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Research Report

Reduced activity of monoamine oxidase in the rat brain following repeated nandrolone decanoate administration

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ABSTRACT

Anabolic androgenic steroids (AAS) are known as doping agents within sports and body-building, but are currently also abused by other groups in society in order to promote increased courage and aggression. We previously showed that 14 days of daily intramuscular injections of the AAS nandrolone decanoate (15 mg/kg) reduced the extracellular levels of the dopaminergic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the nucleus accumbens shell using microdialysis. The aim of the present study was to investigate whether the same dose regimen of nandrolone decanoate may affect the activities of the dopamine-metabolizing enzymes monoamine oxidases A and B (MAO-A and MAO-B). A radiometric assay was used to determine the activities of MAO-A and MAO-B in rat brain tissues after 14 days of daily i.m. nandrolone decanoate injections at the doses 3 and 15 mg/kg. Gene transcript contents of MAO-A, MAO-B and cathecol-*O*-methyltransferase (COMT) were measured with quantitative real-time reverse transcription PCR. 3 mg/kg of nandrolone decanoate significantly reduced the activity of both MAO-A and -B in the caudate putamen. 15 mg/kg of nandrolone decanoate significantly reduced the activity of MAO-A in the amygdala and increased the gene transcript level of MAO-B in the substantia nigra. In conclusion, imbalanced MAO activities may contribute to explain the impulsive and aggressive behaviour often described in AAS abusers. The reduced MAO activities observed are in line with our previously presented findings of decreased extracellular levels of DOPAC and HVA in the rat brain, indicating decreased monoaminergic activity following repeated AAS administration.

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Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; AAS, anabolic androgenic steroids; ACT, β-actin; β-PEA, β-phenylethylamine; COMT, cathecol-*O*-methyltransferase; CYCLO, cyclophilin; DOPAC 3, 4-dihydrophenylacteic acid; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GOI, gene of interest; H3b, histone H3b; HKG, house-keeping gene; HVA, homovanillic acid; MAO, monoamine oxidase; 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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1. Introduction

The non-medical use of anabolic androgenic steroids (AAS) receives attention not only during sports events, but also in connection to reports of violent acts (Petersson et al., 2006; Yesalis and Bahrke, 1995). AAS are abused for several reasons, e.g. to improve physical appearance and to become more bold and courageous (Kindlundh et al., 1999). Abusers of AAS are reported to display increased aggression and irritability (Bahrke et al., 1992). In addition, both hypomania and depression have been demonstrated in AAS-abusing athletes (Pope and Katz, 1994). There are also reports of AAS abusers engaging in criminal activity, violent acts (Thiblin and Parlklo, 2002), and even homicide (Corrigan, 1996). Behavioural studies have reported increased aggression after AAS administration in rats (Johansson et al., 2000; McGinnis et al., 2002), mice (Pinna et al., 2005) and hamsters (Melloni et al., 1997), implicating involvement of e.g. serotonin (Ricci et al., 2006). Loss of impulse control, often reported among AAS abusers, not only underlies aggression, but could also contribute to explain the initiation of drug abuse and its development into drug addiction (Kreek et al., 2005). AAS have been suggested to be addictive in humans and to serve as a gateway to abuse of other illicit drugs (Kanayama et al., 2003). Testosterone has been shown to be self-administered by rodents (Ballard and Wood, 2005) and to induce conditioned place preference, a phenomena, which could be reversed by dopamine D1 and D2 receptor antagonists (Schroeder and Packard, 2000).

Enzymatic alterations in monomaniergic systems contribute to behavioural changes. Reduced platelet monoamine oxidase (MAO) is associated with e.g. male criminality (Oreland et al., 2007) and violent offence (Skondras et al., 2004). Serotonin, noradrenalin and dopamine are all metabolized by MAO, and dopamine and noradrenalin by catechol-O-methyltransferase (COMT) as well. MAO exists in two isoforms, MAO-A and MAO-B, where type A displays highest affinity for serotonin and noradrenalin, and type B for β-phenylethylamine (β-PEA). Both isoforms display similar affinities for dopamine. In human brain, MAO-B is the main dopamine-metabolizing enzyme. Human platelets contain MAO of the B-form and this activity is associated with certain personality traits and behaviours (Oreland, 2004). MAO-B knock-out mice do not express great behavioural changes, while MAO-A knock-out mice show enhanced aggression (Cases et al., 1995). However, there are species variations with respect to the relative importance of MAO-A and MAO-B, respectively, for CNS monoamine metabolism. In contrast to human brain, a much higher proportion of MAO-A than MAO-B contributes to metabolism in the rodent brain (Oreland et al., 1983). In human, presence of a variation in the MAO-A promoter expressing lower MAO-A enzyme activity is associated with an increased vulnerability for a variety of antisocial behaviours (Sjoberg et al., 2008). Similar studies on MAO-B have not been possible to perform because of the seemingly scarcity of functional polymorphisms in the MAO-B gene (Pivac et al., 2006). It is of great interest to investigate whether changes in MAO activity also contribute to AAS abuse.

The current research on MAO activity in relation to AAS abuse is restricted. In one study, investigating the effects of AAS administered once weekly for 6 weeks, oxymetholone increased MAO-A activity in the hypothalamus, whereas no effects were found by nandrolone propionate (Thiblin et al., 1999). On the other hand, studies have been performed in order to determine monoamine levels and their corresponding metabolites in the rat brain after repeated AAS administration. The drawback of the studies is that findings on AAS induced changes in monoaminergic systems are inconsistent. This could be due to differences in dose regimens, routes of administration, and how and when the animals were sacrificed. In male rats, nandrolone propionate subcutaneously administered at a dose of 5 mg/kg once weekly for 6 weeks, caused increased dopamine turnover in the striatum and increased serotonin turnover in the hippocampus, 48 h after the last injection (Thiblin et al., 1999). Daily subcutaneous injections of 15 mg/kg nandrolone decanoate for 14 days produced decreased levels of serotonin and 5-HIAA in the dorsal striatum 24 h after the last injection, but no changes with respect to dopamine (Lindqvist et al., 2002). In another study with the same dose regimen, pre-treatment with nandrolone decanoate prevented amphetamine induced effects upon dopamine turnover in the hypothalamus and hippocampus (Birgner et al., 2006). 5 mg/kg of nandrolone decanoate 5 days per week for 2 weeks increased dopamine turnover and 20 mg/kg increased the levels of serotonin in the hypothalamus. In this last report the animals were sacrificed 72 h after the last injection with carbon dioxide and not decapitation as in the before mentioned studies (Kurling et al., 2005).

We recently performed a microdialysis study where the extracellular levels of dopamine, DOPAC and HVA were measured following intramuscular administration of nandrolone decanoate at a dose of 15 mg/kg/day for 14 days. The basal level of dopamine was unchanged but the metabolite levels were significantly reduced in the nucleus accumbens shell of male Sprague–Dawley rats. This difference persisted during the first hour following an amphetamine challenge (Birgner et al., 2007). In the light of these findings, we hypothesize that these reduced brain levels of DOPAC and HVA after AAS administration could be explained by reduced activity of the dopamine-metabolizing enzymes MAO-A and MAO-B. The specific aim of the present study was to examine whether administration of the AAS nandrolone decanoate, at the doses 3 and 15 mg/kg daily for 14 days, could affect the enzyme activity of MAO-A and MAO-B in rat brain regions regulating behaviours such as aggression and the development of drug dependence. Additionally, gene transcript levels of MAO-A, MAO-B and COMT were investigated in the same animals, using quantitative real-time reverse transcription polymerase chain reaction (qPCR). The amounts of AAS administered by abusers have been reported to widely exceed those used clinically. We consider the doses 3 and 15 mg/kg nandrolone decanoate administered intramusculary once daily for 14 days to rats, to be equivalent of one cycle of human use during early and experienced AAS abuse respectively, based on a one-year follow-up study of AAS abusers (Fudala et al., 2003).

2. Results

2.1. *Enzyme activity*

The enzyme activity of MAO-A is presented in Fig. 1 and of MAO-B in Fig. 2. Nandrolone decanoate at a daily dose of 3 mg/kg,

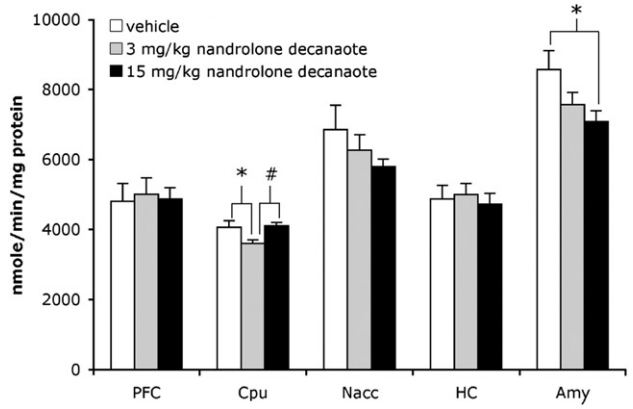

Fig. 1 – Enzymatic activity of monoamine oxidase A in male rat brain tissue, after 14 days of daily nandrolone decanoate administration at the doses 3 mg/kg and 15 mg/kg. Values are expressed as mean ± S.E.M. in nmol/min/mg protein. N = 9–10. *($p < 0.05$) vs vehicle #($p < 0.05$) vs 3 mg/kg nandrolone decanoate.

significantly reduced the MAO-A activity (11%; $F_{2,\ 27}=4.22$; $p=0.025$) and the MAO-B activity (17%; $F_{2,\ 27}=8.73$; $p=0.001$) in the caudate putamen, compared to controls. The MAO-A and MAO-B activities in the group treated with 3 mg/kg nandrolone decanoate also differed significantly from those in the group treated with 15 mg/kg of nandrolone decanoate, which were similar to control values. The activity of MAO-A was significantly reduced in the amygdala after administration of nandrolone decanoate at a dose of 15 mg/kg/day for 14 days, compared to the control group (17%; $F_{2,\ 27}=3.36$; $p=0.049$) and also MAO-B were close to being significantly reduced in the group receiving 15 mg/kg of nandrolone decanoate ($F_{2,\ 26}=3.35$; $p=0.051$). No significant changes were found in the prefrontal cortex, the nucleus accumbens or the hippocampus.

2.2. *qPCR*

The gene expression data of MAO-B and COMT are presented in Table 1. The expression level of MAO-B was significantly increased in the substantia nigra after 14 days of daily intramuscular injections of nandrolone decanoate at the dose 3 mg/kg ($p=0.046$). No significant changes were found in the prefrontal cortex, nucleus accumbens, caudate putamen, hippocampus, hypothalamus, amygdala or the ventral tegmental area. COMT could not be detected in the ventral tegmental area and MAO-A could not be detected in any of the tissues included in this study, due to their low tissue specific mRNA expression.

3. Discussion

In the present study, we have shown reduced activities of MAO-A and MAO-B in the caudate putamen, results which are in line with our earlier findings of decreased extracellular levels of DOPAC and HVA in the nucleus accumbens of nandrolone decanoate treated rats (Birgner et al., 2007). The reduced activity of MAO-A in the amygdala might contribute to an explanation of the impulsive and aggressive behaviours often reported in AAS abusers. The question remains to what extent effects of nandrolone decanoate on MAO activities shown in the current study underlie mechanisms behind previously reported biochemical and behavioural effects of the drug.

In the caudate putamen there was a U-shaped dose-response both with regard to MAO-A and MAO-B. The effects of 15 mg/kg of nandrolone decanoate on MAO activities in the caudate putamen were similar to those of the control group. Biphasic dose-response relationships have indeed been described in the literature for several transmitters and hormones, including androgens (Calabrese, 2001a) and estrogens (Calabrese, 2001b). The reduced MAO activities in the caudate putamen of the group receiving 3 mg/kg of nandrolone decanoate, are interesting since this region plays an important role in the behavioural manifestations of drug addiction (Everitt and Robbins, 2005). Previous studies investigating the impact of 2 weeks of AAS administration on the dopamine system have demonstrated down-regulated dopamine D1 receptor density and mRNA expression and up-regulated dopamine D2 receptor density and mRNA expression (Kindlundh et al., 2001, 2003), as well as an increased density of the dopamine transporter in the striatum using in vitro autoradiography and positron emission tomography (Kindlundh et al., 2002, 2004). Utilizing the advantages of microdialysis, we found that the extracellular levels of DOPAC and HVA were reduced in the nucleus accumbens shell of male Sprague–Dawley rats after 14 days of nandrolone decanoate administration (15 mg/kg) and that also the biochemical response to amphetamine was attenuated (Birgner et al., 2007). A microdialysis study with a similar experimental design has shown attenuated dopamine release and also reduced behavioural response to amphetamine after nandrolone pre-treatment (Kurling et al., in press). Taken together, the current finding of reduced MAO-B activity in the CPU by AAS is in line with previous studies indicating on dopaminergic dysregulation in the striatum following sub-chronic administration of nandrolone decanoate. The

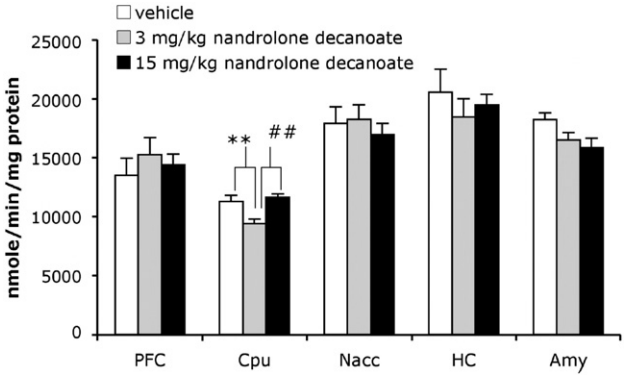

Fig. 2 – Enzymatic activity of monoamine oxidase B in male rat brain tissue, after 14 days of daily nandrolone decanoate administration at the doses 3 mg/kg and 15 mg/kg. Values are expressed as mean ± S.E.M. in nmol/min/mg protein. N = 9–10. **($p < 0.01$) vs vehicle ##($p < 0.01$) vs 3 mg/kg nandrolone decanoate.

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

		Treatment			
Tissue	GOI	Control	Nandrolone decanoate (3 mg/kg)	Nandrolone decanoate (15 mg/kg)	*p*-value
Prefrontal cortex	MAO-B	0.172 (0.136–0.271)	0.194 (0.161–0.215)	0.199 (0.145–0.276)	0.949
	COMT	0.396 (0.319–0.589)	0.344 (0.234–0.566)	0.481 (0.398–0.548)	0.120
Nucleus accumbens	MAO-B	0.381 (0.295–0.476)	0.296 (0.245–0.478)	0.341 (0.272–0.427)	0.506
	COMT	0.342 (0.188–0.366)	0.344 (0.290–0.389)	0.357 (0.242–0.416)	0.665
Caudate putamen	MAO-B	0.457 (0.445–0.472)	0.500 (0.421–0.590)	0.459 (0.376–0.626)	0.835
	COMT	0.642 (0.572–0.670)	0.548 (0.533–0.554)	0.413 (0.376–0.647)	0.295
Hippocampus	MAO-B	0.981 (0.826–1.24)	1.02 (0.98–1.34)	1.15 (0.855–1.23)	0.623
	COMT	0.625 (0.572–0.670)	0.548 (0.533–0.554)	0.413 (0.376–0.647)	0.315
Hypothalamus	MAO-B	0.176 (0.140–0.198)	0.172 (0.107–0.226)	0.149 (0.124–0.196)	0.860
	COMT	nd	nd	nd	
Amygdala	MAO-B	0.464 (0.301–0.540)	0.417 (0.351–0.621)	0.453 (0.329–0.560)	0.934
	COMT	0.530 (0.386–0.594)	0.523 (0.334–0.689)	0.533 (0.302–0.646)	0.941
Ventral tegmental area	MAO-B	0.230 (0.110–0.393)	0.214 (0.084–0.367)	0.240 (0.054–0.330)	0.879
	COMT	nd	Nd	nd	
Substantia nigra	MAO-B	0.208 (0.184–0.248)	0.284 (0.242–0.294)*	0.261 (0.201–0.380)	0.046
	COMT	0.216 (0.198–0.297)	0.273 (0.243–0.352)	0.250 (0.214–0.351)	0.290

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. Attempts were made to also detect the MAO-A transcript, but did not succeed due to low tissue specific expression. Data are expressed as median (interquartile range) and p-values from the Kruskal–Wallis test are presented on the right. N = 8–10 in all regions except the amygdala, hypothalamus and caudate putamen where N = 5–8.
nd: not detectable.
* $p < 0.05$ vs control.

increased gene transcript level of MAO-B in the substantia nigra, indicated in this study, might be a compensatory response to the reduced MAO-B activity observed in the caudate putamen since the cellbodies originating in the substantia nigra extensively innervate the caudate putamen. The lack of linearity between mRNA level and protein level has earlier been shown in the rat brain in studies of MAO-B (Gundlah et al., 2002; Smith et al., 2004).

Two weeks of daily nandrolone decanoate administration reduced the MAO-A activity in a dose-responsive manner in the amygdala, and the MAO-B activity showed a tendency towards reduction ($p = 0.051$). The MAO-A promoter contains androgen response elements with an enhancing effect on enzyme expression (Ou et al., 2006). However, since glucocorticoids, with a stronger effect than androgens, compete with androgens for this site, the net result of increased androgen levels might be a reduced expression of the enzyme (Sjoberg et al., 2008). Ex vivo experiments, using MAO-A inhibitors, showed that rat striatal HVA decreased almost linearly even at an MAO-A inhibition of less than 20%. Interestingly, however, also in this study the levels of the parent amine were not affected, possibly due to compensatory mechanisms in the living organism (Waldmeier, 1987). The efficiency of such compensatory mechanisms might differ between species and between different brain regions and, hence, it cannot be excluded that the reduced MAO-A activity in the amygdala might be of particular interest in relation to the mood changes often reported in AAS abusers.

The amygdala is a region implicated in emotional processing and is considered a part of mammalian aggression circuitries together with the hypothalamus and the periaqeductal grey (Gregg and Siegel, 2001). Adolescent hamsters treated with AAS displayed increased offensive aggression, which could be reduced by either a 5HT1A agonist or a serotonin reuptake inhibitor. These hamsters also had a reduced number of serotonin fibres in the amygdala and the hypothalamus, which was detectable already after 7 days of AAS exposure (Grimes and Melloni, 2002; Grimes et al., 2007). This is of particular interest since partial destruction of the serotonergic system repeatedly has been shown to increase aggressiveness in rodents (Vergnes et al., 1988). Thus, it can be speculated that a sustained increase in the levels of serotonin might have caused such a reduction in density of serotonergic fibres, as is also the case after the increased serotonin levels during foetal life observed in MAO-A knock-out mice (Cases et al., 1995). In contrast to hamsters, it has, however, been difficult to establish a uniform pattern of AAS' effects on rat brain serotonin, possibly due to differences in study designs, further discussed below (Kurling et al., 2005; Lindqvist et al., 2002; Thiblin et al., 1999). However, it has been shown that male rats exhibit even more aggressive behaviour when exposed to testosterone after serotonin depletion, than normal rats receiving the same testosterone treatment (Keleta et al., 2007).

The differences between earlier studies of nandrolone decanoate, regarding experimental design, constitute type of steroid, dose (1–40 mg/kg), dose interval (1–7 days), duration of administration (1 day–6 weeks), route of administration (intramuscular or subcutaneous) and time from last injection to euthanasia (24–72 h). We chose the doses 3 and 15 mg/kg nandrolone decanoate administered intramusculary once daily for 14 days, corresponding to one cycle of use during early and experienced AAS abuse respectively, based on a one-year follow-up study of AAS abusers (Fudala et al., 2003). Nandrolone decanoate has a half-life of 5.4 days in rat and 6 days in human when administered as an intramuscular depot (van der Vies, 1985). A single injection of 20 mg/kg of nandrolone decanoate gave rise to a plasma level of 2.6 μg/l after 4 days and nandrolone was still detectable in plasma 16 days after administration

(1.3 μg/l) (Kurling et al., in press). 24 h after the last injection of nandrolone decanoate administered in this study, the depot is calculated to have contained 6 mg of nandrolone decanoate in the 3 mg/kg group and 30 mg in the 15 mg/kg group and thus still released nandrolone into the circulation (van der Vies, 1985).

In summary, MAO is involved in an array of mood disorders. Reduced activity of platelet MAO-B has, for example, been reported in violent offenders, sensation seekers and type II male alcoholics (Fowler et al., 1980; Skondras et al., 2004; von Knorring et al., 1991). Mood disorders, similar to those associated with reduced MAO activity, are frequently reported in connection to AAS abuse, e.g. hypomania, aggression and violent behaviour. During withdrawal, depression is a common symptom, which often leads to resumed AAS intake (Pope and Katz, 1994). The reduced activities of MAO-A and MAO-B in the caudate putamen shown in this study, are in line with our earlier findings of decreased levels of DOPAC and HVA in the nucleus accumbens of nandrolone decanoate treated rats. The reduced activity of MAO-A in the amygdala might contribute to an explanation of impulsive and aggressive behaviours caused by AAS.

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). 24 h after the last injection, 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, caudate putamen, nucleus accumbens, hippocampus, hypothalamus, amygdala, ventral tegmental area and the substantia nigra were collected. The regions of interest from one hemisphere were put on dry ice, and transferred to Eppendorf tubes when frozen, and later used for the radiometric enzyme assay. The regions from the other hemisphere were immersed in RNAlater (Ambion) allowing the solution to infiltrate the tissue for 1 h in room temperature before taken for analysis with qPCR. The ventral tegmental area and substantia nigra were too small to be divided and were used for qPCR only. 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. *Radiometric assay*

The enzyme activity was determined with a radiometric assay, described in detail elsewhere (Hallman et al., 1987). In short, the brain tissues were homogenized by sonication in 0.01 M sodium phosphate buffer (pH 7.4) and diluted to 2.5% of the wet weight. The homogenates were first pre-incubated for 20 min with an enzyme inhibitor in glass tubes. 100 nM deprenyl, also known as selegiline (Sigma-Aldrich Chemicals Sweden AB), was added to inhibit MAO-B, and 100 nM clorgyline (Sigma-Aldrich Chemicals Sweden AB) to inhibit MAO-A. After the pre-incubation, the reaction was terminated by addition of 3 M HCl in the samples for blank activity, while substrates were added to the samples for total activity. 0.1 nM ^{14}C-serotonin (1.0 μCi/ml, PerkinElmer Sweden AB) was used as a substrate for MAO-A, and 0.05 nM ^{14}C-2-phenylethylamine (0.5 μCi/ml, PerkinElmer Sweden AB) as a substrate for MAO-B. The samples for MAO-A activity were incubated for 20 min and the samples for MAO-B activity for 5 min, in a water bath (37 °C). The reaction was terminated by acidification. The radioactive compound was extracted with a water saturated mixture of toluene:ethylacetate (1:1 vol/vol), and the organic phase was transferred to scintillation vials and mixed with scintillation fluid (Ready Safe, Beckman Coulter Inc., Fullerton, CA, USA). Standard samples were prepared and all samples were then measured for 5 min in a liquid scintillation analyzer (Packard 1900 CA Tri-CARB, PerkinElmer Life and Analytical Sciences Inc., Wellesley, MA, USA). The remaining homogenates were used for protein determination (Lowry et al., 1951). Enzyme activities were then calculated as nmol/min/mg protein. All samples were made in triplicates.

4.3. *qPCR*

The primers used for the genes of interest (GOI) were designed with Beacon Designer (v4.0). Forward and reverse primers, together with accession numbers, for both HKGs and GOIs are presented in Table 2. 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. Primer efficiencies ranged between 80 and 100%.

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 1:50 000, 10 nM fluoroscein, 0.8 pmol/μl

Table 2 – Primer sequences for gene transcripts used in the quantitative real-time reversed transcription PCR

	Gene	Accession	Forward primer	Reverse primer
GOI	MAO-A	XM_343764	5-ccacagccagagcgttcag-3	5-tgagagcctttgcccagattg-3
	MAO-B	NM_013198	5-cagtggaagcagaggagag-3	5-tgctgccatacctgagatg-3
	COMT	NM_012531	5-tgggctggggcttggtga-3	5-gatgcgctgctccttttgtgtc-3
HKG	ACT	NM_031144	5-cactgccgcatcctcttcct-3	5-aaccgctcattgccgatagtg-3
	CYCLO	M19533	5-gagcgttttgggtccaggaat-3	5-aatgcccgcaagtcaaagaaa-3
	GAPDH	X02231	5-acatgccgcctggagaaacct-3	5-gcccaggatgcccttttagtgg-3
	H3	NM_053985	5-attcgcaagctccccttttcag-3	5-tggaagcgcaggtctgttttg-3
	RPL19	NM_031103	5-tcgccaatgccaactctcgtc-3	5-agcccgggaatggacagtcac-3
	SDCA	NM_130428	5-gggagtgccgtggtgtcattg-3	5-ttcgcccatagcccccagtag-3
	TUB	NM_173102	5-cggaaggaggcggagagc-3	5-agggtgcccatgccagagc-3

Abbreviations: ACT, β-actin; CYCLO, cyclophilin; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; GOI, gene of interest; H3, histone H3b; HKG, house-keeping gene; RPL19, ribosomal protein L19; SDCA, succinate dehydrogenase complex A subunit A; TUB, β-tubulin beta 5.

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 except MAO-A for which both 62 °C and 55 °C were tried. The annealing temperature used for the HKGs was 60 °C. 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 $2^{-\text{Delta DeltaCt}}$ 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), discussed in (Lindblom et al., 2006). The HKGs included in the normalization factors were, for the prefrontal cortex: ACT, H3b, TUB; nucleus accumbens: CYCLO, GAPDH, SDCA; caudate putamen: H3b, RPL19, TUB; hippocampus: ACT, H3b, RPL19; hypothalamus: ACT, CYCLO, TUB; amygdala: ACT, GAPDH, RPL19; ventral tegmental area: CYCLO, H3b, RPL19; and substantia nigra: GAPDH, H3b, RPL19 (for abbreviations, see Table 2).

4.4. *Statistics*

Statistical analysis was performed using Prism v4.0 (GraphPad Software Inc.). The results of the enzyme activity test were statistically evaluated with one-way ANOVA followed by Newman–Keuls *post-hoc* test. The differences in normalized gene transcript levels were statistically evaluated with Kruskal–Wallis test and Dunn's multiple comparison test when appropriate. Non-parametric analysis was used to evaluate the qPCR data since several of the data sets did not meet the criteria of Gaussian distribution and equal variance. Results were considered significant when $p<0.05$. Grubb's test was used to identify out-liers.

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