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The effect of the anabolic steroid, nandrolone, in conditioned place preference and D1 dopamine receptor expression in adolescent and adult mice

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Abstract

Adolescents and adults engage in anabolic-androgenic steroid (AAS) misuse seeking their anabolic effects, even though later on, many could develop neuropsychological dependence. Previously, we have shown that nandrolone induces conditioned place preference (CPP) in adult male mice. However, whether nandrolone induces CPP during adolescence remains unknown. In this study, the CPP test was used to determine the rewarding properties of nandrolone (7.5 mg/kg) in adolescent mice. In addition, since D1 dopamine receptors (D1DR) are critical for reward-related processes, the effect of nandrolone on the expression of D1DR in the nucleus accumbens (NAc) was investigated by Western blot analysis. Similar to our previous results, nandrolone induced CPP in adults. However, in adolescents, nandrolone failed to produce place preference. At the molecular level, nandrolone decreased D1DR expression in the NAc only in adult mice. Our data suggest that nandrolone may not be rewarding in adolescents at least during short-term use. The lack of nandrolone rewarding effects in adolescents may be due, in part to differences in D1DR expression during development.

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Conflict of interest

Authors declared that they have no conflicts of interest.

Keywords

Anabolic-androgenic steroids; reward; adolescents; D1 dopamine receptors; nucleus accumbens

1. Introduction

Anabolic-androgenic steroids (AAS) are considered drugs of abuse, being misused at supraphysiological doses by an increasing number of athletes and adolescents (Bahrke et al., 1998). The use of AAS by teenagers has been a primary public health concern because of their potential side effects during a period where remodeling of the brain and behavioral maturation occurs. The lifetime prevalence of AAS misuse for adolescents has been estimated to be 4–6% in males and 1.5–3% in females (Harmer, 2010). This and other epidemiological reports support studies that link early initiation of AAS misuse with increased risk for psychiatric dysfunction, steroid dependence, as well as a higher risk to engage in the use of other illicit drugs (Kanayama et al., 2009). However, during the last decade, studies that assessed rewarding properties of AAS have been mainly done in adult animal models. In fact, a growing body of work in adult rodents shows that androgen compounds have hedonic and reinforcing effects (Clark and Henderson, 2003; Wood, 2004). These effects have been observed using different behavioral paradigms such as conditioned place preference (CPP) or self-administration (Clark and Henderson, 2003; Wood, 2004). Recently, we have demonstrated, in adult mice, that pharmacological doses of the anabolic steroids, testosterone (T) propionate (0.075, 0.75 and 7.5 mg/kg) or nandrolone (0.75 and 7.5 mg/kg but not 0.075 mg/kg), shifted place preference during the CPP (Parrilla-Carrero et al., 2009). Still, the rewarding properties of AAS during adolescence have remained undetermined.

The AAS-induced rewarding effects seem to be mediated directly or indirectly by the corticomesolimbic dopamine reward system (Triemstra et al., 2008). This circuit, which is critical for regulating reward-related associative learning (Kindlundh et al., 2004), is mainly composed of the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC; Koob and Volkow, 2010). In this context, rewarding stimuli lead to increments of dopamine release to the NAc from the VTA. Although D1 and D2 dopamine receptors (D1DR and D2DR) are key players for the signaling process of reward, the D1DR are the most critical in reward-related learning (Beninger and Miller, 1998). In this line of evidence, Kindlundh and colleagues (2001, 2003) have shown that nandrolone altered proteins and transcripts of the dopamine receptors within the striatum and NAc. Moreover, accumbal administration of flupentixol, a D1/D2 dopamine receptor antagonist, blocked the expression of androgen-induced CPP (Packard et al., 1998).

In the present study, the rewarding properties of nandrolone, one of the most abused AAS (Lood et al., 2012), were assessed using an adolescent animal model. Furthermore, we addressed the molecular modulation of nandrolone in the NAc through the expression of D1DR. Knowing that adolescents engage in high reward motivated behaviors (Wahlstrom et al., 2010) due to developmental changes in the striatum that confer reward hypersensitivity (Galvan, 2010), we hypothesized that exposure to nandrolone during this developmental

stage will induce: i) an increase in place preference, and ii) an increase in the levels of accumbal D1DR protein expression.

2. Materials and Methods

2.1. Subjects

C57Bl/6 male mice were purchased from Charles River and housed individually in the Animal Resources Center with food and water available ad libitum. Animals were kept on a reverse 12:12 hour light/dark cycle. The behavioral experiments were performed during the dark phase of the cycle, in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, Medical Sciences Campus.

2.2. Androgen administration

Similar to previous experiments, adolescent and adult experimental groups received intraperitoneal (IP; Hoseini et al., 2009; Witzmann, 1988) injections of nandrolone (7.5 mg/kg; 19-nortestosterone; Sigma Chemical Co. St. Louis, MO), whereas control groups received IP injections of vehicle, which consisted of sesame oil (0.02 cc/10g of body weight). As 1 mg/kg of AAS is sufficient to restore endocrine function and reproductive behaviors in rodents (Clark and Barber, 1994), the dosage used in this experiment reflects a high dose. Since periadolescence in rodents extends from postnatal (PN) day 28 to PN-60, (Spear, 2000), nandrolone injections alternated with vehicle injections, were started at PN-33 until PN-42 (Fig. 1). Furthermore, to confirm our previous experiment in adults (Parrilla-Carrero et al., 2009), adult male mice (PN 88-97) were also tested following the same CPP injection protocol as in adolescents.

2.3. Conditioned place preference (CPP)

This behavioral paradigm was performed as previously described (Parrilla-Carrero et al., 2009) with some modifications. In brief, the apparatus consisted of an acrylic cage separated in two compartments by an opening that can be closed by a removable guillotine door. The chambers comprised of two sides. One side of the chamber has smooth flooring, lateral walls with black and white lines, and anterior/posterior white walls. The other side has grated-texture flooring, lateral walls with black and white checkers, and anterior/posterior black walls.

The CPP was performed using a biased design and under semi dark conditions. Briefly, the CPP protocol lasted 14 days and consisted of four phases (Fig. 1): habituation (days 1–2), baseline (day 3), conditioning (days 4–13) and test day (day 14). During the baseline (15 min), animals were recorded for the time spent in each side of the chamber to determine the non-preferred (NP) side (where the drug will be injected during conditioning). Thereafter, animals were randomly assigned to control or experimental groups. The conditioning phase consisted of a 30 min session per day. The experimental group received nandrolone injections (days 4, 6, 8, 10, 12) while they were restricted to the NP side, or vehicle injections while restricted to the preferred (P) side (days 5, 7, 9, 11, 13). The control group received daily vehicle injections whether they were restricted to the NP or P side of the chamber. Overall, both groups received a total of 10 injections. On the test-day, all animals

were tested for the time spent in the NP-side in the absence of further vehicle or nandrolone injections. The time in the CPP compartments was gathered using the Any-Maze tracking system (Stoelting Co, IL). Data were obtained as the time (sec) the animals spent in the P and NP sides during the test-day. The time spent in the NP side between control and experimental animals was reported and statistically compared.

2.4. Western blot analysis

2.4.1. Sample preparation—Nandrolone (7.5 mg/kg) and vehicle treated adolescent and adult mice were sacrificed by rapid decapitation, and the NAc rapidly dissected as previously described (Sharma and Fulton, 2012). Tissue was homogenized and lysed using CelLytic MT with SIGMAFAST™ Protease Inhibitor Tablets (Sigma-Aldrich, MO). Supernatants were collected to determine total protein concentration using Quick Start™ Bradford Protein Assay (Bio-Rad, CA).

2.4.2. Western blotting—Western blots were performed as previously described (Silva, et al., 1999) with some modifications. Immunodetection of D1 dopamine receptor (D1DR) and β -actin on nitrocellulose membranes was performed using a 1:1000 dilution of a primary goat anti D1DR polyclonal antibody (Santa Cruz Biotechnology, CA) and 1:5000 dilution of a primary rabbit anti β -actin polyclonal antibody (Abcam, MA). Secondary antibodies, anti-goat polyclonal (1:10,000) or anti-rabbit polyclonal (1:15,000; Sigma-Aldrich, MO) were used. Blots were visualized using an enhanced chemiluminescence kit (SuperSignal Femto, Pierce, IL) and images were obtained using a VersaDoc 1000 system (Bio-Rad, CA). Western blots were performed in triplicate from three independent experiments, and densitometric analysis performed using NIH ImageJ (v1.47d).

2.5. Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). The number of animals per experiment is as follows: CPP for adults (vehicle n=5; nandrolone 7.5 mg/kg n=6); CPP for adolescents (vehicle n=15; nandrolone 7.5 mg/kg n=11); Western blot experiments, n=three (3) technical replicates of four (4) independent experiments (n=4 animals) for each group. Student's t-test analyses were employed for both the CPP and Western blots. Statistical significance was established as *P < 0.05.

3. Results

3.1. Conditioned place preference (CPP)

We tested the effect of nandrolone (7.5 mg/kg) in adolescent and adult mice in the CPP task. Similar to our previous findings (Parrilla-Carrero et al., 2009), in the present study we confirmed the rewarding properties of nandrolone in adult mice (Fig. 2A). Specifically, control animals spent 393.54 ± 7.84 sec in the drug-paired side, whereas the nandrolone-treated animals spent 465.63 ± 17.64 sec ($t_9=3.47$; $P=0.007$). On the other hand, nandrolone failed to reveal place preference in adolescent mice (Fig. 2B). Specifically, control animals spent 437.27 ± 30.10 sec in the drug-paired side, whereas nandrolone-treated animals spent 445.77 ± 22.08 sec ($t_{24}=0.21$; $P=0.830$). In order to conclusively state that there are no effects in the adolescent brain we also tested two lower doses of nandrolone. Similar to the

higher dose, no significant effects ($F_{(3, 43)} = 0.079$; $P=0.971$) were observed at 0.75 mg/kg (447.24 ± 44.46) and 0.075 mg/kg (424.97 ± 41.60).

3.2. Western blot analysis

3.2.1. Accumbal D1DR expression—To begin to explore the molecular basis possibly contributing to the differential sensitivity in reward between adults and adolescents after nandrolone exposure (7.5 mg/kg), we performed a Western blot analysis for the expression of D1DR. In adult mice, densitometric quantification of D1DR (74-KD band) after normalization to β -actin showed that nandrolone decreased D1DR protein expression levels within the NAc ($t_4=3.695$; $P=0.0209$; Fig. 2C). On the contrary, nandrolone treatment (7.5 mg/kg) did not affect D1DR in adolescent mice (Fig. 2D). In order to conclusively state that there are no effects in D1DR protein expression levels, we also tested two lower doses of nandrolone (0.75 and 0.075 mg/kg). Similar to the higher dose, no significant effects were observed at any lower dose tested ($F_{(3, 14)} = 2.46$; $P = 0.11$). Regarding control levels of expression, D1DR showed a tendency to be higher in adults than in adolescents ($t_4=2.311$; $P=0.08$).

4. Discussion

The present study demonstrates that in adolescents, nandrolone was not rewarding, even though the contrary has been observed in adult animals. Since adolescents typically engage in high reward motivated behaviors (Ernst et al., 2009), and this age group has been shown to express hypersensitivity to some drugs of abuse (Schepis et al., 2008), we expected to find nandrolone-induced place preference at this developmental age. In general, although the literature suggests that adolescents may be more sensitive to the reinforcing effects of drugs of abuse (Ernst et al., 2009; Galvan, 2010), several studies revealed that such sensitivity during adolescence is drug-dependent. For instance, Schramm-Sapota and colleagues (2009) showed that amphetamine and methamphetamine stimulate less locomotion in early adolescents than their adult counterparts. In contrast, exposure to cocaine or opioids appears to induce greater locomotor sensitization in adolescents than in adults (Caster et al., 2005, 2007; Schramm-Sapota et al., 2004; Spear, et al., 1982).

At the molecular level, we found that nandrolone decreased the expression of D1DR in the NAc of adult animals. Consistently, Kindlundh and colleagues (2001, 2003) showed that 2-week exposure to nandrolone (15 mg/kg) significantly decreased protein and mRNA expression of D1DR in the NAc of adult rats. Likewise, downregulation of dopamine receptors has been shown in studies of psychostimulants and opioids, suggesting a stimulation of the reward circuit through an increase in accumbal dopamine levels (Dumartin et al., 1998). This sustained stimulation produces desensitization through receptor internalization that ultimately produces a decrease in protein expression (Christie, 2008; Dumartin et al., 1998). Besides D1DR modulation, nandrolone elicited an increase in D2DR and dopamine transporter (DAT) expression in the VTA and striatum (Kindlundh et al., 2001, 2003, 2004). Indeed, we have gathered preliminary data suggesting an increase in accumbal DAT from adult mice exposed to nandrolone (7.5 mg/kg). Together, these

findings and ours suggest a homeostatic regulation of dopaminergic activity in the reward circuit in androgen sensitive-afferent neurons.

Certainly, our study showed a lack of nandrolone modulation in the expression of adolescents D1DR, data that goes in accordance with the absence of CPP at this developmental stage. The fact that we observed a tendency for less expression of D1DR in adolescent control mice as compared with their adult counterparts could be interpreted as that the mesolimbic dopaminergic system in adolescents is not sensitive or fully mature to elicit reward-related behaviors in response to nandrolone. Evidence suggests that the dopaminergic system matures during adolescence (Kuhn et al., 2010). Accordingly, dopamine fiber densities in the NAc increase significantly during gerbils' adolescence, suggesting that significant maturation of VTA dopaminergic projections to the NAc occurs at this stage of development (Lesting et al., 2005). In this respect, it seems that although the specific markers for the dopaminergic mesolimbic system, such as D1DR and D2DR increase markedly from PN-5 to PN-40 in the NAc and frontal cortex (Kuhn et al., 2010), the maturation process that occurs until PN-60 might play an important role for saliency and reward, particularly for androgens.

Finally, since the complex metabolism pathways that respond to androgens are not fully developed during puberty (Salas-Ramírez et al., 2008; Sato et al., 2008), we cannot rule out the possibility that developmental differences in metabolic regulation could be responsible for the lack of CPP in adolescents. Regarding nandrolone metabolism, it can be aromatized to 17 β -estradiol by aromatase although to a lesser extent if compared to T (Clark and Henderson, 2003). In fact, although the expression of this enzyme decreases through development (Gillies and McArthur, 2010; Lauber and Lichtensteiger, 1994) it has been shown to be upregulated in adults after nandrolone exposure (Roselli, 1998; Takahashi et al., 2007). In this line of evidence, DiMeo and Wood (2006) showed estradiol self-administration in male hamsters, while the same hormone induced CPP in ovariectomized rats (Frye and Rhodes, 2006), a cellular response that could be mediated through estrogen receptors in the NAc (Walf et al., 2007).

However, the VTA and NAc have few androgen and estrogen receptors (AR and ER; Shughrue et al., 1997). Thus, it is likely that androgens interact with the mesolimbic system through androgen-sensitive afferents such as the bed nucleus of the stria terminalis, amygdala and medial preoptic nucleus (MPN), which have abundant expression of AR and ER (for review see Sato et al 2008; Handa et al., 1996). Therefore, the fact that these brain regions are still undergoing maturation could be a plausible explanation for the lack of effect of nandrolone in adolescents. In this respect, it is noteworthy that a significant increase in the expression of AR occurs between PN-28 to PN-49 in the MPN (Meek et al., 1997), a similar time period in which our conditioning protocol for nandrolone was applied. Similarly, since the ER expression increases onward into adulthood (Gillies and McArthur, 2010; Romeo et al., 1999), a major responsiveness to the activational effects of steroid hormones could be expected in rewarding behaviors in adult mice.

Taken together, it is suggested that differences in the expression of structural components of the mesolimbic reward circuit, as well as differences in metabolic processing are key

candidates to be further studied with the aim to understand androgen sensitivity throughout development.

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Abbreviations

AAS	anabolic androgenic steroids
AR	androgen receptor
CNS	central nervous system
CPP	conditioned place preference
DAT	dopamine transporter
D1DR	D1 dopamine receptor
D2DR	D2 dopamine receptor
ER	estrogen receptor
IP	intraperitoneal
MPN	medial preoptic nucleus
NAc	nucleus accumbens
T	testosterone
PFC	prefrontal cortex
PN	postnatal
NP	non-preferred
P	preferred
VTA	ventral tegmental area

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Highlights

- The anabolic steroid nandrolone induces CPP in adult, but not adolescent mice
- Nandrolone decreases accumbal D1 dopamine receptors only in adult mice
- Rewarding effects of nandrolone are dependent on stages of development

Figure 1. Experimental design and injection protocol for the CPP in nandrolone-treated animals
The CPP was performed for 14 days. On days 1–2, animals were habituated to the CPP chambers for 15 min. On day 3 (baseline), animals were allowed to move freely between the compartments for 15 min. The time spent in each compartment was immediately calculated. During days 4–13 (conditioning), nandrolone-treated animals received alternate injections of drug (days 4, 6, 8, 10, 12) or vehicle (days 5, 7, 9, 11, 13) while placed for 30 min in the non-preferred (NP) or preferred (P) side, respectively. Control animals received daily injections of vehicle, whether they were restricted to the NP or P side of the chamber. On day 14 (test day), injection-free animals were allowed to move freely between the compartments, and the time spent in the NP side was recorded for 15 min.

Figure 2. Effect of nandrolone on CPP and accumbal dopamine receptor-1 (D1DR)

During the CPP test day, the time in the drug-paired side was recorded in adults (**A**) and adolescent (**B**) mice after exposure to alternate injections of nandrolone (7.5 mg/kg). (**C–D**) Representative western blot of protein extracts from control and nandrolone-treated adult and adolescent mice. Densitometric analysis of D1DR levels normalized to β -actin showed a significant decrease in D1DR expression levels in nandrolone-treated adult mice when compared to control mice (**C**), while no changes were observed among adolescents (**D**). For CPP, Adults: vehicle- $n=5$; 7.5 mg/kg- $n=6$. Adolescents: vehicle- $n=15$; 7.5 mg/kg- $n=11$.

** $P < 0.01$, unpaired t-test. For D1DR expression, * $P < 0.05$, unpaired t-test. N=three replicates of four independent experiments for each group. Error bars represent standard error of the mean.