavioral sensitization and CPP to cocaine. We have found that higher doses (30 mg/kg) may induce tolerance and lower doses are not as effective in a behavioral sensitization paradigm that is not context-dependent (Segarra et al., 2014; Menendez-Delmestre and Segarra, 2011).

2.3 Nandrolone Treatment

At postnatal day 28 (PN-28) rats were weighed and randomly distributed into two groups: one that received daily injections of nandrolone (ND) (20mg/kg) (n=20) and the other that received daily injections of sesame oil (Oil) (n=20) for 10 consecutive days. Two cohorts of rats were used for the behavioral studies. One cohort was used for the Open Field Test (OFT) and the sensitization experiments. The second cohort was used for the Elevated Plus Maze (EPM) studies and the CPP experiments. On day 39 one cohort of rats were tested in the Open Field (OFT). From day 40 to 62 they were tested for behavioral sensitization to cocaine and euthanized on day 63. The second cohort of

rats were tested on day 39 on the Elevated Plus Maze (EPM) and from days 40 to 53 they were used to assess Conditioned Place Preference (CPP) to cocaine. This last group of rats were euthanized on day 54. For the CPP and Sensitization tests, animals were assigned to one of 4 groups: Oil-Saline (Oil-Sal), Oil-Cocaine (Oil-Coc), Nandrolone-Saline (ND-Sal), Nandrolone-Cocaine (ND-Coc).

2.4 Elevated Plus Maze

The EPM is a highly used paradigm to measure anxiety-related behaviors (Pellow et al., 1985). Rats previously exposed to anxiogenic drugs decrease the time spent in the open arms of the maze, while anxiolytic drugs increase the time spent in open arms (Biedermann et al., 2017). Our testing apparatus consisted of a plus-shaped custom-made apparatus with two 50 cm open arms and two 50 cm enclosed arms, each with an open roof. The floor of the open arms had a 1 cm ledge to prevent rats from slipping to the floor. We also lined the floor with rugged plastic to avoid slipping. The apparatus was elevated 70 cm from the floor. An infrared video camera was placed in the center above the maze. The camera was connected to a computer containing the ANY-Maze™ software. At the beginning of the test, rats were placed at the junction of the open and closed arms and the video tracking system was activated. The software automatically records the number of entries into the open and closed arms, as well as of the time spent in each arm. Entry into an arm was defined as the time point when more than 95% of the rat is in the arm. This was considered time zero. The test ended after 5 min. The amount of time spent in the closed arms, the time spent in the open arms relative to the closed arms and the number of entries into the open arms was measured. The greater the amount of time spent in the closed arms, the higher the anxiety.

2.5 Open field test

The OFT is an assay of locomotor activity that can be used to measure anxiety, exploratory behavior, risk taking behavior and thigmotaxis. Anxiolytic drugs increase the amount of time rats spend in the center area (Treit and Fundytus, 1988). The locomotor activity chambers (Versamax™) were used to measure Open Field Behavior. These chambers are made from clear acrylic (42 cm × 42 cm × 30 cm). All beams were connected to a Data Analyzer that sent information to a personal computer.

Animals were placed in the Activity Cage and allowed to roam freely for 10 minutes. The breaking of infrared beams determined the position of the rats in the activity cage. The amount of time spent in the center of the cage versus at the periphery was compared, as well as the total distance travelled. Animals that spent less time in the center of the cage were classified as more anxious and as displaying less risk-taking behavior when compared to their counterpart controls (oil-treated).

2.6 Locomotor activity

Horizontal and stereotyped activity were measured with the automated animal activity cage system (Versamax™; AccuScan Instruments, Columbus, Ohio). The cages were made from clear acrylic (42 cm × 42 cm × 30 cm) with 16 equally spaced (2.5 cm) infrared beams across the length and width of the cage at a height of 2 cm (horizontal beams). An additional set of 16 infrared beams were located at a height of 10 cm (stereotyped activity). This system differentiates between horizontal, stereotyped, or rearing activity based on sequential breaking of different horizontal beams, the same beams or vertical beams respectively.

Activity was measured in an isolated room with low illumination. Animals were habituated to the chamber for 1 hour, 1 day prior to injections (Day 0). On days 1,5,13 and 23 rats were placed for 30 mins in the chambers, and basal locomotor activity was recorded. Animals then received a saline or cocaine injection, and locomotor activity was recorded for 60 additional minutes. On days 2–4 animals received a daily injection of 0.9% saline or cocaine (15mg/kg) in their home cages. During days 6–12 and 14-22 animals remained in their home cages undisturbed (Fig 1). Animals were sacrificed the day after the last behavioral test at 63 days of age.

2.6 Conditioned Place Preference

Cocaine-induced CPP was measured using Versamax™ activity cages. Each cage consisted of an acrylic box divided into 2 chambers and placed in the locomotor activity apparatus. For the pre- and post-conditioning sessions, the chambers were separated by an acrylic wall that had an opening; during the conditioning phase, a solid acrylic wall separated the two chambers. Each chamber had different visual and tactile cues. During pre-conditioning, animals were placed in the CPP apparatus for 3 consecutive days and allowed to roam between both chambers for 15 minutes. The amount of time spent in each chamber was recorded. The conditioning phase consisted of alternating saline and cocaine injections for 10 days. Saline was injected in the preferred chamber, cocaine in the non-preferred chamber with 24 hours of separation between injections. Rats were confined to the chamber where they received the injection for 30 min. During post-conditioning, animals were placed in the activity chamber and allowed to roam between the 2 chambers for 15 min (Fig 1). The time spent in each chamber during pre- and post-conditioning was compared. Rats that

showed a significant increase in the time spent in the chamber where they received cocaine displayed conditioned place preference.

2.7 Western Blots

Western blots were used to quantify D2DR levels in the mPFC and NAc. The protein concentration of the samples was determined by the BioRad Protein Assay method (BioRad Laboratories, Hercules, CA, USA). For our gels, 20 µg of protein were mixed in SDS/-mercaptoethanol, vortexed, and heated at 95°C for 7 min prior to separation by 10% SDS-PAGE (BioRad Laboratories). Following electrophoresis, proteins were transferred to a 0.2 µm nitrocellulose membrane using a Trans-Blot Turbo (BioRad Laboratories). Nonspecific binding to the membrane was blocked by incubating in Odyssey Blocking Buffer for 60 min at room temperature. This was followed by overnight incubation at 4°C with a D2 receptor antibody (1:200; Santa Cruz Biotech, Santa Cruz, CA, USA, #sc-5303) and a B-Actin antibody (1:2500; Abcam, MA, #ab8227) dissolved in Odyssey Blocking Buffer. The next day, membranes were washed 3X in TRIS-buffered saline and Polysorbate 20 (PBS-T). After the washes, membranes were incubated for one hour in IRDye 680RD goat anti-rabbit (1:15000; LICOR, Lincoln, NE, USA, #926-68071) and IRDye 800CW goat anti-mouse (1:15000; LICOR, Lincoln, NE, USA, #926-32210). Proteins were detected using the Odyssey CLx infrared imaging system (excitation/emission filters at 700 nm/ 800 nm range, LI-COR Biosciences, Lincoln, NE, USA). Optical density of D2 receptors of each sample was obtained using Odyssey software (LI-COR Biosciences), normalized against background, and then corrected against their own B-Actin levels.

3. Statistical analyses

All data were analyzed using GraphPad Prism version 9.00 for Windows (Graphpad Software, San Diego California USA). An unpaired t-test was used to compare two groups, a Two-way ANOVA was used when comparing more than two groups and a repeated measures MANOVA was used for analyzing repeated measures. To ascertain if rats showed sensitization, the time course of each group was analyzed separately using repeated measures (RM) ANOVA with days (40, 44, 52 and 62) and minutes (35-90) as the repeated factors. Tukey's multiple comparisons were used for post-hoc analysis to compare locomotor and stereotyped activity across time. CPP was analyzed using repeated measures MANOVA, using Pre and Post Conditioning as repeated factors. A Two-Way ANOVA was used to compare D2DR expression in the PFC and the NAc between groups. Results of statistical analysis are included as supplemental material. Data are presented as the mean \pm standard error of the mean (SEM). A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

4. Results

4.1 Open Field and basal locomotor activity

Rats treated with nandrolone spent more time in the center of the open field, and ambulated less, than oil-treated rats (Fig 2A and 2B respectively). Data were analyzed with Student's T-test - Fig 2A: $t=2.361$, $df=36$, $p = 0.0264$; Fig 2B: $t=5.174$, $df=36$, $p < 0.0001$. The decrease in distance travelled corresponds to the first 10 minutes of habituation (sensitization protocol-Day 0). A decrease in total horizontal activity during the first 10 min was also observed in the habituation portion on day 1 of the

sensitization protocol (Fig 1C; Student's T-test: $t=2.152$, $df=18$, $p<0.0453$). This difference was not observed on subsequent testing days (days 5, 13 and 23).

4.2 Elevated Plus Maze

Nandrolone administration decreased the time spent (Fig 3A: Student T-test: $t=2.623$, $df=18$, $p = 0.0173$) into the closed arms of the EPM compared to rats that received oil. In addition, nandrolone decreased the total number of arm entries (Fig 3B: Student's T-test: $t=3.28$, $df=18$, $p=0.0041$), which is considered an indication of decreased locomotor activity.

4.3 Behavioral sensitization

Rats treated with nandrolone showed a higher locomotor response to cocaine on day 5 than that displayed on day 1, i.e. they displayed behavioral sensitization (Fig 4A, 4B, 4C, 4D, 4E). Of the 12 cocaine-induced locomotor activity timepoints measured during the 60 min after injection (5 min intervals), 8 were higher in rats that received nandrolone-cocaine (Fig 4C vs 4D). This pattern was maintained on day 13, 10 out of 12 cocaine-induced locomotor activity timepoints for nandrolone-cocaine treated rats were higher from those on day 1 (Fig 4D). In comparison, oil-cocaine treated rats did not show differences in cocaine-induced locomotor activity between days 1 and 5 in any of the 12 timepoints measured and showed differences in only 5 of the 12 timepoints comparing day 1 with day 13 (Fig 4C). Thus, rats treated with nandrolone-cocaine displayed behavioral sensitization earlier (day 5) than oil-cocaine treated rats (day 13) (Fig.4D: Two-Way RM ANOVA, Days, $F(2.45, 22.08) = 13.03$; $p<0.0001$). Similar results were obtained with Stereotyped activity (Fig.5A-E: Two-Way RM ANOVA, Days, $F(2.58, 23.23) = 10.03$, $p= 0.0003$). In addition, cocaine-induced locomotor activity of

nandrolone-treated rats was greater than that of oil-treated rats in 9 of the 12 timepoints measured on day 5.

4.4 Western Blot Analysis of D2DR in mPFC (Sensitization brains)

Our data show that cocaine decreased D2DR in the mPFC (Fig 6A and Fig 6C). This effect was not altered by preexposure to nandrolone (Fig. 6A and 6C): One Way ANOVA, Tukey's multiple comparisons, $F(3, 12) = 110.5$, <0.0001 ; Oil-Sal vs ND-Sal, $p = 0.1559$; Oil-Sal vs Oil-Coc, $p < 0.0001$; Oil-Sal vs ND-Coc, $p < 0.0001$; ND-Sal vs Oil-Coc, $p < 0.0001$; ND-Sal vs ND-Coc, $p < 0.0001$; Oil-Coc vs ND-Coc, $p = 0.9909$.

4.5 Western Blot Analysis of D2DR in NAc (Sensitization brains)

Rats treated with nandrolone showed increased expression of D2DR in the NAc (Fig 6B and 6D). Cocaine treatment decreased the expression of D2DR in this brain area; this decrease was more pronounced in animals that received nandrolone (Fig.6B and 6D: One Way ANOVA, Tukey's multiple comparisons, $F(3, 12) = 84.90$, < 0.0001 ; Oil-Sal vs ND-Sal, $p = 0.0002$; Oil-Sal vs Oil-Coc, $p = 0.0339$; Oil-Sal vs ND-Coc, $p < 0.0001$; ND-Sal vs Oil-Coc, $p < 0.0001$; ND-Sal vs ND-Coc, $p < 0.0001$; Oil-Coc vs ND-Coc, $p = 0.0002$.

4.6 Conditioned Place Preference

All animals that were tested displayed CPP to cocaine, however nandrolone decreased the time spent in the chamber associated with cocaine (Fig 7). Rats treated with oil-cocaine displayed CPP to cocaine more robustly than nandrolone-cocaine treated rats (Fig.7). During postconditioning, oil treated rats spent 70% of their time in the side

associated with cocaine vs 31% during the preconditioning phase. In contrast, rats treated with nandrolone spent 48% of their time during postconditioning in the chamber associated with the drug vs 30% during preconditioning. Data were analyzed with a Two-Way ANOVA, with pre and post-conditioning as repeated measures = $F(1, 62) = 28.22$, $p < 0.0001$ and Cocaine as the independent factor. Cocaine's effect: $F(3, 62) = 21.97$, $p < 0.0001$; Oil-Coc pre vs post conditioning, $p < 0.0001$; ND-Coc pre vs post conditioning, $p = 0.0397$; Oil-Coc post vs ND- Coc post conditioning, $p = 0.0035$.

4.7 Western Blot Analysis for D2DR in mPFC (CPP brains)

Similar to our previous results, rats that received cocaine had decreased D2DR in the PFC compared to saline treated rats (Fig 8A y 8C). However, the brains from nandrolone-treated males used in the CPP experiments had more D2DR in the PFC than oil-treated rats (Fig 8A y 8C). The difference from these two experiments is that the animals used for the CPP experiments were killed at day 53 and those used for the sensitization experiments were sacrificed 11 days later, at day 64. In addition, for the sensitization studies rats received 7 cocaine injections (days 1-5, 13 and 23), whereas for the CPP experiments rats received 5 cocaine injections (days 2,4,6,8,10). Fig. 8C: One Way ANOVA, Tukey's multiple comparisons, $F(3, 12) = 28.78$, <0.0001 ; Oil-Sal vs ND-Sal, $p = 0.1752$; Oil-Sal vs Oil-Coc, $p = 0.0013$; Oil-Sal vs ND-Coc, $p = 0.0007$; ND-Sal vs Oil-Coc, $p < 0.0001$; ND-Sal vs ND-Coc, $p < 0.0001$; Oil-Coc vs ND-Coc, $p = 0.9825$.

4.8 Western Blot Analysis for D2DR in NAc (CPP brains)

Similar to the results obtained from the brains of animals sensitized to cocaine (Fig 6D) CPP rats treated with nandrolone showed increased expression of D2DR in the NAc. In contrast, cocaine treatment decreased the expression of D2DR in this brain area, similar to our previous results. Fig.8D: One Way ANOVA, Tukey's multiple comparisons, $F(3, 12) = 45.87$, $p < 0.0001$; Oil-Sal vs ND-Sal, $p = 0.0316$; Oil-Sal vs Oil-Coc, $p < 0.0001$; Oil-Sal vs ND-Coc, $p = 0.0001$; ND-Sal vs Oil-Coc, $p < 0.0001$; ND-Sal vs ND-Coc, $p < 0.0001$; Oil-Coc vs ND-Coc, $p = 0.6828$.

5. Discussion

5.1 Summary of results

Nandrolone was administered prior to puberty (PN 28-37) and the effects on anxiety and risk-taking behaviors (days 38 and 39), and on the response to cocaine (day 40) was assessed immediately after nandrolone treatment, not after a withdrawal period. We found that administration of nandrolone decanoate decreased anxiety and altered addictive-like behaviors in male rats.

Prepubertal (PN 28-37) males treated with nandrolone showed reduced anxiety and basal locomotor activity compared to oil-treated males. In contrast, nandrolone increased risk-taking behavior and the locomotor response to cocaine on day 5 and accelerated the development of cocaine sensitization in young animals (i.e., they required less cocaine injections). Nandrolone also attenuated CPP to cocaine. Changes in D2DR in the NAc may partially mediate these behavioral changes: an increase in D2DR in the NAc was observed in nandrolone-treated males, whereas those treated with cocaine or with nandrolone and cocaine had less D2DR in the NAc and PFC.

5.2 Anxiety and risk-behaviors

Previous studies have found that the use of AAS is associated with adverse effects on emotion, cognition, and rewarding behaviors (Grönbladh et al., 2016). Clinical studies indicate that most of the effects of AAS on anxiety appear after cessation of use, which may be at the end of an AAS cycle, or after longer withdrawal periods (Pope et al., 1996). People who feel depressed or are experiencing withdrawal symptoms, are more likely to revert and use anabolic steroids or other drugs of abuse (Malone et al., 1995).

Other studies indicate that 60% of children born of mothers with polycystic ovarian syndrome, which results in increased androgen exposure during fetal development, have a higher prevalence of psychiatric disorders, such as depression and anxiety (Dokras et al., 2012). Additional studies in rodents confirm that exposure to excessive androgens during prenatal development increases the number of offspring that subsequently display anxiety behaviors (Hu et al., 2015). In addition, several psychiatric disorders like anxiety, depression, mood disorders, psychosis and substance abuse tend to manifest themselves close to puberty (Paus et al., 2008)

Very few studies have examined the effects of AAS administered prepubertally on emotional behaviors, although the use of AAS in this population is very high. Our study found that nandrolone exposure prepubertally decreased anxiety-like behaviors. Rats treated with nandrolone showed a decrease in the time spent in the closed arms of an EPM and an increase in the center of an open field compared to oil-treated rats, behaviors associated with decreased anxiety.

This increase in time spent in the center of an open field is associated with increased risk-taking behavior as animals that venture more into the center of an open field are at greater risk of being detected by predators.

Previous studies such as Kouvelas et al (2008) have found that nandrolone administration (15mg/kg) to adult rats decreased anxiety as measured in the EPM. Decreased anxiety following androgen administration has also been reported by others (Aikey et al., 2002; Bitran et al., 1993; Rainer et al., 2014). This anxiety-lowering effect has also been seen with androgens such as testosterone and DHT (Bitran et al., 1993; Edinger and Frye, 2006).

However, it is not unequivocally established whether AAS have anxiolytic or anxiogenic properties since several studies in adult male rats (Rosic et al., 2014) or in adolescent rats tested as adults (Ricci et al., 2012; Morrison et al., 2015) report that AAS exert anxiogenic effects (Ricci et al., 2012; Morrison et al., 2015).

The difference between studies investigating the effect of AAS on anxiety may vary depending on the type of androgen administered, the age of exposure, the duration of treatment, and the dose administered. These last studies used mainly an aromatizable form of androgen, and although the doses used were smaller (5 mg/kg vs 20 mg/kg), as well as the total dose administered (60, 150 or 175 mg/kg vs 200 mg/kg) the duration of treatment was longer (30-35 days vs 10 days). In addition, our current study is unique in that nandrolone was administered prior to puberty (PN 28-37) and the effects on anxiety and risk-taking behaviors assessed the day after the last injection (days 38 and 39) and not after a withdrawal period.

Studies that examined the role of androgens on anxiety report that androgen receptors, especially those in hippocampal tissue, appear to play a significant role in mediating this response (Edinger and Frye, 2004). Kouvelas et al., (2008) found that the anxiolytic effect of AAS was abolished by treatment with flutamide, an androgen receptor inhibitor.

A correlation between high-risk behaviors in adolescents and testosterone has also been established. For example, a positive correlation was found between salivary testosterone and activation of the nucleus accumbens in response to a monetary reward in adolescent boys ages 10 to 16 (Op De MacKs et al., 2011). Not only do testosterone levels correlate positively with receiving a financial reward, but AAS users also engage more frequently in other risky behaviors such as gambling.

5.3 Locomotor activity

Rats treated with nandrolone showed a decrease in distance traveled in an open field and a reduction in the total number of arm entries when tested in an EPM, indicative of lower ambulation. Lower locomotor activity of nandrolone-treated males was also evident during the first 10 min of habituation (day 0) and on day 1 of the sensitization trial (Fig.3C). This effect of nandrolone on locomotor activity appears to be related to novelty-induced exploration since it was not observed upon further testing (days 5, 13 and 23).

Most studies agree that androgens decrease ambulation. In a similar study, prepubertal males treated with nandrolone (15 mg/kg) for 30 days showed reduced locomotor activity in an open field and EPM (El-Shamarka et al., 2020). In adults, nandrolone is reported to decrease activity in the running wheel (Keleta et al., 2007), in

an open field (Rosic et al., 2014; Selakovic et al., 2017) and in the EPM (Minkin et al., 1993). Nonetheless there are some studies that fail to find an effect (Salvador et al., 1999), and others that report a decrease in cocaine-induced locomotor activity (Kurling-Kailanto et al., 2010) after nandrolone withdrawal.

The mechanism by which nandrolone decreases locomotor activity is not clear. Purves-Tyson et al (2012) have reported that testosterone administration to prepubertal rats modulates the expression of enzymes that participate in dopaminergic metabolism in brain regions, such as the substantia nigra, that regulate locomotion and affect. Others report that the addictive properties of AAS may be exerted through the endogenous opioid system that in turn stimulate dopaminergic centers in the brain (Bontempi and Bonci, 2020).

5.4 AAS and cocaine

Several studies indicate that AAS users are likely to develop a dependence on other drugs of abuse (Mędraś et al., 2018; Pope et al., 2014a). These results must be interpreted with caution since, in some cases, AAS users abused other drugs prior to using AAS. In addition, studies in adult rodents investigating the role of androgens in modulating the response to drugs of abuse have provided conflicting reports.

Prior studies from our laboratory and of others indicate that removal of the primary source of androgens by gonadectomy increases the response to psychostimulants (Beatty et al., 1982; Dluzen et al., 1986; Forgie and Wart, 1994; Menéndez-Delmestre and Segarra, 2011; Purves-Tyson et al., 2015), although not all studies have obtained similar results (Caine et al., 2004; Chen et al., 2003; Chin et al.,

2002; Haney et al., 1994; Harrod et al., 2005; Jackson et al., 2006). Furthermore, testosterone administration to adult males is reported to decrease the acute locomotor response to cocaine and amphetamine in gonadectomized (Menéndez-Delmestre and Segarra, 2011; Purves-Tyson et al., 2015) and gonadally intact drug-naive males (Beatty et al., 1982; Long et al., 1994). Indeed, our laboratory has found that testosterone is necessary for adult male rats to develop and express sensitization to cocaine (Menéndez-Delmestre and Segarra, 2011).

Several studies indicate that prepubertal male rats do not become sensitized to cocaine at this early age (Kabbaj et al., 2002; Lepsch et al., 2005; Trzcińska et al., 2002). Indeed, our control rats do not show sensitization until after a withdrawal period and re-exposure to cocaine. Oil-cocaine rats displayed sensitization when they were 52 days of age. At this time, they had received 5 daily cocaine injections, a 7-day withdrawal period and a cocaine challenge (day 13 of test). Sensitization in these oil-treated animals became more robust after a second withdrawal period and re-exposure to cocaine (day 23), when they were 62 days of age (Fig 4C). In contrast, we found that exposure to nandrolone during days 28-37 accelerated the development of sensitization, which was evident after 5 cocaine injections, when they are 44 days of age (Fig 4D).

We also observed that the hyperactivity induced by cocaine is maintained for a longer time period in nandrolone-treated males during the first cocaine challenge (day 13). It is unclear if this effect can be attributed to changes in the metabolism of cocaine. Cocaine and nandrolone are metabolized mainly in the liver, cocaine by esterases and cytochrome P450 enzymes, whereas metabolism of nandrolone occurs mainly by 5 α -

reductase and 3 α - and 3 β -hydroxysteroid dehydrogenase enzymes. Thus, it is not surprising that pharmacokinetic interactions between the compounds have been observed. Synergistic effects on the cardiovascular system (Tseng et al, 1994) and seizures (Long et al (2000), as well as alterations in brain dopaminergic and serotonergic outflow (Kurling-Kailanto et al, 2010), have been reported. Unfortunately, although areas of the mesocorticolimbic pathway are rich in androgen receptors (Tobiansky et al., 2018), very few studies have investigated if androgens alter the metabolism of cocaine (Bowman et al., 1999; Lukas et al., 1996). Another possibility is that exposure to androgens during the prepubertal period accelerates the maturation of some component of the mesocorticolimbic circuitry essential for the display of behavioral sensitization. For example, the cytochrome P450 system is involved in the metabolism of androgens and cocaine (McDonnell and Dang, 2013); thus, both drugs may interact pharmacokinetically and affect the individual response of each drug. A study by Yamamoto et al (2007) indicates that treatment with the antiandrogen flutamide decreases plasma levels of cocaine and its main metabolites, suggesting that testosterone may potentiate the effects of cocaine, as we have previously reported (Menéndez-Delmestre and Segarra, 2011).

Our data also indicates that prior exposure to AAS increases the behavioral response to cocaine, a process known as cross sensitization. Cross sensitization occurs with previous exposure between the same drug and also between different drugs of abuse (Smith et al., 2013). The mechanisms that mediate cross sensitization are not fully understood. Evidence suggests that dopamine is the substrate involved in this process (Kalivas and Stewart, 1991). Studies found that androgens like testosterone

induce cross sensitization to cocaine in prepubertal, but not in adult male rats (Engi et al., 2015).

One scenario may be that AAS promotes cross sensitization by activating gene expression of corticotropin releasing hormone (CRH). The gene that codes for CRH contains androgen and estrogen response elements that modulate expression of CRH (Bao et al., 2006; Bao and Swaab, 2007). Indeed, we have recently reported that altered corticotropin-releasing hormone receptor 1 (CRF-R1) sensitivity may lead to the observed DA hyperresponsiveness observed in socially isolated adolescent rats (Novoa et al., 2021).

5.5 CPP

All rats tested between days 40 to 53 days developed CPP to cocaine. In addition, oil-cocaine rats spent more time in the chamber associated with cocaine during the post-conditioning day compared to nandrolone-treated rats. The increase in time spent in the chamber associated with cocaine was 124% in oil-cocaine rats compared to 57% in those pretreated with nandrolone. Thus, although both groups of rats showed CPP to cocaine, CPP was lower in rats that previously received nandrolone.

Our studies agree with that of others that report a decrease in CPP to other drugs of abuse such as cannabinoids (Celerier et al., 2006) and opioids (Célérier et al. 2003) following AAS administration. In addition, exposure to nandrolone (15 mg/kg) during PN 40-53 decreased sucrose consumption (Rainer et al, 2014) 15 days after withdrawal. Nonetheless, there are studies that indicate that withdrawal from nandrolone increases cannabinoids (Struik et al., 2016) and alcohol (Johansson et al.

2000) intake. Thus, there is no consensus indicating if prior exposure to nandrolone results in an enhancement or an aversion to other drugs of abuse or rewarding stimuli such as sucrose. However, there is evidence that the addictive properties of AAS may be exerted through the endogenous opioid system that in turn stimulate dopaminergic centers in the brain (Bontempi and Bonci, 2020).

The CPP paradigm involves several cognition components such as acquisition, retrieval, and extinction of spatial and contextual memories (Anagnostaras et al., 1999; Gould and Leach, 2014; Riedel et al., 1999). Several lines of evidence link the use of AAS with cognitive dysfunction (Wallin and Wood, 2015) and altered decision-making when studied in paradigms such as the operant discounting task (Cooper et al., 2014; Wallin-Miller et al., 2018; Wallin et al., 2015; Wood et al., 2013).

We cannot discard the possibility that during the CPP test, the reduced time spent in the chamber associated with cocaine is due to a decrease in motivation. However, the increased sensitized locomotor response to cocaine displayed at day 5 by animals that received nandrolone elicit uncertainty on this possibility. Sensitization has been defined as the successive augmentation of locomotor hyperactivity elicited by repeated administration of psychostimulants. It involves neuroadaptations in the mesocorticolimbic system that contribute to changes in the motivational circuitry underlying craving and relapse (Chefer et al., 2005; Kumar et al., 2005; Thomas et al., 2008). Since many investigators relate sensitization with increased motivation, and nandrolone-treated animals displayed increased sensitization, we are inclined to favor alterations in cognitive, and not rewarding, aspects of CPP. In addition, the rewarding properties of AAS are evident in previous studies that find that rodents will self-

administer AAS orally and intracranially (Johnson and Wood, 2001; Wood, 2004), an effect that disappears with administration of an androgen receptor antagonist (Peters and Wood, 2005).

5.6 AAS and D2DR

At the conclusion of the sensitization and CPP experiments, we investigated if D2DR receptors in the NAc and mPFC were affected by prior treatment with nandrolone, cocaine or both. Separate groups of animals were used for the above-mentioned experiments. Rats used for CPP received 5 cocaine injections (15 mg/kg) one every other day for 10 days and killed 24 hrs after the last cocaine injection. Rats used for the sensitization experiments received 7 cocaine injections (15 mg/kg) during a 23 day period: 5 daily injections, a 7 day withdrawal period, 1 challenge injection, a 9 day withdrawal period, a second challenge injection and euthanized 24 hours after the last cocaine injection. Thus, the sensitization group received two additional cocaine injections and underwent two drug-free periods of 7 and 9 days prior to euthanasia.

5.7 D2DR in the NAc of rats used for the sensitization experiments

Rats treated with nandrolone (days 28-37) had a higher concentration of D2DR in the NAc at the day of euthanasia (days 54 and 63) compared to oil-saline rats. In contrast, all groups that received cocaine had lower levels of D2DR compared to oil saline rats. Interestingly, groups that were injected with nandrolone (days 28-37) and used for the sensitization experiments (euthanized at day 63) had the lowest levels of D2DR in the NAc of all groups.

Behavioral sensitization has two phases, initiation, and expression. The initiation comprises rapid neural effects that induce behavioral sensitization, the expression

involves the long-term consequences (Kalivas and Stewart, 1991). Initiation of behavioral sensitization does not require activation of dopamine receptors (White et al., 1998), however its expression does (Steketee and Kalivas, 2011; Thomas et al., 2001; Vanderschuren and Kalivas, 2000). Most studies agree that the NAc, although not essential for the development of locomotor sensitization to cocaine, is necessary for its expression (Di Chiara, 1995). Unfortunately, the role of each dopaminergic receptor subtype in the process of sensitization is still not entirely clear.

Some studies report that D2DR in the NAc do not affect cocaine-induced locomotor activity nor behavioral sensitization (Beyer and Steketee, 2002; Mattingly et al., 1994; Vanderschuren and Kalivas, 2000). However, other studies attest to D2DRs modulation of cocaine-induced sensitization (Di Chiara, 1995). For example, blocking D2DR receptors in the NAc significantly decreased cocaine-induced locomotor activity (Baker et al., 1996; Manvich et al., 2019; Neisewander et al., 1998) and abolished cocaine-induced sensitization (Manvich et al., 2019). Furthermore, deletion of D2DR in medium spiny neurons (MSN) of the NAc in mice results in decreased cocaine-induced locomotor activity (Smith et al., 2013). In contrast, experiments using the conditional mutant mice “autodrd2KO”, which is characterized by a lack of D2DR auto receptors (those in dopamine neurons and terminals), indicate that sensitivity to cocaine is enhanced in these animals (Bello et al., 2011). Mice lacking D2DR auto receptors also show enhanced CPP as well as hyperlocomotion (without altering dopamine transporters function) (Bello et al., 2011).

These data argue that the process by which D2DRs in the NAc modulate the response to cocaine can vary depending if these receptors are located on dopaminergic

terminals or GABAergic MSN in the NAc and may partly explain several conflicting reports. It is possible that the observed decrease in D2DR in the NAc in this study occurred mainly in dopaminergic terminals. Repeated exposure to cocaine during sensitization would transiently result in increased dopamine (DA) that in turn would induce D2DR downregulation. D2DRs have a greater affinity for DA than D1DR (Dreyer et al., 2010; Rice and Cragg, 2008). This would result in less dopaminergic autoinhibition and greater, or extended, DA release, resulting in an exacerbated locomotor response to cocaine, as we observed in the current study. It is also possible that DA, by modulating AMPA trafficking, contributes to the enhancement of sensitization (Boudreau and Wolf, 2005). However, more studies are needed to determine the cell type or terminal where the decrease in D2DR occurred.

In addition, nandrolone treatment increased D2DR in the NAc. This group of animals displayed the highest locomotor activity in response to cocaine, and developed sensitization earlier than oil-treated rats, (after 5 cocaine injections). These data are in accordance with previous studies that show that D2DR availability predicts future drug-seeking behavior (Haney et al., 1994)

5.8 Androgens modulate D2DR

Androgens can modulate dopamine receptors in the mesocorticolimbic circuitry (Aubele and Kritzer, 2012; Bertozzi et al., 2018; Purves-Tyson et al., 2012; Tobiansky et al., 2018). Kindlundh et al (2001) found that administering nandrolone for two weeks at low doses (1 and 5 mg/kg) increased D2DR in the NAc core and shell of male rats but found that a higher dose (15 mg/kg) had no effect. The authors of this last study did not state the age, nor weight, of the rats. In our study, rats were 28 days of age when they

received the first nandrolone injection, and the dose used was 20 mg/kg. Thus, we cannot determine if the difference between these two studies is dose or age-related.

Nandrolone can also alter DA metabolism (Zotti et al., 2014). A decrease in the activity of the dopamine-metabolizing enzymes monoamine oxidase A and B has been reported after nandrolone treatment (Birgner et al., 2008, 2007). Furthermore, levels of tyrosine hydroxylase in the substantia nigra increase prior to puberty, coinciding with the increase in testosterone (Purves -Tyson et al., 2012).

The Akil group, among others, have selectively bred rats according to their initial response to a novel environment and classified them as high responders (HR) and low responders (LR) (Aydin et al., 2021; Birt et al., 2021; Clinton et al., 2012; Turner et al., 2009). These two lines also show differences in behavioral traits relevant to addiction, with HR displaying a greater amount of drug taken, persistence of drug-seeking and drug-induced locomotor activity (Flagel et al., 2016). They also differ in the neural substrates that regulate addictive behaviors. Like the nandrolone-treated males in this study, HRs have higher D2DR in the NAc core compared to LR (Clinton et al., 2012). HRs also have greater fibroblast growth factor 2 (FGF2) and lower D1DR levels in the NAc. Interestingly, previous studies show that testosterone increases plasma levels of FGF2 and of Insulin Growth Factor (Ghanim et al., 2019) and mRNA expression of FGF2 *in vitro* (Saito et al., 1991). Dysregulation of neurotrophic factors like FGF2 is involved in increased vulnerability to drugs (McGinty et al., 2010; Thomas et al., 2008). Indeed, drugs such as amphetamines and cocaine modulate the expression of FGF2 (Mueller et al., 2006; Turner et al., 2009). This may explain the synergistic effect of nandrolone and cocaine in decreasing D2DR in the NAc.

5.9 Prefrontal cortex

The mPFC plays an important role in addictive behaviors such as decision making, memory retrieval and cocaine seeking behaviors and is necessary for the induction of sensitization to cocaine (Cador et al., 1999; Li et al., 1999; Dalley et al., 2004; Moorman et al., 2015). It contains a distinct population of glutamatergic pyramidal neurons that project to the striatum and other subcortical regions that express D1DR and D2DR (Gaspar et al., 1995). Dopaminergic modulation of glutamatergic function contributes to reward, salience, attention, and working memory (Brozoski et al., 1979; Chudasama and Robbins, 2004; Lohani et al., 2019; Yokel and Wise, 1975). Recent evidence indicates that DA modulates ensemble activity, facilitating and strengthening information processing in the PFC (Lohani et al., 2019).

Maturation of PFC circuitry continues after puberty, dopaminergic innervation from the VTA to the PFC increases gradually until approximately PN day 60 (Salas et al., 2016). At approximately this age, males have acquired adult testosterone plasma levels and the ability to display male sexual behavior (Segarra and Strand, 1989). An increase in dendritic spine density following androgen or estrogen administration suggest that gonadal steroids play a role in PFC function (Hajszan et al., 2007). Interestingly, on day 40, an increase in D2DR receptors in the PFC is observed, coinciding with the activation of the HPG axis in male rats (Andersen et al., 2000). It is possible that in our current study, that administration of nandrolone from day 28 to 37 accelerated maturation of the motivational circuitry in the PFC, which in turn could be responsible for the expression of sensitization at a younger age.

Summary:

In summary, this study provides evidence that exposure to the AAS nandrolone prepubertally increases risk-taking behaviors and decreases anxiety and locomotor activity. Nandrolone also exacerbated the locomotor response to cocaine and accelerated the development of cocaine sensitization, occurring at an earlier age and with less cocaine injections. It highlights the role of androgens in accelerating the development of brain structures that participate in the psychomotor response to drugs of abuse, as illustrated by the display of cocaine-induced behavioral sensitization in nandrolone-treated prepubertal males. This study also confirms that the effect of AAS administration prepubertally on the remodeling of neural circuitry may occur quite rapidly after exposure. Changes in accumbal dopaminergic receptors hint at androgenic modulation of mesocorticolimbic substrates during early adolescent development. The high prevalence of AAS abuse worldwide and the long-lasting and detrimental effects on essential physiological processes and neural circuitries that regulate addictive and social behaviors warrants further studies.

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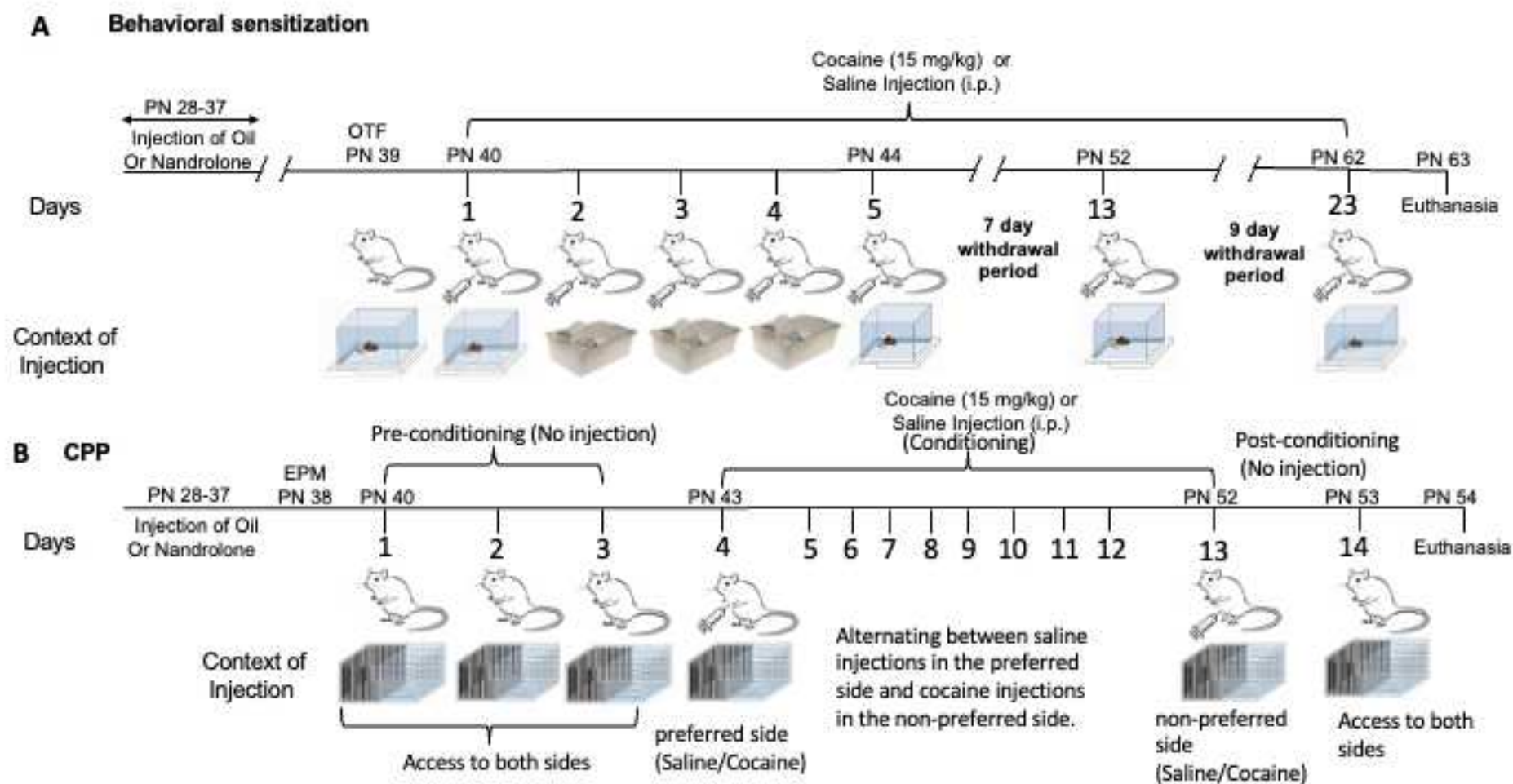


Figure 1. Behavioral sensitization (A) and CPP (B) protocols.

Fig 1A. Five days after rats were weaned from their mother, they received a daily oil or nandrolone injection for ten consecutive days (PN-28 to 37). For the behavioral sensitization experiments, rats were habituated to the locomotor activity chamber for 1 hour (2 days post-nandrolone day, PN-39). The data obtained from the first ten minutes of habituation (Day 0) and of Day 1 was used as data for the open field test (Figs 2B and Fig 2C). From days 1 through 5, on day 13 and on day 23, rats were injected with saline or with cocaine (15 mg/kg). Rats were injected in the locomotor activity chamber (days 1, 5, 13 and 23) or in their home cage (days 2, 3 and 4) (see the context of injection). From days 6 through 12 and from days 14 through day 22, rats remained undisturbed in their home cages.

Fig 1B. Five days after rats were weaned from their mother, they received a daily oil or nandrolone injection for ten consecutive days (PN-28 to 37). On day 2 (PN-38) a second group of rats were tested in the elevated plus maze (EPM). These same rats were used for the CPP experiments. To determine the rat's preference for a particular CPP chamber, rats were allowed to roam freely through both chambers for 3 days (pre-conditioning). The amount of time spent in each chamber was calculated to determine which side it preferred. The following 10 days (days 4–13, conditioning days) rats were injected daily alternating between saline and cocaine (15 mg/kg) injections. Rats received cocaine in the non-preferred chamber and saline in the preferred chamber. Saline animals received saline in both chambers. After the injection, rats were confined for 30 min to the chamber where they received the injection. The last day (day 14, post-conditioning day), the animals were placed in the activity chamber and allowed to roam freely between the two chambers for 30 minutes. The amount of time spent in each chamber was recorded and compared to that of pre-conditioning.

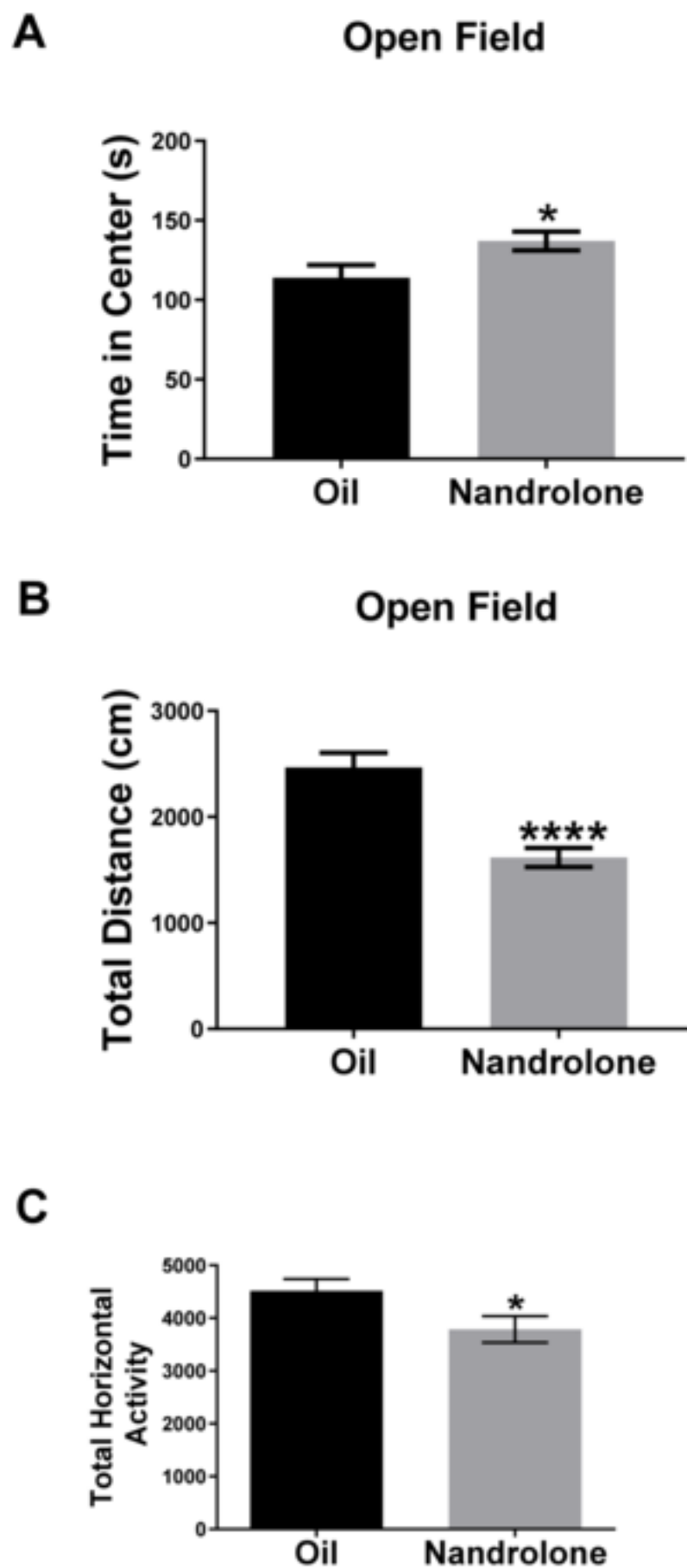


Figure 2. Open field activity of prepubertal rats exposed to nandrolone

Prepubertal males were injected daily with nandrolone (20 mg/kg) or sesame oil from PN 28-37. On days 0 and 1 (PN 39 and PN 40; Fig 1A), rats were tested for open field behavior. Rats were placed in the activity cage and the time spent in the center of the field, as well as the distance travelled, was recorded for 10 min using the Accuscan Versamax monitoring system. Rats treated with nandrolone (20 mg/kg) spent more time in the center of the open field (Fig 2A) and travelled less distance on days 0 (Fig 2B) and 1 (Fig 2C) than oil treated rats. Data are presented as mean \pm SEM (n=10) and were analyzed with a Student's t-Test, asterisks represent a significant difference compared to Oil group. See Table 1 for detailed statistical analysis.

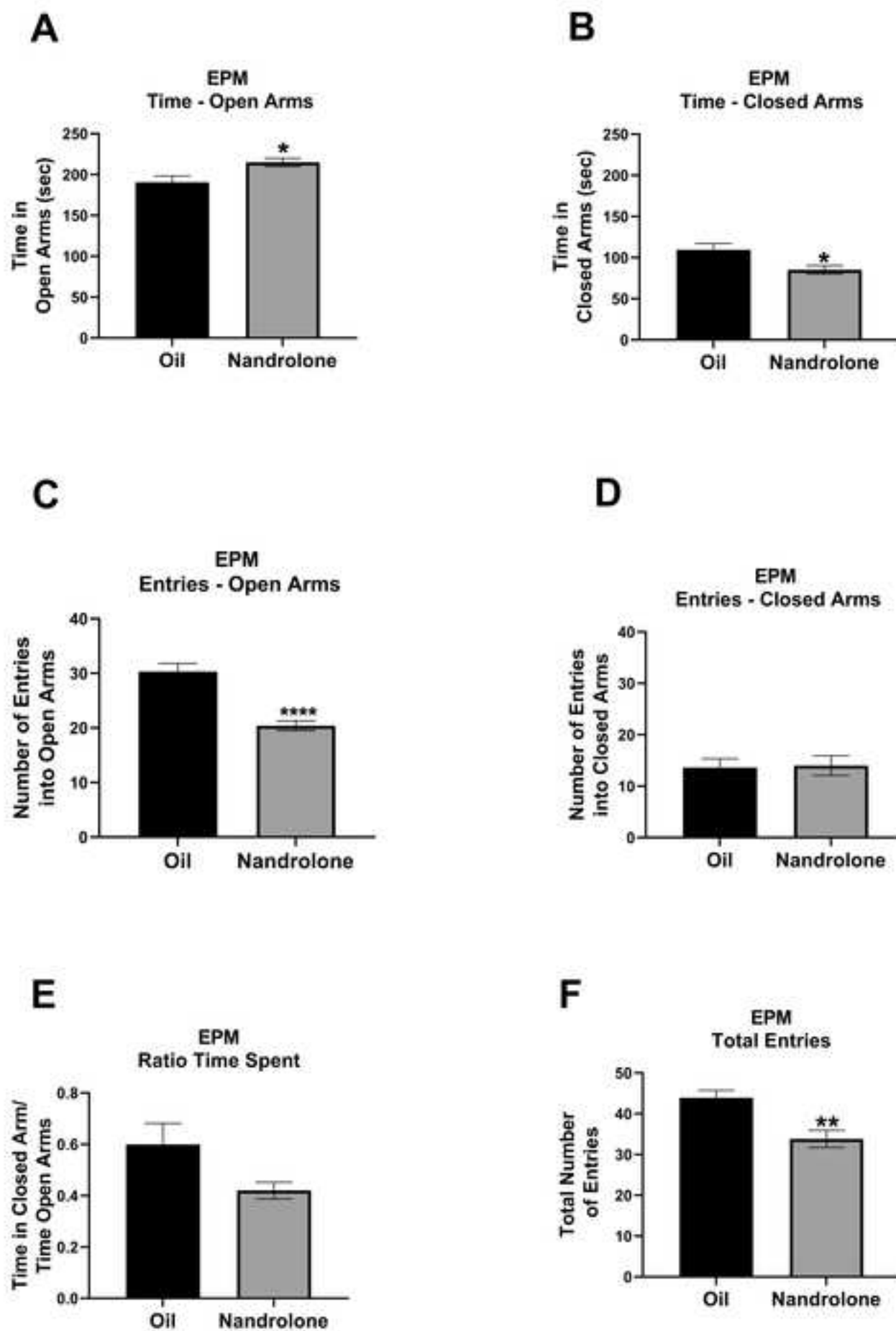


Figure 3. Results from the EPM test of prepubertal rats treated with nandrolone

Male rats were injected daily with nandrolone (20 mg/kg) or sesame oil from PN 28-37 and tested in the elevated plus maze on day 38. Rats were placed at the junction between the open and closed arms and behavioral activity was recorded for 5 min using the ANY-Maze™ tracking software. Rats treated with nandrolone spent less time in the closed arms (Fig 3A), an indication of decreased anxiety. In addition, rats that received nandrolone had fewer total entries into both arms than oil-treated rats (Fig 3B), an indication of reduced ambulation. Data are presented as mean \pm SEM (n=10) and analyzed with a Student t-Test. Asterisks represent a significant difference compared to Oil treated rats.

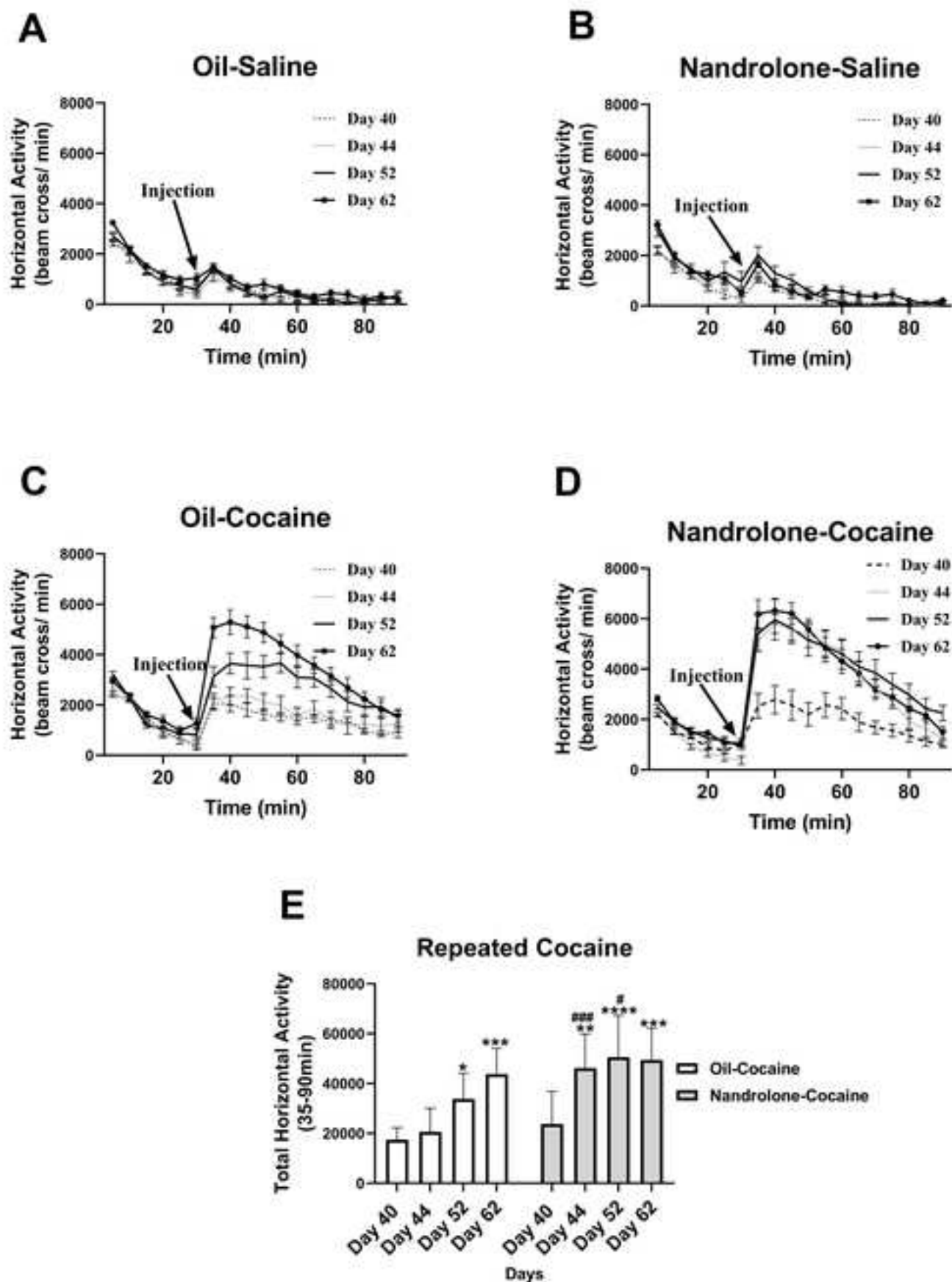


Figure 4. Cocaine-induced locomotor activity of rats exposed to nandrolone during postnatal days 28-37.

Cocaine-induced locomotor activity of prepubertal rats injected daily from PN 28-37 with nandrolone (20 mg/kg) or sesame oil and tested for behavioral sensitization to cocaine from PN 40-62. **Figs 4A and 4B.** Timecourse of locomotor activity of saline (Fig 4A) and nandrolone (Fig 4B) treated males. No differences between oil and nandrolone-treated males were observed. **Fig 4C.** A significant increase in cocaine-induced locomotor activity was observed when comparing the timecourses of day 40 vs day 52 and day 40 vs day 62 in Oil-treated males. Thus, these males displayed behavioral sensitization by day 52. **Fig 4D.** A significant increase in cocaine-induced locomotor activity was observed when comparing the timecourses of day 40 vs day 44, vs day 52 and vs day 62 in nandrolone-treated males. These results suggest that nandrolone accelerates the maturation of the neural circuitry that regulates behavioral sensitization. Data are presented as mean \pm SEM and analyzed with a Repeated Measures ANOVA using Tukey's multiple comparison for post-hoc analysis. (See Supplemental Tables 1, 2, and 4 for complete statistical analysis values). **Fig 4E.** Rats that received nandrolone show a robust increase in cumulative cocaine-induced locomotor activity by day 44, an increase that is maintained after two withdrawal periods. In contrast, it is at day 52 that oil-treated rats show a difference that is further increased by day 62. Data are presented as mean \pm SEM (n=10). Repeated Measures Two-Way ANOVA: $F_{(3,27)} = 4.173$, $p = 0.0150$. **Oil-Coc:** Day 40 vs Day 44, $p = 0.9968$; Day 40 vs Day 52, $p = 0.0288$; Day 40 vs Day 62, $p = 0.0001$. **ND-Coc:** Day 40 vs Day 44, $p = 0.0011$; Day 40 vs Day 52, $p = <0.0001$; Day 40 vs Day 62 $p = 0.0002$. **Oil-Coc vs ND-Coc:** Day 40, $p = 0.8684$; Day 44, $p = 0.0002$; Day 52, $p = 0.0230$; Day 62 $p = 0.9209$. $P < 0.05$ was considered statistically significant Day 40 vs. Day 44, Day 52 and Day 62. (*): significantly different within groups; (#): significantly different between groups (Oil-Cocaine vs Nandrolone-Cocaine).

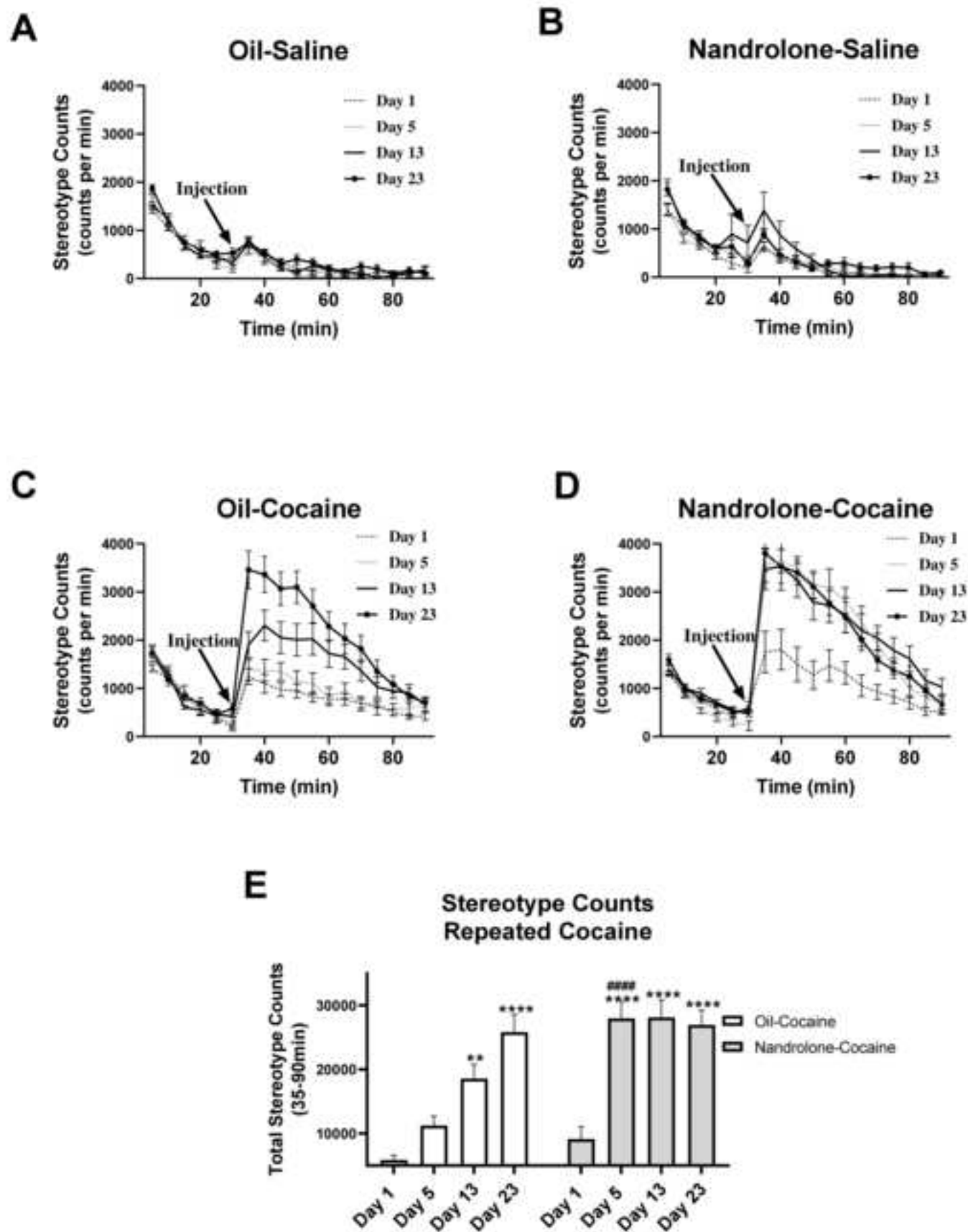


Figure 5. Cocaine-induced stereotyped activity of rats exposed to nandrolone during postnatal days 28-37.

Cocaine-induced stereotyped activity of prepubertal rats injected daily from PN 28-37 with nandrolone (20 mg/kg) or sesame oil and tested for behavioral sensitization to cocaine from PN 40-62. **Figs 5A and 5B.** Timecourse of stereotyped activity of saline (Fig 5A) and nandrolone (Fig 5B) treated males. No differences between oil and nandrolone-treated males were observed. **Fig 5C.** A significant increase in cocaine-induced stereotyped activity was observed when comparing the timecourses of day 40 vs day 52 and day 40 vs day 62 in Oil-treated males. Thus, saline males displayed behavioral sensitization by day 52. **Fig 5D.** A significant increase in cocaine-induced locomotor activity was observed when comparing the timecourses of day 40 vs day 44, vs day 52 and vs day 62 in nandrolone-treated males. Similar to what we observed with total horizontal activity, these results indicate that nandrolone accelerates the development of behavioral sensitization. Data are presented as mean \pm SEM (n=10) and analyzed with a Repeated Measures Two-Way ANOVA using Tukey's multiple comparison for post-hoc analysis. See Tables 1, 2, and 3 for complete statistical analysis values. **Fig 5E.** Rats that received nandrolone show a robust increase in cumulative cocaine-induced stereotyped activity by day 44, an increase that is maintained after two withdrawal periods. In contrast, it is at day 52 that oil-treated rats show a difference that is further increased by day 62. Data are presented as mean \pm SEM. Repeated Measures Two-Way ANOVA: $F_{(3,27)} = 6.746$, $p = 0.0015$. **Oil-Coc:** Day 40 vs Day 44, $p = 0.5042$; Day 40 vs Day 52, $p = 0.015$; Day 40 vs Day 62, $p < 0.0001$. **ND-Coc:** Day 40 vs Day 44, $p < 0.0001$; Day 40 vs 52, $p = <0.0001$; Day 40 vs Day 62, $p < 0.0002$. **Oil-Coc vs ND- Coc:** Day 40, $p = 0.9224$; Day 44, $p < 0.0001$; Day 52, $p = 0.0281$; Day 62 $p = 0.9999$. $P < 0.05$ was considered statistically significant Day 40 vs. Day 44, Day 52 and Day 62. (*): significantly different within groups; (#): significantly different between groups (Oil-Cocaine vs Nandrolone-Cocaine).

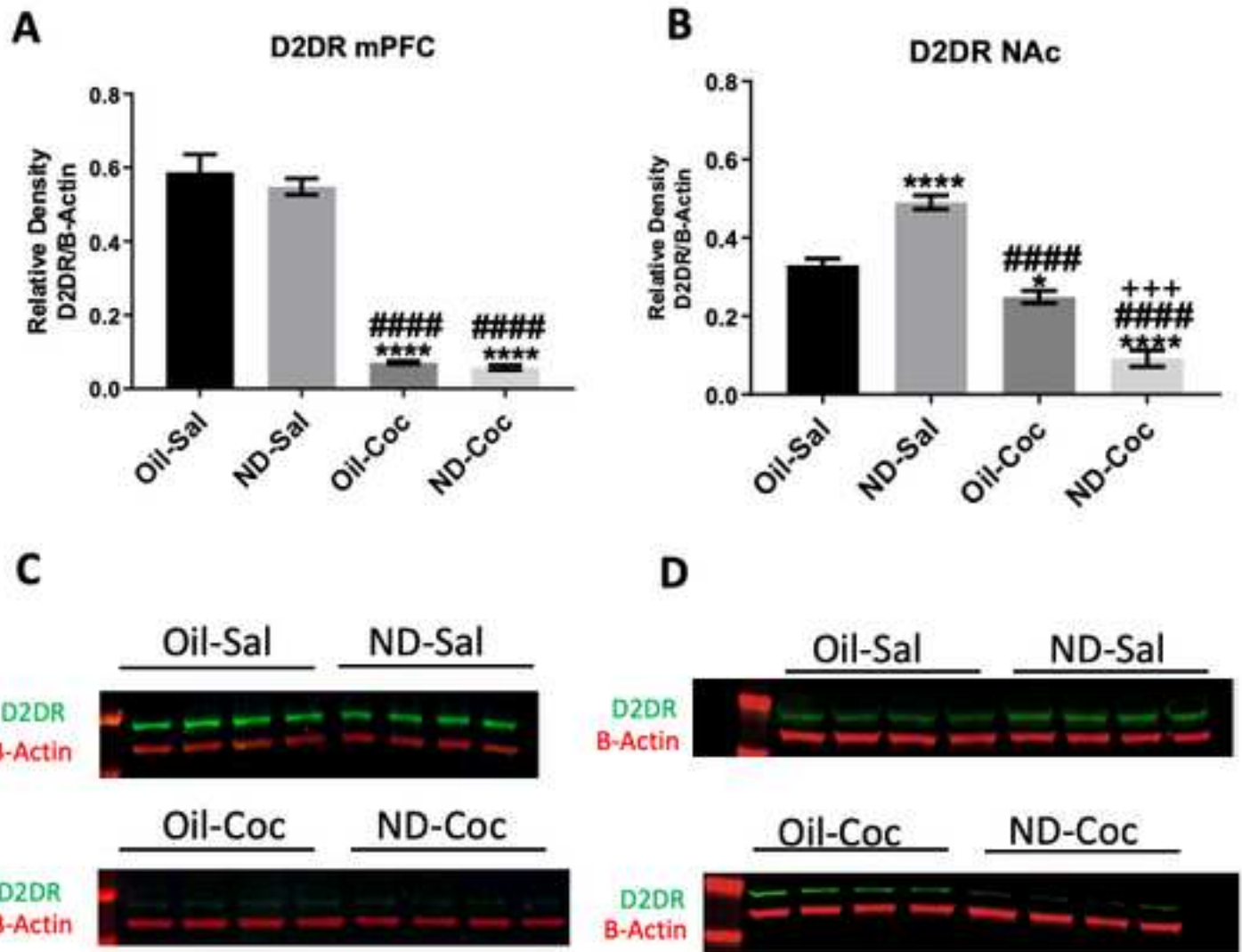


Figure 6. D2DR in the mPFC and NAc of rats exposed to nandrolone during postnatal days 28-37 and tested for behavioral sensitization

Fig. 6A and Fig 6C: Nandrolone did not affect D2DR expression in the mPFC, however a decrease in D2DR expression in the mPFC was observed in animals treated with cocaine. **Fig. 6B and Fig 6D:** In contrast, nandrolone treatment increased D2DR expression in the NAc and similar to what was observed in the PFC, cocaine reduced D2DR in the NAc. Data are presented as mean \pm SEM (n=4). Representative western blots of D2DR and B-Actin in the mPFC (**Fig 6C**) and NAc (**Fig 6D**) of rats exposed to nandrolone during postnatal days 28-37, tested for behavioral sensitization and sacrificed on PN 64. Data were analyzed using One-Way ANOVA. (See Supplemental Table 1 for statistical analysis).

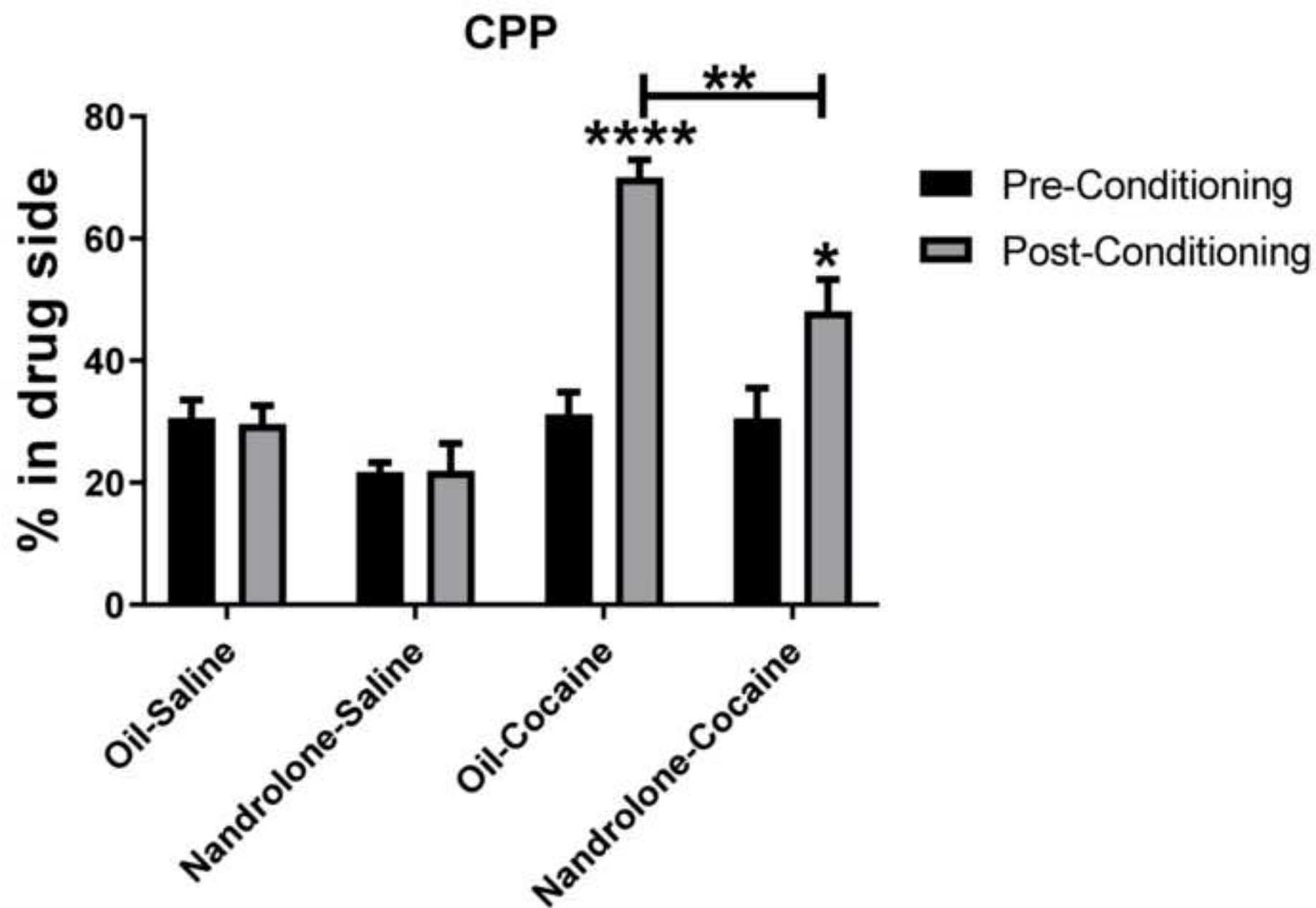


Figure 7. Conditioned Place Preference to cocaine of rats exposed to nandrolone during postnatal days 28-37.

Adolescent male rats were injected daily from PN 28-37 with nandrolone (20 mg/kg) or sesame oil and tested for CPP to cocaine from PN 40-53. Nandrolone-treated males showed a decrease in the time spent in the chamber associated with cocaine compared to oil-treated males. During the postconditioning test, nandrolone-treated rats spent 48% of their time in the chamber associated with cocaine compared to 70% spent by oil-treated males. Rats injected with saline did not show a change in the time spent in the chamber where they were injected with saline. Although oil and nandrolone treated males conditioned to cocaine, conditioning was more robust in Oil-treated males. Data are presented as mean \pm SEM (n=8) and analyzed by a Two Way ANOVA. See Supplemental Table 1 for statistical analysis details.

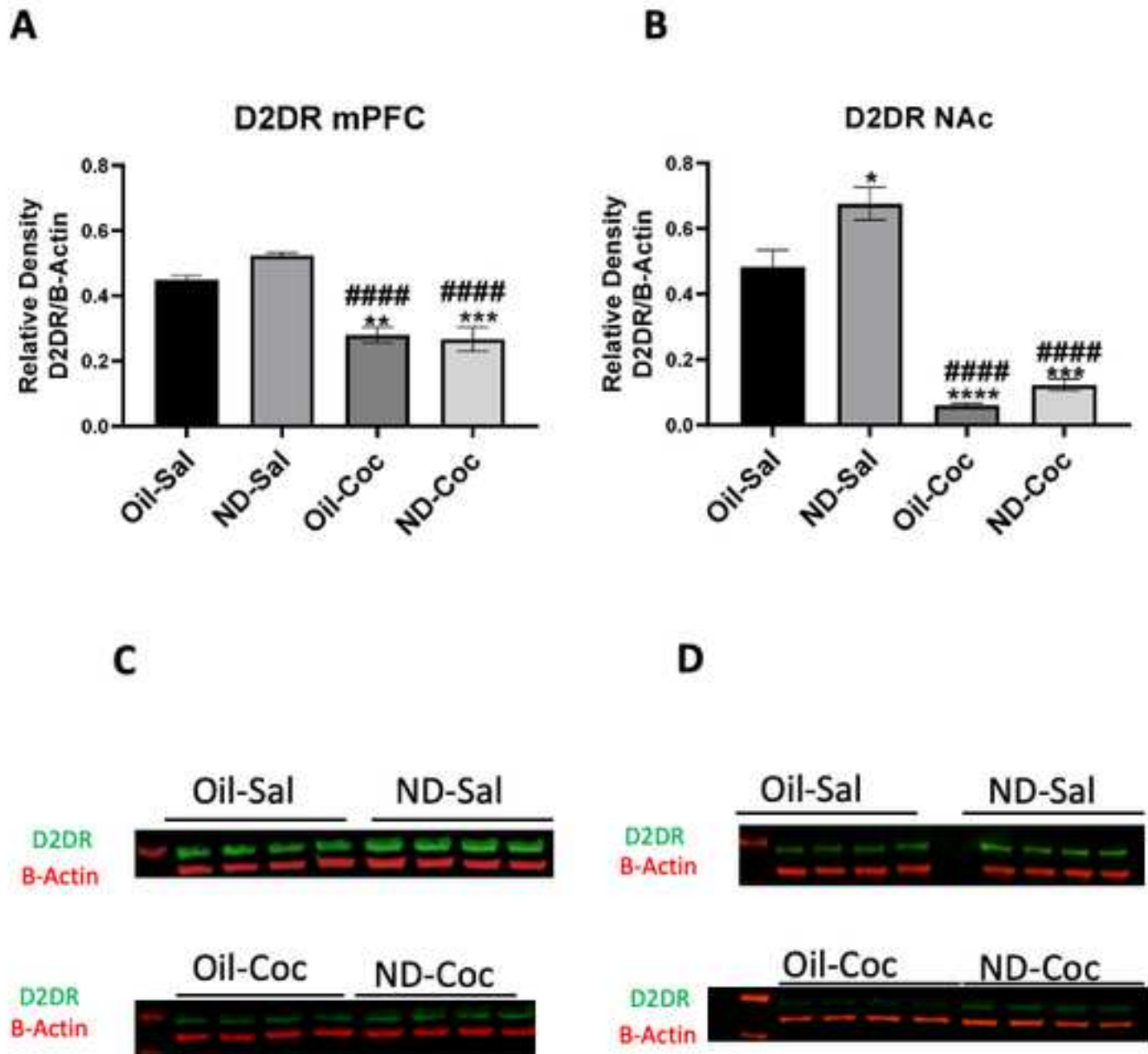


Figure 8. D2DR in the mPFC and NAc of rats exposed to nandrolone during postnatal days 28-37 and tested for CPP.

Fig. 8B and 8D: Nandrolone increased D2DR expression in the mPFC and NAc of prepubertal rats. **Fig. 8B and Fig 8D:** In contrast, cocaine decreased D2DR expression in both brain areas (mPFC and NAc), similar to what was observed in brains obtained from animals that were tested for behavioral sensitization and killed at a later age (PN 64). Representative western blots of D2DR and B-Actin in the mPFC (**Fig 8C**) and NAc (**Fig 8D**) of rats exposed to nandrolone during postnatal days 28-37, tested for CPP and sacrificed on PN 53. Data are presented as mean \pm SEM (n=4) and analyzed using One-Way ANOVA. (See Supplemental Table 1 for detailed statistical analysis).

Tables

Table 1 Statistical values for data analyzed by Student *t* test and ANOVA

Figure	Factor ID and Type	Test Name	F (or T) values + df	P value
Fig 2.2A - Open Field	Time in Center	T-Test	$t=2.31$, $df=36$	0.0264
Fig 2.2B - Open Field	Total Distance	T-Test	$t=5.17$, $df=36$	<0.0001
Fig 2.2C - Open Field	Total Horizontal Activity	T-Test	$t=2.15$, $df=18$	0.0453
Fig 2.3A - EPM	Time in Open Arms	T-Test	$t=2.62$, $df=18$	0.0173
Fig 2.3B - EPM	Time in Closed Arms	T-Test	$t=2.62$, $df=18$	0.0173
Fig 2.3C - EPM	Entries Open Arms	T-Test	$t=5.62$, $df=18$	<0.0001
Fig 2.3D- EPM	Entries Closed Arms	T-Test	$t=0.15$, $df=18$	0.8794
Fig 2.3E - EPM	Time Closed Arms/Time Open Arms	T-Test	$t=2.04$, $df=18$	0.0556
Fig 2.3F - EPM	Total Entries	T-Test	$t=3.28$, $df=18$	0.0041
Fig 2.4A - Oil-Sal	Minutes - (Repeated factor)	Two-Way RM ANOVA	$F(17, 162) = 68.44$	0.5509
	Days - (Repeated factor)		$F(2.83, 459.6) = 28.31$	<0.0001
	Minutes x Day		$F(51, 486) = 0.9621$	<0.0001
Fig 2.4B - ND-Sal	Minutes - (Repeated factor)	Two-Way RM ANOVA	$F(4.03, 36.33) = 76.01$	<0.0001
	Days - (Repeated factor)		$F(2.24, 20.22) = 8.739$	0.0014
	Minutes x Day		$F(5.01, 45.09) = 2.968$	0.02124
Fig 2.4C - Oil-Coc	Minutes - (Repeated factor)	Two-Way RM ANOVA	$F(2.55, 23.01) = 35.82$	<0.0001
	Days - (Repeated factor)		$F(2.01, 18.17) = 25.01$	<0.0001
	Minutes x Day		$F(4.44, 39.98) = 6.809$	0.0002
Fig 2.4D - ND-Coc	Minutes - (Repeated factor)	Two-Way RM ANOVA	$F(2.46, 22.19) = 63.94$	<0.0001
	Days - (Repeated factor)		$F(2.45, 22.08) = 13.03$	<0.0001
	Minutes x Day		$F(4.73, 42.62) = 6.396$	0.0002
Fig 2.4E - Repeated	Treatment - (Independent)	Two-Way RM	$F(1, 9) = 10.32$	0.0106

Cocaine Horizontal Activity	Days - (Repeated factor)	ANOVA	F (3, 27) = 26.05	<0.0001	
	Days x Treatment		F (3, 27) = 4.173	0.015	
Fig 2.5A - STRCNT Oil-Sal	Minutes - (Repeated factor)	Two-Way RM ANOVA	F (3.55, 31.95) = 103.8	<0.0001	
	Days - (Repeated factor)		F (2.03, 18.27) = 5.256	0.0154	
	Minutes x Day		F (6.90, 62.10) = 1.418	0.2149	
Fig 2.5B - STRCNT ND-Sal	Minutes - (Repeated factor)	Two-Way RM ANOVA	F (2.71, 24.41) = 51.34	<0.0001	
	Days - (Repeated factor)		F (1.56, 14.04) = 4.673	0.0349	
	Minutes x Day		F (2.27, 20.46) = 2.049	0.15	
Fig 2.5C - STRCNT Oil-Coc	Minutes - (Repeated factor)	Two-Way RM ANOVA	F (2.58, 23.24) = 30.63	<0.0001	
	Days - (Repeated factor)		F (1.82, 16.46) = 18.34	<0.0001	
	Minutes x Day		F (4.76, 42.91) = 8.519	<0.0001	
Fig 2.5D - STRCNT ND-Coc	Minutes - (Repeated factor)	Two-Way RM ANOVA	F (2.57, 23.18) = 65.65	<0.0001	
	Days - (Repeated factor)		F (2.58, 23.23) = 10.03	0.0003	
	Minutes x Day		F (4.74, 42.68) = 5.314	0.0008	
Fig 2.5E - Repeated Cocaine STRCNT	Treatment - (Independent)	Two-Way RM ANOVA	F (1, 9) = 11.00	0.009	
	Days - (Repeated factor)		F (3, 27) = 35.44	<0.0001	
	Days x Treatment		F (3, 27) = 6.746	0.0015	
Fig 2.6C - Western Blot mPFC	D2DR	One-Way ANOVA	F (3, 12) = 110.5	<0.0001	
Fig 2.6D - Western Blot Nac	D2DR	One-Way ANOVA	F (3, 12) = 84.90	<0.0001	
Fig 2.7 - CPP	Treatment	Two-Way ANOVA	F (3, 27) = 20.00	<0.0001	
	Pre and Post Conditioning		F (1,9) = 42.23	0.0001	
	Treatment x Pre and Post Conditioning		F(3,17) = 18.84	<0.0001	
Fig 2.8C- Western Blot mPFC	D2DR	One-Way ANOVA	F (3, 12) = 25.78	0.001	
Fig 2.8D - Western Blot Nac	D2DR	One-Way ANOVA	F (3, 12) = 45.87	0.0012	

Table 2 Two-Way ANOVA Post-hoc Analysis of Horizontal Activity Days

Compared	Minutes	Oil-Sal	ND-Sal	Oil-Coc	ND-Coc
		p-value	p-value	p-value	p-value
Day 40 vs Day 44	35	0.5041	0.7727	0.9855	0.006
	40	0.9744	0.9977	0.9475	0.0022
	45	0.9297	0.5096	0.5515	0.0094
	50	0.2566	0.9827	0.6503	0.004
	55	0.3532	0.4234	0.6792	0.0241
	60	0.5303	0.9463	0.9982	0.0109
	65	0.7148	0.7011	0.7029	0.04
	70	0.5451	0.9973	0.9982	0.0141
	75	0.3414	0.9196	0.948	0.0679
	80	0.5418	0.8949	0.8702	0.5677
	85	0.2339	0.4924	0.8913	0.5947
	90	0.3941	0.5489	0.8611	0.9218
Day 40 vs Day 52	35	>0.9999	0.0791	0.2432	0.0324
	40	0.9985	0.1566	0.1476	0.0407
	45	0.9041	0.1367	0.1361	0.0084
	50	0.8632	0.6621	0.0444	0.0081
	55	0.3705	0.7808	0.0076	0.0470
	60	>0.9999	0.9570	0.0253	0.0592
	65	0.8277	0.4163	0.0184	0.0540
	70	0.9991	0.9323	0.0311	0.0124
	75	0.4547	0.7780	0.3878	0.0326
	80	0.9699	0.9988	0.0938	0.0218
	85	0.8248	0.5259	0.1041	0.0317
	90	0.9636	0.9825	0.5770	0.0055
Day 40 vs Day 62	35	0.9324	0.0147	0.0009	0.0004
	40	0.9431	0.7838	0.0051	0.0010
	45	0.9642	0.8711	0.0011	0.0011
	50	0.3203	0.9971	0.0003	0.0018
	55	0.1046	0.0229	0.0006	0.0104
	60	0.8872	0.2543	0.0013	0.0614
	65	0.4582	0.5879	0.0007	0.0126
	70	0.2275	0.1479	0.0024	0.0458
	75	0.9547	0.2065	0.042	0.0305
	80	0.9995	0.5344	0.008	0.1538
	85	0.8776	0.9809	0.0929	0.0455

Day 44 vs 52	90	0.9532	0.8818	0.2877	0.2409
	35	0.0880	0.0524	0.1338	0.9687
	40	0.9809	0.1344	0.0775	0.9948
	45	0.9975	0.6215	0.1614	0.9996
	50	0.3878	0.8998	0.0667	0.9716
	55	0.1597	>0.9999	0.0134	0.9932
	60	0.3982	0.8390	0.0210	0.9956
	65	0.3800	0.7432	0.0319	0.9688
	70	0.5035	0.8640	0.0336	0.5698
	75	0.5400	0.6442	0.0393	0.2890
	80	0.5553	0.8195	0.0678	0.0339
	85	0.6938	0.9101	0.0585	0.2255
	90	0.2769	0.7762	0.8486	0.0898
Day 44 vs 62	35	0.4551	0.0217	0.0019	0.3872
	40	0.4217	0.8331	0.0050	0.8484
	45	0.1737	0.8287	0.0019	0.6558
	50	0.0069	0.8810	0.0013	0.9789
	55	0.0262	0.1471	0.0091	0.9852
	60	0.1354	0.4932	0.0085	0.8792
	65	0.1955	0.2892	0.0176	>0.9999
	70	0.0563	0.1950	0.0320	0.9990
	75	0.1764	0.2011	0.0163	0.9687
	80	0.2883	0.4273	0.0389	0.8228
	85	0.2649	0.5004	0.1626	0.7020
	90	0.5272	0.3290	0.7352	0.9534
Day 52 vs 62	35	0.9973	0.7277	0.0023	0.6189
	40	0.8441	0.4630	0.0062	0.9517
	45	0.7218	0.1595	0.0044	0.6856
	50	0.0365	0.2713	0.0043	0.8629
	55	0.9030	0.1661	0.1659	0.9994
	60	0.9753	0.1473	0.2038	0.9242
	65	0.9253	0.1914	0.3259	0.9453
	70	0.1339	0.2006	0.4607	0.6551
	75	0.2500	0.4096	0.5022	0.6722
	80	0.9829	0.5000	0.3408	0.7791
	85	0.7685	0.4836	0.9473	0.9322
	90	0.8241	0.7045	0.9600	0.3009

Table 3. Two-Way ANOVA Post-hoc Analysis of Stereotyped Counts

Days Compared		Minutes	Oil-Sal	ND-Sal	Oil-Coc	ND-Coc
			p-value	p-value	p-value	p-value
Day 40 vs Day 44		35	0.4012	0.3118	0.8325	0.0285
		40	0.9849	0.6575	0.9473	0.018
		45	0.9354	0.5454	0.8506	0.0134
		50	0.2374	0.9971	0.4979	0.0094
		55	0.3738	0.3587	0.8752	0.004
		60	0.5800	0.9240	0.7075	0.0458
		65	0.7850	0.7184	0.9958	0.0015
		70	0.6207	0.9930	0.8483	0.033
		75	0.3082	0.8829	>0.9999	0.0232
		80	0.5577	0.8773	0.9947	0.0997
		85	0.2727	0.5288	0.9859	0.6277
		90	0.441	0.6132	0.7909	0.539
Day 40 vs Day 52		35	0.9276	0.1358	0.7386	0.0751
		40	0.9855	0.3922	0.1586	0.5083
		45	0.5645	0.1385	0.2683	0.1000
		50	0.7737	0.6071	0.0737	0.0133
		55	0.5045	0.8988	0.104	0.0186
		60	0.9883	0.6477	0.1085	0.0914
		65	0.8679	0.4173	0.0449	0.0871
		70	0.9867	0.9895	0.049	0.0862
		75	0.386	0.8444	0.0557	0.0114
		80	0.9428	0.9814	0.0803	0.0490
		85	0.9845	0.5779	0.3800	0.0310
		90	0.9843	0.9619	0.0895	0.0552
Day 40 vs Day 62		35	0.7243	0.2288	0.0949	0.8752
		40	0.9809	0.9455	0.6839	0.0066
		45	>0.9999	0.8899	0.0151	0.0219
		50	0.3376	0.9942	0.0026	0.0087
		55	0.1505	0.0607	0.0041	0.0013
		60	0.9036	0.2899	0.0015	0.0193
		65	0.6803	0.6992	0.0009	0.0357
		70	0.2038	0.1897	0.0042	0.0251
		75	0.9432	0.1876	0.0039	0.085
		80	0.999	0.4452	0.0088	0.0854
		85	0.8974	0.9979	0.0105	0.1701
		90	0.9552	0.9518	0.0336	0.0261
		35	0.1864	0.055	0.0194	0.1950

Day 44 vs 52	40	0.9997	0.3227	0.1337	0.9976
	45	0.9925	0.5854	0.2368	0.9936
	50	0.4519	0.739	0.6581	0.8827
	55	0.2259	0.9677	0.1719	0.6592
	60	0.4096	0.6731	0.0875	0.7239
	65	0.4286	0.803	0.1319	0.9068
	70	0.5158	0.9285	0.0432	0.9765
	75	0.7389	0.6735	0.0988	0.9714
	80	0.5536	0.8546	0.0437	0.6587
	85	0.6393	0.8311	0.1078	0.0462
Day 44 vs 62	90	0.2732	0.8022	0.0751	0.0961
	35	0.696	0.6059	0.2083	0.4662
	40	0.6621	0.7646	0.0769	0.952
	45	0.6908	0.8864	0.1791	0.9927
	50	0.0105	0.9364	0.9999	0.9901
	55	0.0445	0.5631	0.0774	0.999
	60	0.1799	0.6359	0.0089	0.8221
	65	0.2246	0.3566	0.0071	0.8341
	70	0.0932	0.2464	0.0023	0.7047
	75	0.2359	0.1768	0.0006	0.7933
Day 52 vs 62	80	0.3141	0.3539	0.0229	0.9936
	85	0.2588	0.3751	0.0153	0.8755
	90	0.5422	0.2591	0.0704	0.9531
	35	>0.9999	0.9955	0.0413	0.8168
	40	0.8198	0.5510	0.0424	0.9918
	45	0.7672	0.3150	0.0970	0.9788
	50	0.0497	0.2451	0.4310	0.9696
	55	0.8245	0.2238	0.9917	0.6538
	60	0.9995	0.1416	0.6197	0.9989
	65	0.9931	0.2092	0.0106	0.9991
Day 52 vs 62	70	0.2024	0.2470	0.0052	0.8898
	75	0.276	0.4028	0.0048	0.5158
	80	0.8853	0.3761	0.0010	0.5820
	85	0.9071	0.3750	0.1064	0.7355
	90	0.8265	0.6621	0.1559	0.8604

Table 4. Two-Way ANOVA Post-hoc Analysis Oil-Coc vs ND-Coc

Horizontal Activity	Minutes	Oil-Coc vs ND-Coc	Stereotyped Counts	Minutes	Oil-Coc vs ND-Coc
		p-value			p-value
Day 40	35	0.9998	Day 40	35	0.9980
	40	0.9967		40	0.9487
	45	0.9959		45	0.9819
	50	0.9972		50	0.9993
	55	0.7364		55	0.7978
	60	0.6912		60	0.6056
	65	0.9984		65	0.9973
	70	0.9952		70	0.9837
	75	>0.9999		75	0.9971
	80	0.9722		80	0.9844
	85	0.9968		85	0.9973
	90	>0.9999		90	>0.9999
Day 44	35	<0.0001	Day 44	35	0.0018
	40	<0.0001		40	<0.0001
	45	<0.0001		45	<0.0001
	50	<0.0001		50	0.0006
	55	<0.0001		55	0.0109
	60	<0.0001		60	0.0003
	65	0.0004		65	0.0378
	70	0.0011		70	0.0111
	75	0.0177		75	0.0319
	80	0.9094		80	0.7744
	85	0.9992		85	0.9999
	90	>0.9999		90	>0.9999
Day 52	35	0.2279	Day 52	35	0.0003
	40	0.3583		40	0.0109
	45	0.3057		45	0.0160
	50	0.6197		50	0.4261
	55	0.8734		55	0.5827
	60	0.6344		60	0.3668
	65	0.9578		65	0.9095
	70	0.7585		70	0.6581
	75	0.5990		75	0.4511
	80	0.7202		80	0.6685
	85	0.9998		85	>0.9999

Day 62	90	0.8402	Day 62	90	0.9977
	35	0.9077		35	0.9983
	40	0.9582		40	>0.9999
	45	0.8230		45	0.9986
	50	0.9932		50	>0.9999
	55	>0.9999		55	>0.9999
	60	>0.9999		60	>0.9999
	65	>0.9999		65	>0.9999
	70	>0.9999		70	>0.9999
	75	>0.9999		75	>0.9999
	80	>0.9999		80	>0.9999
	85	>0.9999		85	>0.9999
	90	>0.9999		90	>0.9999