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Research Report

The anabolic androgenic steroid nandrolone decanoate affects mRNA expression of dopaminergic but not serotonergic receptors

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ABSTRACT

The abuse of anabolic androgenic steroids (AASs) at supratherapeutic doses is a problem not only in the world of sports, but also among non-athletes using AASs to improve physical appearance and to become more bold and courageous. Investigations of the possible neurochemical effects of AAS have focused partially on the monoaminergic systems, which are involved in aggressive behaviours and the development of drug dependence. In the present study, we administered nandrolone decanoate (3 or 15 mg/kg/day for 14 days) and measured mRNA expression of dopaminergic and serotonergic receptors, transporters and enzymes in the male rat brain using quantitative real-time polymerase chain reaction. Expression of the dopamine D1-receptor transcript was elevated in the amygdala and decreased in the hippocampus while the transcript level of the dopamine D4-receptor was increased in the nucleus accumbens. No changes in transcriptional levels were detected among the serotonin-related genes examined in this study. The altered mRNA expression of the dopamine receptors may contribute to some of the behavioural changes often reported in AAS abusers of increased impulsivity, aggression and drug-seeking.

1. Introduction

Anabolic androgenic steroids (AASs) are abused at supratherapeutic doses to improve athletic performance and physical appearance (Yesalis and Bahrke, 1995) and AAS abusers are reported to fulfill the DSM-IV criteria for drug dependence (Copeland et al., 2000; Pope and Katz, 1994). In rodents, AASs cause conditioned-place preference, which is reversed by dopamine antagonists (Packard et al., 1998; Schroeder and Packard, 2000). Additionally, rodents self-administer AASs (Ballard and Wood, 2005; DiMeo and Wood, 2004; Wood et al., 2004). AAS abusers self-report increased aggression and

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Abbreviations: AAS, anabolic androgenic steroids; ACT, β-actin; CYCLO, cyclophilin; DOPAC, 3,4-dihydrophenylacteic acid; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; GOI, gene of interest; H3b, histone H3b; HKG, house-keeping gene; HVA, homovanillic acid; LTD, long-term depression; LTP, long-term potentiation; MDMA, 3,4-methylenedioxy-methamphetamine; qPCR, quantitative real-time polymerase chain reaction; RPL19, ribosomal protein L19; SDCA, succinate dehydrogenase complex A subunit; TUB, β-tubulin beta 5

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irritability (Bahrke et al., 1992) and are associated with violent crimes (Thiblin and Parlklo, 2002). Therefore, a number of animal studies have been performed in order to examine long-term effects of AASs upon monoamines, their receptors and metabolites in neurocircuitries involved in these behaviours.

In some studies, sub-chronic AAS administration at supratherapeutic doses reduces dopaminergic function (Birgner et al., 2007; Kurling et al., 2008). In one of the microdialysis studies, the extracellular basal levels of the dopamine metabolites 3,4-dihydrophenylacteic acid (DOPAC) and homovanillic acid (HVA) are significantly reduced in the nucleus accumbens after 14 days of nandrolone decanoate administration (15 mg/kg/day i.m.). This difference persists during the first hour after an amphetamine challenge (Birgner et al., 2007). In a similar study, pre-treatment with nandrolone decanoate (5 and 20 mg/kg/48 h for 10 days) attenuates amphetamine- and 3,4-methylenedioxy-methamphetamine (MDMA)-induced behavioural response and dopamine release (Kurling et al., 2008). In addition to this, nandrolone decanoate pre-treatment prevents the amphetamine-induced effect on hippocampal and hypothalamic DOPAC/dopamine ratios (Birgner et al., 2006). Furthermore, 2 weeks of AAS administration decreases dopamine D1-like receptor density and increases dopamine D2-like receptor- and dopamine transporter densities in the striatum (Kindlundh et al., 2001, 2004). These dopamine receptor changes have been confirmed for the dopamine D1- and D2-receptor subtypes in the striatum using *in situ*-hybridization (Kindlundh et al., 2003b). The dysfunctional dopamine system suggested by these findings could be related to the late stages in the development of dependence (Koob, 2006).

AASs increases aggression in several rodent species (Johansson et al., 2000; McGinnis et al., 2002; Melloni et al., 1997; Pinna et al., 2005) and the serotonin system may be implicated in such behavioural changes (Ricci et al., 2006). In hamsters treated with a steroid cocktail consisting of 2 mg/kg/day of testosterone cypionate, 2 mg/kg/day of nandrolone decanoate and 1 mg/kg/day of boldenone undecylenate for 30 days aggressive behaviour was accompanied by decreased density of 5HT1A-receptors in the anterior hypothalamus (Ricci et al., 2006). A 5HT1A-receptor agonist can reverse the aggression (Ricci et al., 2006), consistent with earlier behavioural studies reporting reduced aggressive and impulsive behaviours due to either 5HT1A- or 5HT1B-receptor agonist administration (Simon et al., 1998). In studies on rats, AASs decrease the density of 5HT1B-receptors in the CA1 field of the hippocampus (Kindlundh et al., 2003a). However, effects upon brain levels of serotonin and its metabolites are inconclusive, possibly due to differences in dosing paradigms (Kurling et al., 2005; Lindqvist et al., 2002; Thiblin et al., 1999).

Previous studies have mainly examined the effect of AASs on the monoaminergic systems by measuring transmitter and metabolite levels or by detecting protein densities of receptors, enzymes and transporters. Knowledge of AAS-induced effects on mRNA expression of dopaminergic receptors is limited to the dopamine D1- and D2-receptor subtypes in the *striatum* (Kindlundh et al., 2003a). The documented literature of AAS-induced effects on mRNA expression of dopaminergic and serotonergic receptors and transporters in other brain areas regulating cognitive functions, control of behaviour versus disinhibitory behaviour (impulsivity) and emotions is restricted. The prefrontal cortex and the hippocampus are crucial for cognition and memory and implicated in reward-related associative learning (Di Chiara, 1999; Thierry et al., 2000). The nucleus accumbens is not only characterized by its acute increase of dopamine release (Pontieri et al., 1995) in response to drugs of abuse, but also, together with the amygdala, a component of the "extended amygdala" (Di Chiara et al., 1999). This area is important for e.g. emotional conditioning. The prefrontal cortex is also important for the regulation of inhibitory control of behaviour and therefore plays a role in disinhibition and impulsivity (Jentsch and Taylor, 1999; Tekin and Cummings, 2002). The primary aim of the present study was therefore to investigate whether gene-transcript expressions of dopaminergic and serotonergic receptor subtypes, transporters and enzymes, are implicated in AAS-induced effects in brain regions predominantly regulating cognitive functions, memory, inhibitory behaviour and emotions (the prefrontal cortex, hippocampus, nucleus accumbens and the amygdala). Nandrolone decanoate was administered to male rats for 14 days at doses of 3 or 15 mg/kg/day. Based on a one-year follow-up study of AAS abusers (Fudala et al., 2003), the dosing paradigm aimed to mimic one cycle of human abuse during light and experienced AAS abuse, respectively.

2. Results

Two weeks of daily nandrolone decanoate administration significantly elevated the dopamine D1-receptor mRNA expression in the amygdala at a dose of 15 mg/kg ($F_{2,\ 20}=8.16$, $p=0.003$), but decreased it in the hippocampus at doses of 3 and 15 mg/kg, respectively ($F_{2,\ 25}=6.72$, $p=0.005$) (Table 1). The dopamine D4-receptor mRNA expression was significantly increased in the nucleus accumbens by nandrolone decanoate at a dose of 3 mg/kg/day for 14 days, both compared to controls and the group of rats administered the highest dose of 15 mg/kg/day ($F_{2,\ 22}=4.93$, $p=0.017$) (Table 1). No statistically significant changes regarding dopamine-related transcripts were detected in the prefrontal cortex, ventral tegmental area and substantia nigra. None of the serotonin-related transcript levels examined were statistically changed (Table 2).

3. Discussion

The major findings in the present study are that 2 weeks of daily nandrolone decanoate administration (3 or 15 mg/kg) causes alterations in mRNA expression of dopamine receptors, but not in any of the investigated serotonin receptors.

In the nucleus accumbens, the lower nandrolone decanoate dose of 3 mg/kg increased the transcription level of the dopamine D4-receptor, whereas no alterations were observed at the highest dose (15 mg/kg) (Table 1). Biphasic dose–response relationships have, indeed, been described in the literature for several transmitters and hormones, including androgens (Calabrese, 2001b) and estrogens (Calabrese, 2001a). Increased mRNA expression of the dopamine D4-receptor in the nucleus

Table 1 – Expression of dopamine-related transcripts after sub-chronic treatment with nandrolone decanoate at two different doses

Tissue	Gene of interest	Treatment		
		Control	Nadrolone decanoate (3 mg/kg)	Nanrolone decanoate (15 mg/kg)
Prefrontal cortex	D1	0.446±0.040	0.445±0.085	0.370±0.027
	D2	0.291±0.044	0.398±0.097	0.341±0.059
	D3	0.167±0.064	0.177±0.064	0.162±0.058
	D4	Nd	Nd	Nd
	D5	Nd	Nd	Nd
	DAT	Nd	Nd	Nd
	VMAT2	Nd	Nd	Nd
Nucleus accumbens	D1	0.384±0.032	0.356±0.028	0.359±0.047
	D2	0.386±0.032	0.331±0.038	0.380±0.042
	D3	0.564±0.072	0.559±0.058	0.449±0.081
	D4	0.277±0.050	0.457±0.064 [a]	0.232±0.042 [c]
	D5	Nd	Nd	Nd
	DAT	Nd	Nd	Nd
	VMAT2	Nd	Nd	Nd
Hippocampus	D1	0.403±0.033	0.259±0.021 [b]	0.314±0.025 [a]
	D2	0.541±0.031	0.465±0.046	0.493±0.032
	D3	0.521±0.045	0.646±0.099	0.502±0.029
	D4	Nd	Nd	Nd
	D5	0.392±0.043	0.336±0.049	0.431±0.079
	DAT	0.414±0.053	0.402±0.052	0.388±0.058
	VMAT2	0.628±0.055	0.645±0.070	0.608±0.045
Amygdala	D1	0.368±0.041	0.341±0.029	0.560±0.048 [b,c]
	D2	0.393±0.093	0.279±0.035	0.408±0.084
	D3	0.403±0.076	0.450±0.077	0.402±0.018
	D4	Nd	Nd	Nd
	D5	0.585±0.118	0.660±0.087	0.548±0.073
	DAT	Nd	Nd	Nd
	VMAT2	Nd	Nd	Nd
Ventral tegmental area	D1	0.178±0.022	0.166±0.009	0.176±0.015
	D2	0.234±0.036	0.225±0.021	0.290±0.039
	D3	Nd	Nd	Nd
	D4	Nd	Nd	Nd
	D5	Nd	Nd	Nd
	TH	0.299±0.041	0.356±0.061	0.367±0.074
	AAAD	0.277±0.034	0.371±0.056	0.275±0.033
	DAT	0.343±0.050	0.426±0.044	0.387±0.055
	VMAT2	0.136±0.030	0.164±0.038	0.229±0.063
Substantia nigra	D2	0.173±0.026	0.169±0.030	0.142±0.023
	D3	0.209±0.037	0.199±0.036	0.180±0.026
	TH	0.299±0.049	0.287±0.043	0.297±0.0378
	AAAD	0.289±0.034	0.236±0.018	0.236±0.020
	DAT	0.165±0.016	0.187±0.025	0.168±0.011
	VMAT2	0.172±0.015	0.176±0.012	0.171±0.014

Expression of dopamine-related transcripts in male rat brain tissue after administration of nandrolone decanoate at the doses 3 and 15 mg/kg for 14 days. Data are expressed as mean±S.E.M. and statistically evaluated with one-way ANOVA followed by Newman–Keuls Multiple Comparison Test when appropriate. Nd: not detectable.

[a] $p< 0.05$ vs. control.

[b] $p<0.01$ vs. control.

[c] $p<0.05$ vs. nandrolone decanoate (3 mg/kg).

accumbens may explain some of the behavioural changes reported in AAS abusers. For example, the dopamine D4-receptor has been implicated in novelty seeking (Dulawa et al., 1999) and impaired behavioural inhibition (Avale et al., 2004), behavioural features that both are related to schizophrenia and substance abuse. Dopamine D4-receptor antagonists display antipsychotic effects (Boeckler et al., 2004) and several atypical neuroleptics have high affinity for the dopamine D4-receptor (Seeman et al., 1997). D4-receptor knock-out mice display decreased basal levels of dopamine, DOPAC and HVA in the striatum, as measured by microdialysis (Thomas et al., 2007), and unaltered or even increased sensitivity to ethanol, cocaine and methamphetamine, compared to wild type mice (Rubinstein et al., 1997). This is consistent with the results of increased dopamine D4 transcription in the present study and decreased sensitivity to other drugs, shown repeatedly after AAS treatment (Birgner et al., 2006, 2007; Celerier et al., 2003, 2006; Kurling et al., 2008; Lindqvist et al., 2002). However, chronic AAS

administration also decreases extracellular levels of dopamine, DOPAC and HVA (Birgner et al., 2007; Kurling et al., 2008), which is inconsistent with the dopamine D4 knock-out phenotype. Therefore, the role of dopamine D4-receptor mRNA expression in the mechanism behind AAS-induced alterations remains to be evaluated.

The transcription level of the dopamine D1-receptor was increased in the amygdala after administration of 15 mg/kg of nandrolone decanoate (Table 1). The amygdala is innervated by the ventral tegmental area, and is together with the nucleus accumbens involved in stimulus-reward learning, as part of the extended amygdala (Di Chiara, 1999; Koob, 2003). In the basolateral amygdala, dopamine D1-receptor activation contributes to acquisition of cocaine-cue association (Berglind et al., 2006) and drug-seeking behaviour (See et al., 2001). Thus, the increased transcription of the dopamine D1-receptor in the amygdala might contribute to drug-seeking behaviour often seen among AAS abusers. In the hippocampus, on the other hand, the transcription level of the dopamine D1-receptor was decreased by both doses of nandrolone decanoate (Table 1). In addition to glutamate, dopamine has the ability to modulate hippocampal plasticity, in the form of long-term potentiation (LTP) and long-term depression (LTD), particularly during novelty detection (Lisman and Grace, 2005). D1/D5 antagonists impair late-phase LTP (Lemon and Manahan-Vaughan, 2006) and long-term memory (O'Carroll et al., 2006) whereas D1/D5 agonists facilitate late-phase LTP (Li et al., 2003). Spatial memory and hippocampal plasticity were unaffected in studies using a steroid cocktail consisting of 2 mg/kg/day of testosterone cypionate, 2 mg/kg/day of nandrolone decanoate and 1 mg/kg/day of boldenone undecylenate for either 4 or 12 weeks (Clark et al., 1995; Smith et al., 1996), and impaired memory function after 15 mg/kg/day of nandrolone decanaote for 6 weeks (Kouvelas et al., 2008). The possible effects of AAS on learning and memory need to be further evaluated.

Regarding serotonin receptors, the gene-transcript level of the 5HT2A-receptor in the nucleus accumbens showed a trend towards an increase, but this did not reach statistical significance (Table 2). Earlier, 2 weeks of nandrolone decanoate administration to male Sprague–Dawley rats upregulated 5HT2-receptor densities in the nucleus accumbens shell (at 1, 5 and 15 mg/kg) and the ventromedial hypothalamus (at 1 and 5 mg/kg) using *in vitro*-autoradiography (Kindlundh et al., 2003a). Interestingly, testosterone replacement in castrated male rats has been shown to increase the 5HT2A-receptor mRNA content in the dorsal raphe nucleus (Fink et al., 1999) and ventromedial hypothalamus (Zhang et al., 1999). Previously, the 5HT2-receptor density was also shown to be decreased in the frontal cortex, hippocampus and amygdala (Kindlundh et al., 2003a). No corresponding alterations in mRNA levels of 5HT2-receptors could be detected with qPCR in this study. However, since the radioligand used in the *in vitro*-autoradiography study labelled the 5HT2 receptors

Table 2 – Expression of serotonin-related transcripts after sub-chronic treatment with nandrolone decanoate at two different doses

Tissue	Gene of interest	Treatment		
		Control	Nadrolone decanoate (3 mg/kg)	Nanrolone decanoate (15 mg/kg)
Prefrontal cortex	5HT1A	0.173±0.013	0.156±0.015	0.213±0.032
	5HT1B	0.258±0.037	0.337±0.052	0.334±0.037
	5HT2A	0.268±0.052	0.323±0.042	0.364±0.039
	5HT2C	0.201±0.030	0.177±0.031	0.196±0.039
	5HT3	0.199±0.021	0.202±0.023	0.267±0.042
	5HT6	0.387±0.038	0.355±0.051	0.363±0.023
Nucleus accumbens	5HT1A	0.220±0.038	0.201±0.039	0.194±0.031
	5HT1B	0.411±0.049	0.396±0.036	0.469±0.469
	5HT2A	0.245±0.052	0.271±0.034	0.347±0.028
	5HT2C	0.484±0.053	0.377±0.031	0.508±0.089
	5HT3	0.172±0.042	0.170±0.041	0.191±0.051
	5HT6	0.517±0.060	0.421±0.078	0.359±0.049
Hippocampus	5HT1A	0.552±0.049	0.557±0.057	0.522±0.076
	5HT1B	0.444±0.027	0.459±0.053	0.438±0.040
	5HT2A	0.427±0.051	0.409±0.072	0.392±0.062
	5HT2C	0.164±0.021	0.212±0.027	0.267±0.059
	5HT3	0.444±0.077	0.458±0.072	0.446±0.070
	5HT6	0.357±0.031	0.394±0.040	0.353±0.034
Amygdala	5HT1A	0.667±0.103	0.653±0.078	0.558±0.025
	5HT1B	0.099±0.019	0.084±0.026	0.096±0.021
	5HT2A	0.453±0.097	0.579±0.053	0.610±0.062
	5HT2C	0.379±0.051	0.334±0.035	0.450±0.067
	5HT3	Nd	Nd	Nd
	5HT6	0.467±0.069	0.422±0.033	0.467±0.038

Expression of serotonin-related transcripts in male rat brain tissue after administration of nandrolone decanoate at the doses 3 and 15 mg/kg for 14 days. Data are expressed as mean±S.E.M. and statistically evaluated with one-way ANOVA followed by Newman–Keuls Multiple Comparison Test when appropriate. Nd: not detectable.

unselectively, whereas the primers used in the current qPCR are specific towards different 5HT2-receptor subtypes, findings from the different studies are not comparable. The data indicate that changes in protein levels can be due to posttranslational processing. The lack of linearity between mRNA level and protein level in the serotonin system has been reported earlier, e.g. for the serotonin transporter in the macaque brain following ovarian steroid treatment (Lu et al., 2003; Smith et al., 2004).

In conclusion, sub-chronic administration of the AAS nandrolone decanoate significantly altered the gene-transcript levels of dopamine D1- and D4-receptors in the nucleus accumbens, amygdala and hippocampus. These findings may explain behavioural changes often observed in AAS abusers such as impulsivity and drug-seeking.

4. Experimental procedures

4.1. *Animals*

Ten-week-old male Sprague–Dawley rats (B&K, Sollentuna, Sweden) were housed three in each cage at constant conditions (22 °C, 60% humidity, a twelve-hour light/dark circle, and food and water provided *ad libitum*). After being allowed to adapt to the new environment for 7 days, the rats were randomly divided into three groups ($n=10$). Two groups were administered intramuscular injections of nandrolone decanoate (Deca-Durabol®, Organon, Oss, Netherlands) at a dose of 3 or 15 mg/kg once daily for 14 days, and the control group received intramuscular injections of the vehicle (arachidis oleum, Apoteket AB, Umeå, Sweden). On day 15, animals were sacrificed by decapitation and the brains were rapidly removed and dissected using a rat brain matrix (Activational Systems, Warren, MI, USA). The prefrontal cortex (Bregma>+4.2), nucleus accumbens (Bregma +2.2 to + 1.0), hippocampus (Bregma −1.8 to −5.3), amygdala (Bregma −1.8 to −3.3), ventral tegmental area (−5.2 to −5.6) and the substantia nigra (−4.8 to −6.04) were collected. Tissues were immersed in RNAlater (Ambion) allowing the solution to infiltrate the tissue for 1 h in room temperature. All samples were stored in −80 °C until prepared for analysis. The experimental procedure was approved by the Animal Care and Ethical Committee in Uppsala, Sweden.

4.2. *qPCR*

The used method, qPCR, offers a variety of advantages, such as the normalization to a stable set of house-keeping genes (HKGs) and the possibility to quantify a large number of transcripts in the same individual. The primers were designed with Beacon Designer (v4.0). Forward and reverse primers, together with accession numbers, for both house-keeping genes (HKGs) and genes of interest (GOIs) are presented in Table 3. The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used to verify that no homologies were shared between amplified sequences and other cDNA in the database.

Table 3 – Primer sequences for gene transcripts used in the quantitative real-time reversed transcription PCR

	Gene	Accession	Forward primer	Reverse primer
GOI	5HT1A	genbank:NM_012585	5′-ccgcacgcttccgaatcc-3′	5′-tgtccgttcaggctcttcttg-3′
	5HT1B	genbank:NM_022225	5′-caccctttcttctggcgtcaag-3′	5′-accgtggagtagaccgtgtag-3′
	5HT2A	genbank:NM_017254	5′-aacggtccatccacagag-3′	5′-aacaggaagaacacgatgc-3′
	5HT2C	genbank:NM_012765	5′-ttggactgagggacgaaagc-3′	5′-ggatgaagaatgccacgaagg-3′
	5HT3	genbank:NM_024394	5′-caaggaagggtcaggatgg-3′	5′-aaggaacagtgtggctctc-3′
	5HT6	genbank:NM_024365	5′-gccgcatccactcagttc-3′	5′-cctaccacctcctagtctcag-3′
	AAAD	genbank:U31884	5′-ctggggaaggggaggagtg-3′	5′-gcagctggcggatcattttag-3′
	D1	genbank:M35077	5′-cgggctgccagcggagag-3′	5′-tgcccaggagagtggacagg-3′
	D2	genbank:NM_012547	5′-agacgatgagccgcagaaag-3′	5′-gcagccagcagatgatgaac-3′
	D3	genbank:NM_017140	5′-acggcaccgggcagagc-3′	5′-gagggcaggacacagcaaaag-3′
	D4	genbank:NM_012944	5′-ggcgtgtggctgctgag-3′	5′-ttgaagatggaggcggtgc-3′
	D5	genbank:NM_012768	5′-ccactgcctccatcctgaatc-3′	5′-vgctactcgctgggtcatcttg-3′
	DAT	genbank:S76145	5′-gctgcgtcactggctgttgc-3′	5′-ctgtccccgctgttgtgaggt-3′
	TH	genbank:NM_012740	5′-gcccccacctggagtattttg-3′	5′-agacacccgacgcacagagc-3′
	VMAT2	genbank:NM_013031	5′-ggacgcacggacagagac-3′	5′-atcgcagcagcaccagatc-3′
HKG	ACT	genbank:NM_031144	5′-cactgccgcatcctcttcct-3′	5′-aaccgctcattgccgatagtg-3′
	CYCLO	genbank:M19533	5′-gagcgttttgggtccaggaat-3′	5′-aatgcccgcaagtcaaagaaa-3′
	GAPDH	genbank:X02231	5′-acatgccgcctggagaaacct-3′	5′-gcccaggatgcccttttagtgg-3′
	H3b	genbank:NM_053985	5′-attcgcaagctccccttttcag-3′	5′-tggaagcgcaggtctgttttg-3′
	RPL19	genbank:NM_031103	5′-tcgccaatgccaactctcgtc-3′	5′-agcccgggaatggacagtcac-3′
	SDCA	genbank:NM_130428	5′-gggagtgccgtggtgtcattg-3′	5′-ttcgcccatagcccccagtag-3′
	TUB	genbank:NM_173102	5′-cggaaggaggcggagagc-3′	5′-agggtgcccatgccagagc-3′

Abbreviations: 5HT1A, serotonin receptor 1A; 5HT1B, serotonin receptor 1B; 5HT2A, serotonin receptor 2A; 5HT2C, serotonin receptor 2C; 5HT3, serotonin receptor 3; 5HT6, serotonin receptor 6; AAAD, aromatic L-amino acid decarboxylase; ACT, β-actin; CYCLO, cyclophilin; D1, dopamine D1 receptor; D2, dopamine D2 receptor; D3, dopamine D3 receptor; D4, dopamine D4 receptor; D5, dopamine D5 receptor; DAT, dopamine transporter; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; GOI, gene of interest; H3b, histone H3b; HKG, house-keeping gene; RPL19, ribosomal protein L19; SDCA, succinate dehydrogenase complex A subunit A; TH, tyrosine hydroxylase; TUB, β-tubulin beta 5; VMAT2, vesicular monoamine transporter 2.

Total RNA was isolated from individual brain tissue samples by phenol–chloroform extraction. Tissue samples were homogenized in 500 μl TRIzol® (Invitrogen AB, Stockholm, Sweden) by ultrasonication with a Branson sonifier. 100 μl chloroform was added and the homogenate was centrifuged at 12000 ×*g* for 15 min (4 °C). The supernatant was transferred to a new tube and RNA was precipitated in isopropanol. The pellet was washed twice with 75% ethanol, thereafter air-dried and dissolved in DNAase buffer. DNAase treatment was performed at 37 °C for 2 h in order to remove DNA contamination, followed by inactivation of the DNAase at 75 °C for 15 min. RNA purity was validated by PCR and gel-electrophoreses using primers for a 300 bp cDNA of GAPDH. RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (Saveen & Werner AB, Limhamn, Sweden). cDNA synthesis was performed with M-MLV reverse transcriptase according to the manufacturer's protocol, using random hexamer primers (GE Healthcare, Sweden). The cDNA synthesis was validated by PCR and gel-electrophoreses.

qPCR was performed in a final reaction volume of 25 μl (20 mM Tris–HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, SYBR Green 1:50000, 10 nM fluoroscein, 0.8 pmol/μl each of reverse and forward primer, 0.02 U/μl Taq DNA polymerase) using an iCycler real-time detection instrument (Bio-Rad Laboratories, Sundbyberg, Sweden). 50 cycles were performed. Annealing temperatures were 62 °C for all GOIs and 60 °C for the HKGs. Melting point curves were included to confirm that only one product was formed. Each assay included individual samples in duplicate and a negative control in triplicate. iCycler IQ v3.0 software was used to analyse qPCR data where the starting quantity means were normalized to the maximum sample value of each plate, resulting in values falling between 0 and 1. For the nucleus accumbens, caudate putamen, ventral tegmental area, hippocampus and prefrontal cortex a standard curve of four dilution points in triplicates was included, and for the hypothalamus, amygdala and substantia nigra the sample values were corrected for primer efficiency using the LinRegPCR protocol (Ramakers et al., 2003). Sample values were then divided by the normalization factors created according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The most stable set of HKGs out of seven tested in each tissue was selected using the GeNorm protocol (Vandesompele et al., 2002), an approach which we have previously validated (Lindblom et al., 2006). The HKGs included in the normalization factors were, for the prefrontal cortex: ACT, H3b, TUB; nucleus accumbens: CYCLO, GAPDH, SDCA; hippocampus: ACT, H3b, RPL19; amygdala: ACT, GAPDH, RPL19; ventral tegmental area: CYCLO, H3b, RPL19; and substantia nigra: GAPDH, H3b, RPL19 (for abbreviations, see Table 3).

4.3. *Statistics*

Statistical analysis was performed using Prism v4.0 (GraphPad Software Inc.). The differences in normalized gene-transcript levels were statistically evaluated with one-way ANOVA and Newman–Keuls Multiple Comparison Test when appropriate. Results were considered significant when $p<0.05$. Grubb's test was used to identify outliers.

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