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Blockade of androgen or estrogen receptors reduces nandrolone's ability to modulate acute reward-related neurochemical effects of amphetamine in rat brain

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ABSTRACT

Previously we have reported that sub-chronic administration of nandrolone modifies reward-related neurochemical effects of psychomotor stimulant drugs of abuse. The aim of the present study was to evaluate whether the ability of nandrolone (19-nortestosterone) to attenuate the effects of amphetamine depends on activation of androgen (AR) or estrogen receptors (ER). We used an *in vivo* microdialysis technique in fully conscious rats to monitor whether administration of the AR-antagonist flutamide (7×50 mg/kg) or the ER-antagonist clomiphene (7×20 mg/kg), attenuates nandrolone-induced modulation of dopaminergic and serotonergic effects of acute injections of amphetamine (1 mg/kg). Dopamine (DA), 5-hydroxytryptamine (5-HT) and their metabolites were measured from the samples using high performance liquid chromatography (HPLC). Blocking the androgen receptors with flutamide abolished the attenuating effect of nandrolone pre-treatment on amphetamine-induced elevation of extracellular DA concentration. Blocking the estrogen receptors with clomiphene did the same but to a lesser extent. In conclusion, the results of this study show that the ability of nandrolone to attenuate the effects of amphetamine depends on activation of androgen receptors or to a lesser extent, on estrogen receptors.

1. Introduction

The misuse of anabolic androgenic steroids (AASs) — synthetic derivates of testosterone — has become a prevalent substance abuse problem. The primary use of AASs is for muscle building effects, but there also appears to be secondary gain associated with improvements in appearance and/or subjective rewarding effects (Kindlundh et al., 1998, 1999; Yesalis and Bahrke, 1995). According to the literature, abuse of AASs may lead to abuse of other drugs. Indeed, AAS-abusers are shown to share personality factors with the abusers of the psychotropic substances cannabis, amphetamine and cocaine (Arvary and Pope, 2000; Kanayama et al., 2003; Lukas, 1996; Skarberg et al., 2008). In addition, animal studies indicate that AASs can evoke neurochemical changes in dopaminergic and serotonergic neuronal systems related to reward, as well as numerous other behavioral responses in rats (Bitar et al., 1991; Kurling et al., 2005, 2008; Thiblin et al., 1999; Vermes et al., 1979).

Although AASs are abused by humans, their neurochemical effects remain largely unknown. Alterations of DA and 5-HT functions, as well as, in steroidal system, are indicated as a possible neurochemical basis for these effects. However, the effects of supratherapeutic doses of AASs on these systems are not yet fully evaluated. In order to understand the characteristics of addictive behavior and provide novel pharmacological treatment strategies for addiction diseases, it is essential to understand the neurochemical mechanisms mediating the rewarding properties of drugs of abuse in receptor level. Previously we have reported that sub-chronic administration of nandrolone changes the neurochemical and behavioral effects of amphetamine, MDMA (Kurling et al., 2008). Nevertheless, in these studies nandrolone failed to affect the basal levels of DA, 5-HT and their metabolites in extracellular space in the NAc. Thus it seems that steps of DA and 5-HT transmission, in all likelihood, are not the primary target of nandrolone action, but rather in some other mechanisms mediated by transporter proteins or postsynaptic receptors. Regarding nandrolone's fate, it binds avidly to the AR and it can also be aromatized to estrogens (Roselli, 1998; Ryan, 1959). Interestingly, there are several studies showing that accumbal DA systems, which are considered central brain regions in drug reward, respond to both androgens (Alderson and Baum, 1981; Hernandez et al., 1994; Mitchell and Stewart, 1989) and estrogens (Becker, 1999; Di Paolo et al., 1985; Lammers et al., 1999; Landry et al., 2002; Thompson and Moss, 1994). One possible mediator of those effects, as well as the nandrolone-induced attenuation of psychomotor stimulant drug response observed in our previous studies, could be androgen receptors (AR) or estrogen receptors (ER). The aim of this study was to evaluate whether the ability of nandrolone to modulate reward-related effects of amphetamine is dependent on activation of AR or ER. We used an *in vivo* microdialysis technique in fully conscious rats to monitor whether administration of AR-antagonist

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flutamide (50 mg/kg) or ER-antagonist clomiphene (20 mg/kg) affects the ability of sub-chronic nandrolone treatment to modulate the dopaminergic and serotonergic effects of acute injections of amphetamine.

2. Experimental procedures

2.1. Animals

Studies were performed on adult male Wistar rats (Harlan Netherlands B.V., The Netherlands), weighing 300 to 350 g at the beginning of the experiments. The animals were housed in clear plastic boxes (Techniplast Eurostandard type IV case: 595×380×200 mm, floor area 1820 cm²) under standard conditions with constant temperature and humidity, with lights on 06.00 a.m.–06.00 p.m., during which time all the experiments were conducted. The animals had free access to tap water and standard laboratory chow (Altromin Nr. 1314; Chr. Petersen A/S, Ringsted, Denmark) throughout the experiments. The rats were housed three per cage except after microdialysis surgery when they were housed individually. The State Provincial Office of Southern Finland Animal Experiment Board approved the animal experiments, and they were conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

2.2. Drugs and treatments

Flutamide 2-methyl-*N*-[4-nitro-3-(trifluoromethyl) phenyl]-propanamide and clomiphene 2-(4-(2-chloro-1,2-diphenylethenyl) phenoxy)-N,N-diethyl-ethanamine were purchased from Sigma Chemical Co., St Louis, MO, USA. Nandrolone decanoate was a commercial preparation (Deca-Durabolin®) from NV Organon (Oss, the Netherlands). The matching vehicle for nandrolone decanoate preparation, a mixture of arachinoid oil and benzylalcohol, was prepared by the University Pharmacy (Helsinki, Finland) for control purposes. Flutamide was administered as 25 mg/ml water and Tween® 20 suspension (Fluka Sigma-Aldrich Chemie GmbH, CH-9471 Buchs, Germany), and clomiphene as 10 mg/ml water solution. Water was used as an intragastric vehicle for both flutamide and clomiphene.

Flutamide (50 mg/kg), clomiphene (20 mg/kg) or vehicle (water) was administered intra gastric (i.g.) at days 1, 3, 5, 8, 10, 12 and 15 from the beginning of the experiment between 8 and 9 a.m. Nandrolone decanoate (20 mg/kg, calculated as free base) was administered intramuscularly (i.m.) 6 h after receptor antagonist or water dosing at days 1, 3, 5, 8 and 10 from the beginning of the experiment. The injections were given in the left and right hind leg alternately. The two doses of the receptor antagonists at days 12 and 15 were administered to ensure sufficient receptor blockage during the combined half-life (ester hydrolysis, distribution, and elimination) of nandrolone decanoate (4.3 days; unpublished data). The animals were weighed every morning before dosing to adjust the exact dose. The doses and schedule of administration of flutamide were chosen on the basis of its ability to block the steroidal effects of androgens on androgen-dependent gene expression, male rat play behavior and HPA functions (Hotchkiss et al., 2002; Kelce et al., 1997; McCormick and Mahoney, 1999). The doses and schedule of clomiphene administration were chosen on the basis of its ability to block estradiol effect on the DA neurons, rat organ weight changes and estrogen antagonism (Bowman et al., 1981; Hart, 1990; McCall et al., 1988).

Amphetamine sulfate (Sigma Chemical co, St Louis, MO, USA) was dissolved in saline (0.9% NaCl) at a concentration of 1 mg/kg (calculated as free base). The drug was a racemic mixture and was injected intraperitoneally (i.p.) during the microdialysis experiment.

The treatment groups ($n=5$–6 rats per treatment) were as follows: 1. water–oil–saline, 2. water–oil–amphetamine, 3. water–nandrolone–saline, 4. water–nandrolone–amphetamine, 5. flutamide–oil–saline, 6. flutamide–oil–amphetamine, 7. flutamide–nandrolone–saline, 8. flutamide–nandrolone–amphetamine, 9. clomiphene–oil–saline, 10. clomiphene–oil–amphetamine, 11. clomiphene–nandrolone–saline, 12. clomiphene–nandrolone–amphetamine.

2.3. Microdialysis surgery and experiments

The rats were anesthetized using 5% halothane gas (Halothane Liquid BP; Rhodia Organique Fine Ltd., Bristol, UK) and mounted in a stereotactic frame (day 11). A guide cannula (CMA/12; CMA Microdialysis, Solna, Sweden) was implanted 2 mm above the NAc [A, +1.9; L, −1.0; D, −6.0 as calculated relative to the bregma and skull surface according to Paxinos and Watson (1986)] and secured with two small screws and dental cement (Aqualox; VOCO, Cuxhaven, Germany). During surgery halothane gas was administred at a concentration of 2.5%. The animals received subcutaneously 0.05 ml of buprenorphine preparation (Temgesic®, 0.3 mg/ml; Schering-Plough Europe, Brussels, Belgium) to alleviate the pain, and were allowed to recover from the surgery for 5 days.

One day before the experiment (day 15), the rat was allowed to habituate to the test box and a microdialysis probe (CMA/12, membrane length 2 mm; CMA Microdialysis, Solna, Sweden) was inserted through the guide cannula into the NAc shell. The next day (day 16), the rat was placed in test cage and probe was connected to a CMA/100 microinjection pump and perfused with modified Ringer's solution (147 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, pH 6) at a flow rate of 2 µl/min. In order to prevent degradation of monoamine transmitters, a 6.5-µl aliquot of an antioxidant solution (1.0 mM oxalic acid, 3.0 mM L-cysteine, 0.1 mM acetic acid) was added to each vial before collecting the dialysate samples (Kankaanpaa et al., 2001).

The perfusate was discarded during the first 60 min, after which the samples were collected at 20 min intervals. Amphetamine or saline was injected (i.p.) after collection of four basal samples (2 h 20 min from beginning of the perfusion). At the end of the experiment the animals were anesthetized with 5% halothane gas and decapitated. The brains were removed and immersed in buffered 10% formalin solution to verify the correct placement of the probes. Only data from animals with accurate probe placements were included in statistical analyses.

2.4. Analytical procedures

DA, 5-HT and their metabolites DOPAC, HVA and 5-HIAA were analyzed with HLPC by using a method described earlier (Kurling et al., 2008). Briefly, dialysate samples were injected into high performance liquid chromatography (HPLC) apparatus equipped with an Inertsil ODS-3V 5 µm (4.6×250 mm ID) reverse-phase column (GL-Sciences Inc., Tokyo, Japan) and a coulometric ESA Coulochem III detector. The mobile phase was a mixture of a buffer containing 50 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 2.3 mM octanesulfonic acid, and acetonitrile (14% v/v in the final solution),with the pH adjusted to 3.0 with orthophosphoric acid (H_3PO_4). The flow rate was 1.2 ml/min and the detector potentials of the two electrodes were −175 mV and +250 mV.

2.5. Statistics

In the microdialysis experiments the mean of the four samples before the drug treatments was considered as basal release (100%), according to which relative changes after the injections were calculated. The absolute basal releases were calculated on the real values. For statistical evaluations the neurochemical data were calculated as areas under the curves (AUCs) with the trapezoidal

Table 1
Area under the curve (AUC) from a microdialysis study of dopamine, 5-HT and their metabolites.

	DA	DOPAC	HVA	5-HT	5-HIAA
Wat/Veh/Sal	517 ± 18	1200 ± 103	821 ± 95	437 ± 38	1180 ± 79
Wat/Nand/Sal	512 ± 11	1272 ± 50	905 ± 32	438 ± 30	1228 ± 40
Flu/Veh/Sal	523 ± 14	1211 ± 92	821 ± 70	441 ± 24	1251 ± 98
Flu/Nand/Sal	538 ± 31	1139 ± 102	828 ± 49	495 ± 13	1235 ± 105
Clom/Veh/Sal	550 ± 21	1146 ± 61	837 ± 25	384 ± 36	1136 ± 66
Clom/Nand/Sal	575 ± 41	1161 ± 33	801 ± 18	439 ± 30	1182 ± 34
Wat/Veh/Amf	4084 ± 290***	820 ± 49*	643 ± 33	502 ± 29	1318 ± 52

AUCs (% of baseline) for dopamine, DOPAC, HVA, 5-HT and 5-HIAA treatment with water (Wat) or flutamide 50 mg/kg (Flu) or clomihpene 20 mg/kg (Clom) with vehicle oil (Veh) or nandrolone 20 mg/kg (Nand) and a subsequent single injection of amphetamine 1 mg/kg (Amf). Values are expressed as mean ± SEM.
* Significance level $p<0.05$.
*** Significance level $p<0.001$.

method. The microdialysis data were then subjected to one-way analysis of variance (ANOVA) followed by Tukey's test. The results are presented as means ± SEM (standard error of the mean) and the level of statistical significance was set at $p \leq 0.05$.

3. Results

The absolute basal concentrations of DA, 5-HT and their metabolites did not differ significantly between treatment groups. The means (± SEM; $n = 69$) of the basal concentrations in the NAc dialysate were as follows: DA 10.2 ± 0.7 fmol/40 µl; 5-HT 7.0 ± 0.7 fmol/40 µl; DOPAC 18.7 ± 1.0 pmol/40 µl; HVA 8.5 ± 0.5 pmol/40 µl and 5-HIAA 9.3 ± 0.6 pmol/40 µl. The means of the first and the last (1 and 4) basal sample concentrations differ in DA 2.6%, 5-HT 16.4%, DOPAC 1.3%, HVA 2.6% and 5-HIAA 0.1%.

3.1. Effects of the drugs alone

Sub-chronic pre-treatment with nandrolone at a dose of 20 mg/kg (i.m.), pre-treatment with flutamide at a dose of 50 mg/kg or with clomiphene at a dose of 20 mg/kg (i.g.) did not affect spontaneous release of the transmitters or their metabolites *per se* (Table 1). The basal levels also remained unaltered after acute saline injection during the microdialysis experiments. Administration of amphetamine (1 mg/kg i.p) caused a 1100% increase in extracellular DA concentration in the NAc as compared to control groups ($p<0.001$ ANOVA). Amphetamine treatment also decreased the extracellular DOPAC-levels ($p = 0.03$ ANOVA). In addition, administration of amphetamine seemed to decrease the extracellular HVA-levels, but without the statistical significance. Amphetamine dosing had no effect on spontaneous release of 5-HT and 5-HIAA in the NAc.

3.2. Effects of nandrolone pre-treatment on amphetamine-induced neurochemical changes

As evident from Fig. 1, the pre-treatment with nandrolone decreased the amphetamine-induced elevation of extracellular DA

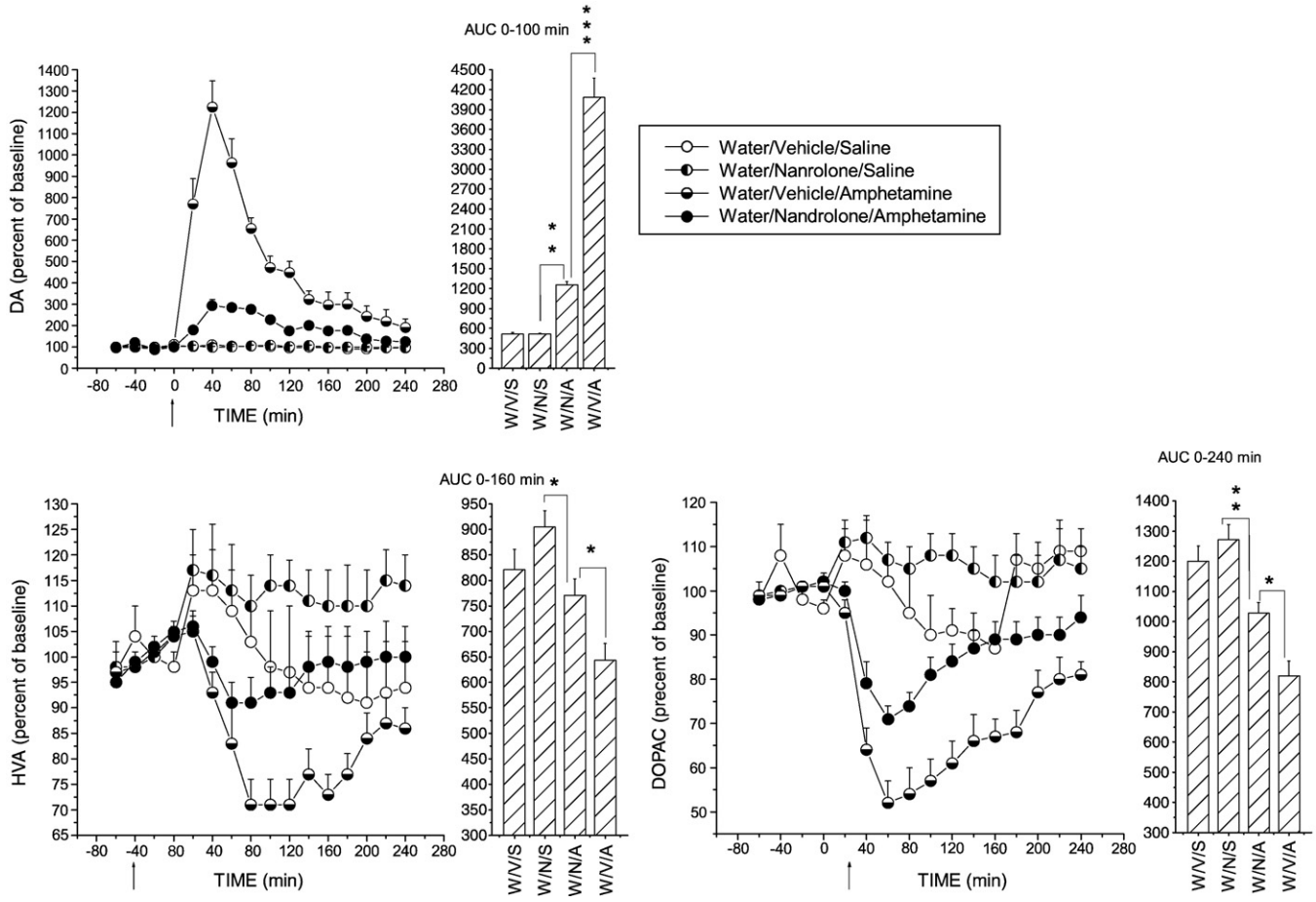

Fig. 1. The effects of nandrolone (20 mg/kg) pre-treatment and acute amphetamine (1 mg/kg) injections on extracellular DA and 5-HT and their metabolite DOPAC, HVA and 5-HIAA levels in the NAc. The times of the amphetamine injections are indicated by arrows. Data expressed as percentages of basal release are given as means ± S.E.M. ($n = 6$). Histograms represent the area under the curve (AUC) after injection of the drug, and the minutes where AUC are shown in the figure. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, *T*-test. Water–vehicle–saline group is added in the figure as a baseline reference. W/V/S = water + vehicle + saline, W/N/S = water + nandrolone + saline, W/N/A = water + nandrolone + amphetamine, W/V/A = water + vehicle + amphetamine.

levels as compared to vehicle treatment ($p<0.000$ Tukey) in the NAc. Nandrolone treatment modified also the effects of amphetamine on extracellular DOPAC ($p=0.025$ Tukey) and HVA ($p=0.030$ Tukey) levels as compared to the vehicle. Co-administration of nandrolone and amphetamine did not have a significant effect on extracellular levels of 5-HT and 5-HIAA (data not shown).

3.3. Effects of receptor antagonists on nandrolone-induced attenuation of amphetamine's action

As seen in Fig. 2, administration of the AR antagonist flutamide (50 mg/kg i.g.) prevented the attenuating effect of nandrolone on amphetamine-induced elevation of extracellular DA levels in the NAc ($p<0.001$ ANOVA). Flutamide also reduced the attenuating effect of nandrolone on amphetamine-induced decrease of extracellular levels of DOPAC ($p=0.008$ Tukey) and HVA ($p=0.007$, Tukey) as compared to the vehicle. Co-administration of flutamide, nandrolone and amphetamine did not have a significant effect on extracellular levels of 5-HT and 5-HIAA compared to the controls (data not shown).

As shown in Fig. 3, administration of the ER-antagonist clomiphene (20 mg/kg i.g.) reduced the attenuating effect of nandrolone on amphetamine-induced elevation of extracellular DA levels in the NAc ($p<0.001$ ANOVA). Clomiphene also reduced the attenuating effect of nandrolone on amphetamine-induced decrease in extracellular levels of HVA ($p=0.01$, Tukey). Clomiphene seems to have the same kind of effect on DOPAC concentration, but without the statistical significance. Co-administration of clomiphene, nandrolone and amphetamine did not have a significant effect on extracellular levels of 5-HT and 5-HIAA compared to the controls (data not shown).

4. Discussion

The main finding of the present study is that blocking of ARs or ERs abolished or reduced nandrolone-induced attenuation of amphetamine's action on extracellular levels of DA and its metabolites in the NAc.

The literature concerning the role of the ARs and ERs in drug reward is scarce. In the study by Frye (2007), flutamide (10 mg/kg, s.c.) failed to attenuate conditioned place preference (CPP) induced by a testosterone secondary metabolite, 3α-diol (10 mg/kg s.c). These results are, however, not directly comparable to ours due to differences between the two studies (e.g. shorter duration and different route of administration, only 2 h interval between receptor antagonist and agonist and lower antagonist dosing). Even though, both methods may give an estimate of addictive potential of a drug. Furthermore the receptor blocker flutamide itself was found addictive (positive CPP), which makes the results extremely difficult to interpret.

Intracellular ARs (the so-called "classical" receptors), are found in the NAc, but their density is shown to be relatively low (Balthazart et al., 1998; Stumpf and Sar, 1976). Therefore, it is possible that the site of action could be outside the NAc, although the number of receptors in the brains of the animals of this study may be higher due to the sub-chronic treatment with nandrolone, which has been shown to up-regulate intracellular (nuclear) AR in male rats (Menard and

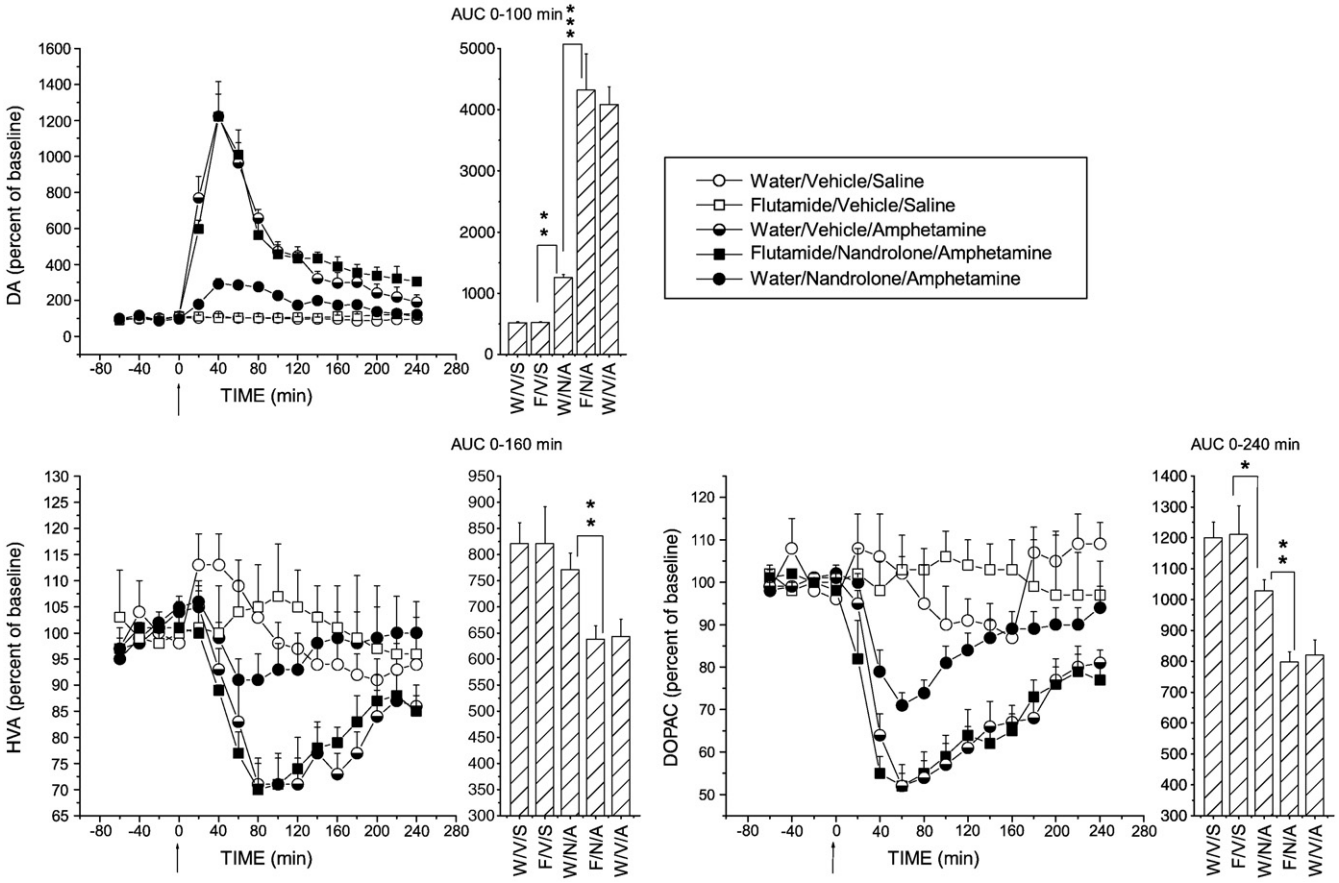

Fig. 2. The effects of pre-treatment with androgen receptor antagonists, flutamide (50 mg/kg), and nandrolone (20 mg/kg) together with acute amphetamine (1 mg/kg) injections on extracellular DA and 5-HT and their metabolite DOPAC, HVA and 5-HIAA levels in the NAc. The times of the amphetamine injections are indicated by arrows. Data expressed as percentages of basal release are given as means ± S.E.M. ($n=6$). Histograms represent the area under the curve (AUC) after injection of the drug, and the minutes where AUC are calculated are shown in the figure. $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$, *T*-test. Water–Vehicle–saline group is added to the figure as a baseline reference. W/V/S = water + vehicle + saline, F/V/S = flutamide + vehicle + saline, W/N/A = water + nandrolone + amphetamine, F/N/A = flutamide + nandrolone + amphetamine, W/V/A = water + vehicle + amphetamine.

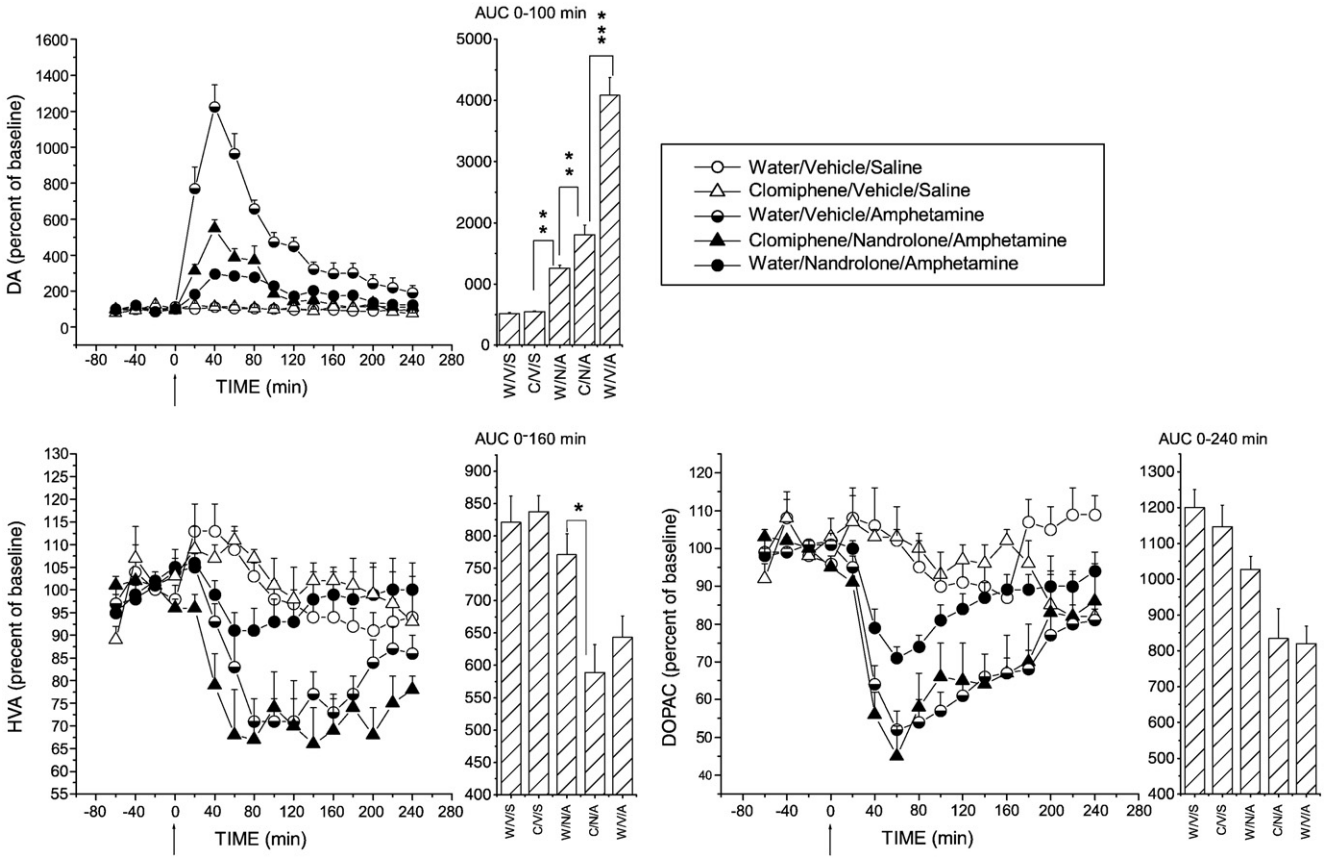

Fig. 3. The effects of pre-treatment with estrogen receptor antagonists, clomiphene (20 mg/kg), and nandrolone (20 mg/kg) together with acute amphetamine (1 mg/kg) injections on extracellular DA and 5-HT and their metabolite DOPAC, HVA and 5-HIAA levels in the NAc. The times of the amphetamine injections are indicated by arrows. Data expressed as percentages of basal release are given as means ± S.E.M. ($n = 5–6$). Histograms represent the area under the curve (AUC) after injection of the drug, and the minutes where AUC are calculated are shown in the figure. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, *T*-test. Water–vehicle–saline group is added in the figure as a baseline reference. W/V/S = water + vehicle + saline, C/V/S = clomiphene + vehicle + saline, W/N/A = water + nandrolone + amphetamine, C/N/A = clomiphene + nandrolone + amphetamine, W/V/A = water + vehicle + amphetamine.

Harlan, 1993; Wesson and McGinnis, 2006). Outside the NAc, steroids may regulate monoamine release indirectly by modulating the activity of ascending inputs, from monoaminergic nuclei that project to the NAc (e.g. the circuits of the bed nucleus of stria terminalis and the medial preoptic area, projecting to the ventral tegmental area (VTA)). Androgen sensitive cells in the lateral hypothalamus project to the VTA as well (Sato et al., 2008). These projections provide an opportunity for androgens to modify the activity of the mesocorticolimbic DA system. Finally, many of the brain areas that supply afferents to midbrain DA nuclei are themselves rich in classical steroid receptors, and are in a position to provide powerful, albeit indirect, genomic influence over the midbrain system (Kritzer, 1997). Recent studies in rats have also demonstrated rapid cellular effects of androgens in brain regions that possess only relatively few classical receptors (Mermelstein et al., 1996). These faster androgen actions are thought to be mediated by "non-classical" receptors, the membrane steroid receptors (Sato et al., 2008). Whereas the distribution of classical steroid receptors in the rodent brain is relatively limited, the potential brain targets for androgen action via membrane receptors are much broader. Therefore, the mechanisms by which AASs induce alterations in neuronal excitability and signal transduction in brain regions implicated in addiction may involve either classical or non-classical ARs, or even both.

The actions of AASs in the brain are complex and the final response depends not only on their interaction with ARs, but also on their ability to regulate and/or act as substrate for aromatase. Aromatase transforms androgens to estrogens through oxidation and subsequent elimination of a methyl group (Schade and Schubert, 1979). In fact, nandrolone is shown to be a substrate for aromatase enzyme (Ryan, 1959) and shown to stimulate the aromatase activity, which is related to the ability of estrogens of nandrolone origin to bind ERs in rat brain (Roselli, 1998). This is in line with our finding that blockade of ERs also reduces the nandrolone's ability to attenuate amphetamine-induced increase in extracellular DA in the NAc. The present data shows, that the effects of AR blockade are more pronounced than those induced by ER blockade, even though the doses are chosen to have equal potential. The less pronounced effect of ER blocking could also result from the fact that ER-immunoreactive neurons send only relatively few projections to the NAc. This gives support to our findings. It is suggested that the modifications of both ARs and ERs in the NAc by AASs, and likely AAS metabolites, are responsible for behavioral complexity associated with abuse of anabolic steroids (van de Poll et al., 1986). Our results confirm that nandrolone's steroidal action involves activation of AR and implicates that nandrolone or its metabolites require aromatization. So, the mechanism of action of nandrolone in brain is likely through a combination of AR and ER as it binds weakly to, but exhibits transactivation of both these receptors.

It has to be remembered that synthetic AASs and their metabolites do not only bind to ARs and ERs but also, with higher doses, to glucocorticoid and progestin receptors (Janne, 1990). They have also been shown to interact with GABA receptors (Masonis and McCarthy, 1995) and 5-HT receptors (Kindlundh et al., 2003). Consequently, the effects of ASSs are far from purely androgenic and may involve actions at multiple genomic and non-genomic substrates. However, the mechanisms other than those involving ARs and ERs by which AASs may affect the actions of amphetamine in the brain remain in many respects elusive and hypothetical.

The reduced activation of dopaminergic neurons may correspond to the increased prevalence of illicit drug usage among people who self-administer AASs (DuRant et al., 1995; Meilman et al., 1995; Scott et al., 1996) and the fact that abusers of AASs may require larger doses of drugs to achieve the desired effects. While AAS may not have the addictive potency of cocaine or morphine, we are just beginning to understand the potential for androgen reinforcement and addiction. Despite increased awareness by both the public and scientific communities of the profound neural changes, experimental study of the neurobiology has been limited. However, since pre-treatment with nandrolone substantially decreases the efficacy of amphetamine on extracellular DA in the present study in the rat, it is suggested that compounds acting like steroids at central receptors may be a useful complement in the research of stimulant treatment. Nonetheless, more research is needed to investigate how hormone exposure affects an individual's risk for psychopathology and multiple drug use.

In conclusion, the present study demonstrates that blockade of androgen receptor abolished, and blockade of estrogen receptor reduced, nandrolone-induced attenuation of amphetamine's action on extracellular levels of DA and its metabolites in the NAc. Therefore, it seems that the previously shown ability of nandrolone to modulate reward-related effects of psychostimulant drugs, at least amphetamine, is dependent on activation of the ARs or ERs.

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