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Effect of Dopamine Uptake Inhibition on Brain Catecholamine Levels and Locomotion in Catechol-*O*-methyltransferase-Disrupted Mice

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ABSTRACT

Two different uptake processes terminate the synaptic action of released catecholamines in brain: the high-affinity uptake to presynaptic nerve terminals (uptake$_1$, followed by oxidation by monoamine oxidase, MAO) or glial cells uptake (uptake$_2$, followed by *O*-methylation by catechol-*O*-methyltransferase, COMT, and/or oxidation by MAO). For dopaminergic neurons, uptake by the high-affinity dopamine transporter (DAT) is the most effective mechanism, and the contribution of glial COMT remains secondary under normal conditions. In the present study we have characterized the role of COMT using COMT-deficient mice in conditions where DAT is inhibited by 1-[2-[bis(4-fluorophenyl)methoxy-]ethyl]-4-(3-phenylpropyl)-piperazine (GBR 12909) or cocaine. In mice lacking COMT, GBR 12909 results in total brain tissue dopamine levels generally higher than in wild-type mice but no such potentiation was ever seen in striatal extracellular fluid. Dopamine accumulation in nerve endings is more evident in striatum and hypothalamus than in cortex. Both GBR 12909 and cocaine induced hyperlocomotion in mice lacking COMT. Unexpectedly, hyperactivity induced by 20 mg/kg GBR 12909 was attenuated only in male COMT knockout mice, i.e., they had an inability to sustain the hyperactivity induced by DAT inhibition. Furthermore, attenuation of hyperlocomotion was observed also after cocaine treatment in both C57BL/6 (at 5 and 15 mg/kg) and 129/Sv (at 30 mg/kg) genetic background COMT-deficient male mice. Despite the possible interaction between DAT and extraneuronal uptake (and subsequently COMT), the role of COMT in dopamine elimination is still minimal in conditions when DAT is inhibited.

The dopamine transporter (DAT) and monoamine oxidase (MAO) play key roles in dopamine elimination and are expected to have pleiotropic effects on susceptibility to a wide range of behavioral/psychiatric disorders and symptoms associated with dysregulation of dopamine transmission. Due the extraneuronal location in the brain (astrocytes, capillary walls, and postsynaptic dendritic spines; Karhunen et al., 1995), the significance of catechol-*O*-methyltransferase (COMT) on dopamine metabolism remains secondary under normal conditions.

Inhibition of COMT activity in rodents either via pharmacological intervention or via disruption of the *Comt* gene (Gogos et al., 1998) reveals only minor changes in their locomotor behavior and brain dopamine and noradrenaline levels in normal conditions (Gogos et al., 1998; Huotari et al., 2002). However, the role of COMT might be more important in areas such as prefrontal cortex, where both decreased responsiveness to dopamine uptake inhibitors and lower terminal density and decreased number of DATs per terminal, have been described previously (Sesack et al., 1998). Also, because L-DOPA is a substrate of COMT, COMT inhibition has a profound L-DOPA-potentiating effect in both animals and patients with Parkinson's disease (Männistö and Kaakkola, 1989, 1999). The same L-DOPA potentiation has been found in COMT-deficient mice (Huotari et al., 2002).

In addition to natural activity variations of DAT and COMT, manipulation of the activity of these proteins has

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ABBREVIATIONS: DAT, dopamine transporter; MAO, monoamine oxidase; COMT, catechol-*O*-methyltransferase; GBR 12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)-piperazine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; HPLC, high-performance liquid chromatography; DHPG, dihydroxyphenylglycol; MHPG, methoxyhydroxyphenylglycol; AUC, area under the curve; ANOVA, analysis of variance.

been accomplished by generation of specific inhibitors. A series of new, very potent, highly selective, and orally active COMT inhibitors have been developed to improve the bioavailability and brain penetration of L-DOPA (Männistö and Kaakkola, 1989, 1999) with applications in the treatment of Parkinson's disease. On the other hand, DAT inhibitors include psychostimulants and addictive drugs (Moghaddam and Bunney, 1989; Kuhar et al., 1991; Giros et al., 1996).

DAT inhibition in the brain enhances extracellular dopamine concentration and produces a typical dopaminergic hyperactivity and stereotypy (Heikkila and Manzino, 1984; Irifune et al., 1995; Nakachi et al., 1995). Increased extracellular dopamine concentration and hyperactivity have been observed in mutant mice in which expression of DAT is genetically reduced to 10% of wild-type mice (Zhuang et al., 2001), as well as in DAT null knockout mice (Giros et al., 1996; Jones et al., 1998; Gainetdinov et al., 1999). Similar effects on behavior and extracellular dopamine levels have been observed after pharmacological inhibition of DAT through 1-[2-[bis(4-fluorophenyl)methoxy-]ethyl]-4-(3-phenylpropyl)piperazine (GBR 12909), a highly potent and selective inhibitor of DAT, and its chemical analogs (Heikkila and Manzino, 1984; Andersen, 1989; Nakachi et al., 1995). GBR 12909 inhibits DAT in a low nanomolar range, whereas its affinity for noradrenaline and serotonin carriers is about 100-fold lower (Andersen, 1989), and it has virtually no effect on dopamine release (Heikkila and Manzino, 1984; Westerink et al., 1987). In addition, GBR 12909 has been shown to inhibit dopamine uptake into brain synaptic vesicles in 1 order of magnitude higher concentration than neuronal dopamine uptake (Reith et al., 1994). Finally, cocaine inhibits dopamine uptake, leading to activation of dopamine receptors in the limbic forebrain and striatum (Koob, 1992; Segal and Kuczenski, 1994; Self and Nestler, 1995; Nestler and Aghajanian, 1997).

In previous studies with rats, the selective COMT inhibitor tolcapone was able, to some extent, to potentiate GBR 12909-induced elevation of striatal extracellular dopamine concentration (Budygin et al., 1999; Huotari et al., 1999). In the present study, we have investigated the effects of the dopamine uptake inhibition on locomotor activity, the levels of catecholamines and their metabolites in three different brain regions, and dopamine concentration in striatal extracellular fluid of COMT-deficient mice of both sexes. Even though the inhibition of DAT increases dramatically the extracellular dopamine concentrations, we hypothesized that the role of COMT on dopamine levels would remain small even in these extraordinary circumstances. Effects of COMT deficiency on DAT protein and binding levels were also investigated.

Materials and Methods

Animals. COMT-deficient mice were generated as described in Gogos et al. (1998). Through a series of five backcrosses, the COMT mutation was introduced into pure C57BL/6 genetic background. Mice were bred in the National Laboratory Animal Center (Kuopio, Finland) or at the Rockefeller University's Laboratory Animal Research Center (New York, NY) and kept under standard conditions (temperature 22 ± 1°C), in small groups (3–12 mice/group), on a 12-h dark/light cycle (lights on at 7:00 AM). They had free and continuous access to fresh tap water and food pellets. Tests were performed on 3- to 6-month-old animals. The estrus phase was not determined in female mice. All procedures were reviewed by the Animal Ethics Committee of the University of Kuopio and approved by the local Provincial Government, as well as by the Rockefeller University Institutional Review Board.

Microdialysis of Anesthetized Mice. A total of 52 mice of both sexes and all three genotypes were used in the striatal microdialysis experiment, which was carried out as described previously (Huotari et al., 2002). Males weighed 22 to 35 g and females 18 to 28 g.

Mice were anesthetized with chloral hydrate (350 mg/kg; Merck, Darmstadt, Germany), placed in a Kopf stereotaxic apparatus with an appropriate mouse adapter and a dialysis probe having 220-μm outer diameters, and a 2-mm exposed membrane (AgnTho's, Lidingö, Sweden) was implanted through a burr hole in striatum. After 60-min baseline collection, the mice were given 20 mg/kg GBR 12909 i.p. (Tocris-Cookson, Bristol, UK). Thereafter, 20-min fractions were collected for 300 min and stored at −80°C. Perfusate samples were analyzed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using HPLC-electrochemical detection (ESA, Chelmsford, MA). The HPLC system consisted of a 582 pump and a 542 autosampler with cooler. The CoulArray Detector (an eight-channel analytical cell) was equipped with 5014B microdialysis cell. The potentials applied were −175 mV (channel 1), +0.200 (channel 2), and +0.400 V (channel 4), respectively. The analytes were separated on a reverse phase column (Inertsil ODS-3, 5 μm, 4.0 × 150 mm; GL Sciences Inc., Tokyo, Japan) with two different isocratic runs: The base of mobile phase was 75 mM sodium phosphate buffer containing 15% acetonitrile (Rathburn Chemicals Ltd., Walkenburn, Scotland). For dopamine analysis it was augmented with 1.5 mM SDS (Sigma-Aldrich, St. Louis, MO) and 12% methanol (Lab-Scan Ltd., Dublin, Ireland) (pH 5.6, flow rate 0.9 ml/min), and for DOPAC and HVA analysis with 1.0 mM SDS and 10.5% methanol (Lab-Scan Ltd.) (pH 2.5, flow rate 1.0 ml/min). Detection limits were about 8 to 9 fmol for dopamine (leading about 10–12 fmol/20-min sample) and about 10 fmol for DOPAC and HVA.

Analysis of Catecholamines and Their Metabolites in Mouse Brain Samples. The brain samples (striatum, frontal cortex, and hypothalamus) were taken and prepared as described previously (Huotari et al., 2002). A total of 60 mice of both sexes and all three genotypes were injected i.p. with 20 mg/kg GBR 12909 and decapitated 90 min later.

Biogenic amines and their metabolites were determined using HPLC-electrochemical detection as described above. Noradrenaline and its metabolites dihydroxyphenylglycol (DHPG) and methoxyhydroxyphenylglycol (MHPG) and dopamine and its metabolites DOPAC and HVA were separated with a reverse phase C18 column (Ultrasphere ODS, 4.6 × 250 mm, 5-μm particle size; Beckman-Coulter, Inc., Fullerton, CA) using a gradient run. The potentials applied were −0.175 V (channel 1), +0.250 (channel 2), and +0.400 V (channel 3), respectively. The detection limits were 10 fmol for dopamine, DOPAC, noradrenaline, DHPG, and MHPG and 25 fmol for HVA. The mobile phase, pH 3.6, consists of 0.1 M sodium acetate buffer, 0.1 M citric acid (Riedel-de Haën, Seelze, Germany), 0.18 mM sodium octyl sulfate (Sigma-Aldrich, Steinheim, Germany), and methanol (7.75–15%; Lab-Scan Ltd.).

Locomotor Activity. To see the effect of COMT activity on GBR 12909-induced hyperlocomotion, 30 mice of all three genotypes and both sexes were tested. The animals were housed individually in test cages (42.5 × 25 × 15 cm) and allowed to acclimate to their surroundings overnight. Food and water were available ad libitum. The next morning, mice were injected i.p. with NaCl or two doses of GBR 12909 (10 or 20 mg/kg) in a volume of 10 ml/kg. Activity was measured using a 10-channel IRS Actometer System, at 10-min consecutive periods for a total of 300 min postinjection. The total locomotor activity was recorded by measuring the movements of the heat radiation source (mouse). For this assay, data are represented as motility (0–100) during each motility integration time (10 min). The area underneath the mobility curve (AUC) was used to estimate the effect of GBR 12909 administration (AUC_{0-300} min) on locomotor activity.

To see the effect of COMT activity on cocaine-induced locomotor activity, 8 to 12 male homozygous mutant and wild-type mice of 8

weeks of age were tested. For the cocaine experiment, in addition to the C57BL/6 genetic background COMT mice used so far, we also included mice where the COMT mutation had been introduced on 129/Sv genetic background (through five generations of backcrossing). Animals were housed individually for 1 week before the experiment. Mice were tested during their inactive (light) phase. Each animal was weighed before the experiment and received saline i.p. on the first day and 5 mg/kg i.p. cocaine the next day. Two and 4 weeks later, the experiment was repeated (i.p. administration of saline on the first day, followed by 15 and 30 mg/kg cocaine the next, respectively). Injected animals were placed in the center of the open field arena, a clear acrylic chamber (40.5 × 40.5 × 30 cm) equipped with infrared sensors for the automatic recording of horizontal activity (Digiscan model RXYZCM; AccuScan Instruments, Inc., Columbus, OH), which was illuminated with a white light and locomotor activity was measured for 30 min. Data were collected continuously using the DIGIPRO program (AccuScan Instruments, Inc.).

Dopamine Transporter Autoradiography and Western Blot. We followed a modified protocol for dopamine transporter ligand binding (Lucas et al., 2000). Slides were removed from −70°C freezer storage, and brain sections were allowed to thaw for 10 min at room temperature. To determine specific DAT binding, sections were incubated in buffer A (137 mM NaCl, 2.8 mM KCl, 10 mM Na_2HPO_4, and 10 mM KH_2PO_4, pH 7.4) with 10 mM NaI plus 1.5 nM [^{125}I]RTI-121 (NEX318; PerkinElmer Life Science, Boston, MA), for 60 min at room temperature. Nonspecific binding was determined by incubating sections that matched those used for specific binding with buffer A plus 1.5 nM [^{125}I]RTI-121 and 50 μM cocaine HCl (Sigma-Aldrich) for 60 min at room temperature. After incubation for 1 h, sections were washed twice for 20 min each time with buffer A at 4°C. Salts were removed from sections with a quick rinse in ice-cold distilled water and slides were dried under forced air. Sections were exposed on Kodak XAR film for 2 h. X-ray film results were examined with a desktop illuminator (Northern Light, St. Catherines, Canada) and a charge-coupled device video camera (DAGE/MTI model 72) with a Micro Nikkor lens (Nikon, Tokyo, Japan) attached. Microscale ^{125}I microscale standards (Amersham Biosciences, Piscataway, NJ) for RTI-121 were exposed on Kodak XAR film for 2 h. After this, autoradiographs were digitized using computer-assisted densitometry (Imaging Research, St. Catherines, Canada). Background illumination was digitally subtracted and gray level/optical density calibration was done using the exposed microscale standards ladder and plotted as a function of microscale calibration values. It was determined that all subsequent optical density values of digitized autoradiographic images fell within the linear range of the function. Five regions per half-section were selected for optical density quantitation in the region of the striatum: dorsolateral and dorsomedial caudate-putamen, core and shell of the nucleus accumbens, plus a background reading taken from the corpus callosum. Four sections per mouse were analyzed in each hemisphere, and a mean optical density value was registered for each region in the striatum. Background optical density values were subtracted from striatal regions of interest. Data are represented as mean number of binding sites (femtomoles per milligram) ± S.E.M.

Western blotting was done with rabbit anti-rabbit DAT antibody and goat anti-rabbit IgG-horseradish peroxidase as a secondary antibody. SuperSignal West Femto chemiluminescent substrate was used according to manufacturer's recommendations (Pierce Chemical, Rockford, IL).

Statistics. For the microdialysis studies, the area under the concentration curve ($AUC_{0-300\ min}$), and for the locomotor studies, the area under the mobility curve ($AUC_{0-300\ min}$), were calculated to estimate the effect of GBR 12909 administration. One-way analysis of variance (ANOVA) followed by Bonferroni's correction of the Newman-Keuls test was used to analyze the effect of GBR 12909 on three genotypes. The statistical significance of the effect of genotype and sex as well as the effects of GBR 12909 and cocaine on the locomotor activity and the brain catecholamines/metabolites levels were tested using two-way ANOVA. Changes in the concentrations of amines and their metabolites as well as in locomotor activity were compared between sexes using independent sample *t* test (with Bonferroni's correction) as a post hoc test. The results are shown as mean ± S.E.M.

For the DAT measurement, optical density measures were analyzed by a two-way repeated measures ANOVA with genotype as between-subject and striatal regions as within-subject factors. Post hoc follow-up tests of main effects (Student-Newman-Keuls) and interaction effects were done as required.

Results

Effect of COMT Deficiency on GBR 12909-Induced Changes of Striatal Extracellular Dopamine, DOPAC, and HVA Levels. In vivo microdialysis was used to monitor extracellular levels of dopamine, DOPAC, and HVA in the striatum of anesthetized wild-type, heterozygous, and homozygous COMT knockout mice. The results of the microdialysis experiment are shown in Fig. 1. In this experiment striatal dopamine baseline levels were no more than 10 to 12 fmol/20-min sample, i.e., very close to the detection limit of our HPLC system.

After GBR 12909 injection, the highest dopamine levels were reached 60 to 80 min postinjection, and remained high throughout the 300-min collection period. However, GBR 12909-induced similar dopamine total overflow ($AUC_{0-300\ min}$; data not shown), and no genotype and/or sex effects were observed. In male mice, peak dopamine levels (at 80 min postinjection) were 181.1 ± 60.1 fmol/20-min sample (mean ± S.E.M.) in wild-type mice, 190.3 ± 60.3 fmol/20-min sample in heterozygous mice, and 121.2 ± 60.4 fmol/20-min sample in homozygous mutant mice (Fig. 1A). In female mice, the highest dopamine levels were 92.8 ± 47.2 fmol/20-min sample in wild-type mice (at 60 min postinjection), 133.4 ± 44.9 fmol/20-min sample in heterozygous mice (at 80 min), and 165.4 ± 50.8 fmol/20-min sample in homozygous mutant animals (at 80 min; Fig. 1D).

As expected, DOPAC levels were increased (Fig. 1, B and E) and HVA was undetectable (Fig. 1, C and F) in the striatal perfusates of either sex lacking the *Comt* gene. After administration of GBR 12909, extracellular DOPAC levels were decreased by 34 to 62% and HVA levels by 23 to 49% in all three genotypes and in both sexes.

Levels of Catecholamine and Their Metabolites in Brain Homogenates after GBR 12909 Treatment. Detailed comparisons of the amines and metabolites between the genotypes and sex are shown in Table 1.

In contrast to striatal extracellular levels, total tissue dopamine content after GBR 12909 injections was relatively, and in some cases significantly, higher in mice lacking COMT compared with wild-type controls. This dopamine accumulation into nerve endings was perceived more clearly in striatal and hypothalamic than in cortical tissues.

As expected, GBR 12909 induced only minor changes in dopamine metabolism and there were no indications of genotype and/or sex interactions. After GBR 12909 administration, we observed moderate decrease in striatal extracellular DOPAC levels. In addition, HVA levels were practically unchanged.

Finally, after GBR 12909 injection, noradrenaline content in striatum was higher in homozygous mutant mice com-

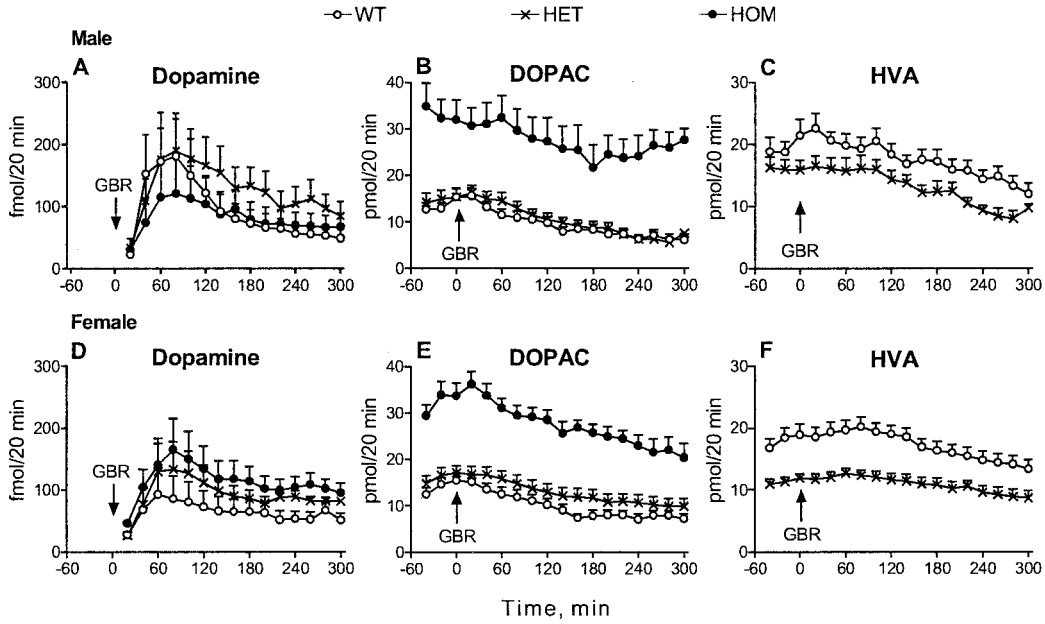

Fig. 1. Striatal extracellular dopamine (A and D), DOPAC (B and E), and HVA (C and F) levels of all three genotypes (WT, wild-type; HET, heterozygote; HOM, homozygote) of both sexes of COMT-deficient mice. Three 20-min baseline samples were collected before GBR 12909 (20 mg/kg i.p.) injection and thereafter 20-min fractions were collected for 300 min. Data are means ± S.E.M. of 8 to 12 animals.

TABLE 1

Brain homogenate levels (nanograms per gram wet tissue) of DA, DOPAC, HVA, noradrenaline (NA), DHPG, and MHPG of both sexes of GBR 12909-treated (20 mg/kg, i.p.) *Comt* gene-disrupted mice

DHPG levels of WT and HET, and cortical HVA levels were under the detection limit.

	Male			Female		
	WT	HET	HOM	WT	HET	HOM
Striatum						
DA	13,160 ± 606	16,051 ± 743^{a}	17,184 ± 736^{aa}	13,745 ± 786	15,886 ± 742	16,795 ± 800
DOPAC	501 ± 30	859 ± 46^{aa}	2,743 ± 119^{aaa,ccc}	460 ± 28	677 ± 48^{a,b}	2,429 ± 72^{aaa,ccc}
HVA	1,840 ± 107	1,527 ± 84	N.D.	1,381 ± 52^{bb}	1,247 ± 75^{b}	N.D.
NA	59.4 ± 5.6	59.6 ± 2.7	77.7 ± 4.7^{c}	58.4 ± 4.0	59.0 ± 4.5	98.8 ± 7.6
DHPG	N.D.	N.D.	158 ± 5	N.D.	N.D.	143 ± 6
MHPG	61.2 ± 1.7	66.8 ± 2.5	N.D.	59.6 ± 2.1	59.8 ± 1.6	N.D.
Cortex						
DA	36.8 ± 4.6	51.0 ± 3.7^{a}	52.7 ± 3.4	39.0 ± 2.3	48.4 ± 2.6	48.4 ± 3.6
DOPAC	14.4 ± 4.0	24.3 ± 1.7	130.8 ± 9.4^{aaa,ccc}	11.4 ± 1.0	22.1 ± 2.9	140.7 ± 17.5^{aaa,ccc}
NA	374 ± 21	442 ± 15	408 ± 15	385 ± 14	418 ± 13	332 ± 20^{cc,b}
DHPG	N.D.	N.D.	223 ± 7	N.D.	N.D.	197 ± 11
MHPG	48.5 ± 18.4	47.4 ± 5.8	N.D.	26.3 ± 3.6	40.1 ± 10.8	N.D.
Hypothalamus						
DA	330 ± 21	445 ± 37	795 ± 181^{a}	448 ± 42	515 ± 56	637 ± 66
DOPAC	108 ± 8	206 ± 8	792 ± 68^{aaa,ccc}	108 ± 5	197 ± 22	796 ± 51^{aaa,ccc}
HVA	315 ± 13	252 ± 10^{aa}	N.D.	259 ± 12^{bb}	252 ± 25^{aa}	N.D.
NA	1,419 ± 74	1,455 ± 83	1,575 ± 128	1,326 ± 93	1,426 ± 100	1,517 ± 114
DHPG	N.D.	N.D.	482 ± 33	N.D.	N.D.	452 ± 21
MHPG	128 ± 12	125 ± 8	N.D.	100 ± 9	106 ± 15	N.D.

WT, wild-type; HET, heterozygous; HOM, homozygous; N.D., not detectable.

Statistics: $^{a}p < 0.05$, $^{aa}p < 0.01$, and $^{aaa}p < 0.001$ indicate the difference between the corresponding WT groups. $^{b}p < 0.05$ and $^{bb}p < 0.01$ indicate the difference between the corresponding male. HOM marked with $^{c}p < 0.05$, $^{cc}p < 0.01$ and $^{ccc}p < 0.001$ were compared with corresponding HET group. Data are means ± S.E.M. of 8 to 11 animals.

pared with heterozygous and wild-type animals. No such difference was evident in hypothalamus and cortex. DHPG was detectable only in homozygous mice. MHPG was not detectable in homozygous but was detectable at similar levels in wild-type and heterozygous mice.

Effects of COMT Deficiency on GBR 12909-Induced Hyperlocomotion. A single injection of GBR 12909 significantly stimulated locomotor activity, compared with saline-treated controls (total activity; Fig. 2). Locomotor activity peaked at 40 to 70 min postinjection, followed by a gradual decline, with male mice exhibiting the highest score (time curves, Fig. 3). In general, 20 mg/kg GBR 12909 stimulated locomotor activity significantly more than 10 mg/kg. Ho-

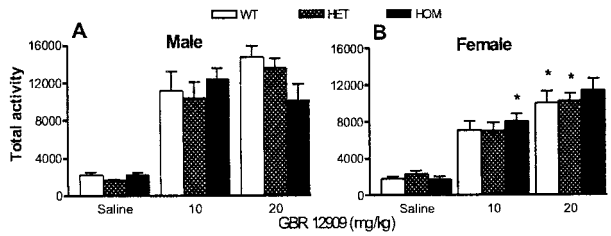

Fig. 2. $AUC_{0-300\ min}$ of all three genotypes (WT, wild-type; HET, heterozygote; HOM, homozygote) and both sexes of COMT-deficient mice. The mice were injected i.p. with NaCl or two doses of GBR 12909 (10 or 20 mg/kg, dissolved in NaCl), and total motor activity was monitored for 300 min. Statistics: *, $p < 0.05$ differs significantly from corresponding male. Data are means ± S.E.M. of 9 to 10 animals.

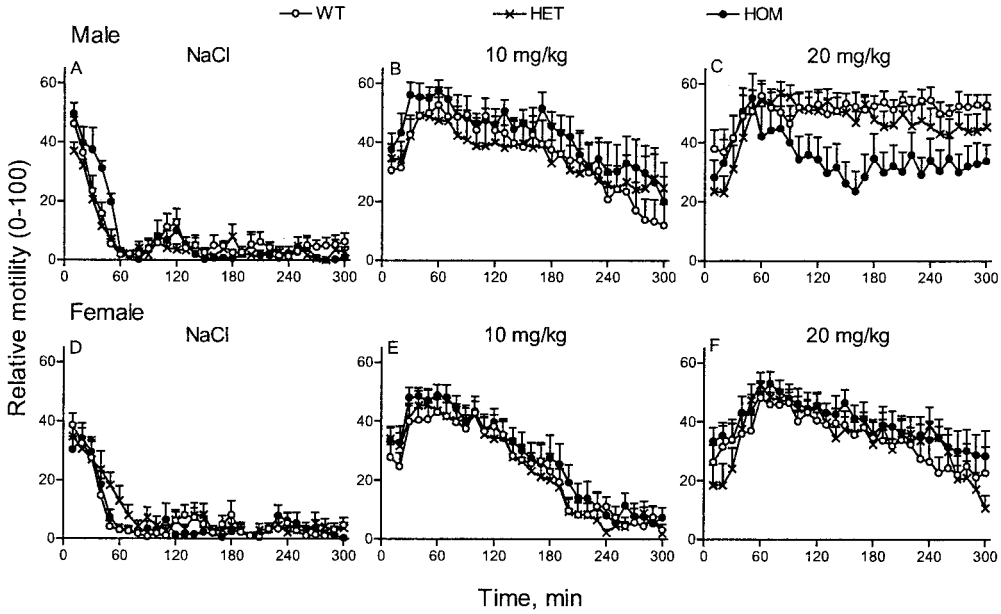

Fig. 3. Time course effects of NaCl (A and D), 10 (B and E), and 20 mg/kg (C and F) GBR 12909 on locomotor activity of all three genotypes (WT, wild-type; HET, heterozygote; HOM, homozygote) and both sexes of COMT-deficient mice. Lack of COMT (NaCl-injected controls) did not affect locomotor activity. The mice were injected i.p. with NaCl or GBR 12909 and total motor activity was monitored for 300 min at 10-min intervals. Data are means ± S.E.M. of 9 to 10 animals.

mozygous male mice were the only exception in that no such a clear dose-response effect was observed. In these mice, locomotor response to 20 mg/kg GBR 12909 was attenuated at levels below the ones induced by 10 mg/kg (Figs. 2 and 3). After 20 mg/kg GBR 12909 the total locomotor activity of wild-type and heterozygous male mice remained elevated throughout the whole 300-min observation period. Strikingly, in homozygous male mice, locomotor activity was initially indistinguishable from that of wild-type controls, but started to decline 60 min postinjection (Fig. 3C). This is also seen in the total locomotor activity (Fig. 2A).

Effects of COMT Deficiency on Cocaine-Induced Hyperlocomotion. To address the potential contribution of the genetic background to the motor phenotype, we extended our analysis at this stage to include two mouse strains (129/Sv and C57BL/6 genetic backgrounds). Figure 4 shows the dose-response effects of cocaine on ambulation in these two strains of wild-type and COMT knockout mice.

As expected, cocaine stimulated locomotor activity in both wild-type and homozygous mice in all doses (5, 15, and 30 mg/kg). In general, cocaine produced greater locomotor activation in C57BL/6 background mice than in 129/Sv background mice. A maximal locomotor response was induced by cocaine at a dose of 30 mg/kg for the 129/Sv wild-type mice and at 15 mg/kg for the C57BL/6 wild-type mice. Consistent with the GBR 12909 effects, cocaine produced significantly less locomotor responses in male COMT knockout mice at doses of 5 and 15 mg/kg for the C57BL/6 genetic background ($p < 0.05$; Fig. 4A) and at 30 mg/kg for the 129/Sv genetic background ($p < 0.05$; Fig. 4B).

Effect of COMT Deficiency on DAT Protein and Binding Levels. Amount of DAT protein was the same in wild-type and knockout mice (Fig. 5A). We then determined the effects of COMT deficiency on striatal DAT using quantitative autoradiography with [^{125}I]RTI-121 as a specific ligand. Ligand binding was indistinguishable between wild-type and COMT knockout mice (Fig. 5B). Although there were no significant differences in dopamine transporter bind-

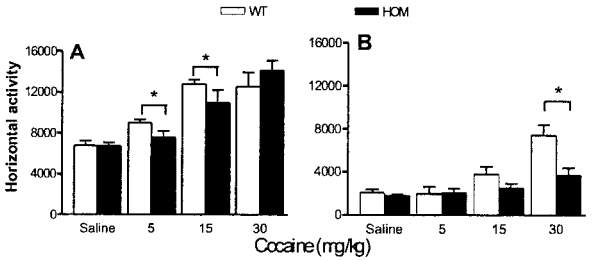

Fig. 4. Locomotor activity of male wild-type (WT) and COMT-deficient (HOM) mice after cocaine administration. The mice were injected i.p. with saline followed 1 day later by cocaine (at doses of 5, 15, and 30 mg/kg). The mice tested carry the COMT mutation on a C57BL/6 (A) or 129/SV (B) genetic background. Asterisk (*) indicates $p < 0.05$. Data are means ± S.E.M. of 8 to 12 male animals from each tested genotype.

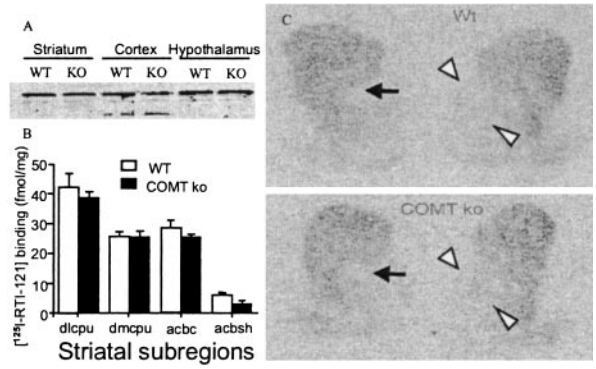

Fig. 5. A, dopamine transporter expression in different brain tissues from wild-type (WT) and COMT-knockout (KO) mouse determined by Western immunoblotting. Five micrograms of total tissue protein per lane was loaded. Anti-DAT binding was detected by standard techniques using horseradish peroxidase-anti rabbit IgG conjugate. Dopamine transporter concentration on striatal mouse tissue as determined by [^{125}I]RTI-121 binding. B, distribution of bound ligand in several striatal subregions, including the dorsolateral caudate putamen (dlcpu), dorsomedial caudate putamen (dmcpu), core of the nucleus accumbens (acbc), and shell of the nucleus accumbens (acbsh). No significant differences were observed in any of the subregions between wild-type (WT) and COMT knockout (COMT ko) mice. C, pair of representative sections of dopamine transporter binding in a Wt and COMT ko mouse. Arrows indicate location of anterior commissure for orientation. Arrowheads delineate the boundaries of the shell of the nucleus accumbens. A small increase in optical density can be observed in the WT shell of the nucleus accumbens.

ing between wild-type and knockout groups, there was a trend toward a decrease in the shell of the nucleus accumbens, where we found a nearly 50% decrease in binding in COMT knockouts compared with wild-type mice (Fig. 5C).

Discussion

Previous studies with mice deficient in COMT (Gogos et al., 1998; Huotari et al., 2002), MAO-A (Cases et al., 1995), MAO-B (Chen et al., 1999), and DAT (Giros et al., 1996), as well as a series of studies using MAO-A and -B (Butcher et al., 1990; Kaakkola and Wurtman, 1992) and COMT inhibitors (Kaakkola and Wurtman, 1992; Li et al., 1998) suggest that 1) for dopaminergic neurons, uptake by the DAT is the most effective mechanism for terminating the synaptic actions of dopamine; 2) dopamine oxidation is a preferable metabolic route to methylation; and 3) dopamine levels are generally refractory to changes in activity of both MAO and COMT. The contribution of glial COMT remains, therefore, secondary under normal conditions at least in the striatal regions. However, when DAT is inhibited, the role of extraneuronal dopamine uptake, although having lower affinity but significantly higher capacity than DAT, is expected to become more important. It also should keep in mind that noradrenaline transporter has even greater affinity to dopamine than DAT itself, and therefore it is an important player for clearing dopamine in brain regions with low levels of DAT, e.g., in the frontal cortex (Moron et al., 2002). In this study, we have investigated the effects of GBR 12909, a well characterized and selective dopamine reuptake inhibitor, on dopamine metabolism and motor activity in COMT-deficient mice. Effects of cocaine on locomotion in COMT knockout mice with two different genetic backgrounds (C57BL/6 and 129/Sv) were also investigated.

GBR 12909 has been shown to induce stereotypy and hyperlocomotion (Heikkila and Manzino, 1984; Westerink et al., 1987; Irifune et al., 1995; Nakachi et al., 1995). These behavioral changes have been linked to activation of dopamine receptors, because GBR 12909-induced hyperactivity can be inhibited by dopamine receptor antagonists such as haloperidol (Heikkila and Manzino, 1984; Westerink et al., 1987) and raclopride, and sulpiride (Rahman et al., 2001). Moreover, hypolocomotion induced by low doses of apomorphine (at concentrations that activate mainly dopamine D_2 autoreceptors) can be reversed by GBR 12909 (Irifune et al., 1995). In COMT-deficient mice, GBR 12909 induced clear, long-lasting elevation of dopamine concentration in striatal microdialysis fluid without any clear genotype-related changes.

Although COMT deficiency as such does not alter either brain extracellular or tissue dopamine concentrations, dopamine metabolism is dramatically changed. In homozygous mice lacking COMT activity, HVA levels were never detectable and DOPAC levels were severalfold higher than in the wild-type mice. On the other hand, heterozygous mice with about half of normal COMT activity in brain (Huotari et al., 2002) showed only slightly and irregularly decreased HVA and increased DOPAC levels. By preventing the intraneuronal restorage and/or degradation of dopamine, uptake inhibitors would be assumed to produce pronounced changes in the pattern of dopamine metabolism. However, pure dopamine uptake inhibitors such as GBR 12909, lacking the dopamine-releasing effect, have only slight effects on DOPAC and HVA levels (Westerink et al., 1987; Irifune et al., 1995). In our previous study we found elevated DOPAC levels in brain homogenates and striatal extracellular space in untreated homozygous mice (Huotari et al., 2002). However, in the present study there was a trend of decreased DOPAC levels in both sexes after GBR 12909 administration within each genotype despite the greatly increased basal DOPAC levels found in homozygous mice. The respective HVA levels changed even less. Taken together, GBR 12909 induced only minor changes in dopamine metabolism and there were no indications of potentiation by COMT deficiency on these effects. These results fit quite well with previous findings in rats, where GBR 12909 alone had no effect on DOPAC and HVA striatal extracellular and brain tissue concentrations (Westerink et al., 1987; Irifune et al., 1995; Budygin et al., 1999; Huotari et al., 1999). However, when tolcapone, a selective and potent COMT inhibitor (Borgulya et al., 1989), was given together with 20 mg/kg GBR 12909, striatal extracellular DOPAC levels were slightly increased (Huotari et al., 1999), whereas with 10 mg/kg GBR 12909 DOPAC levels remained unaltered (Budygin et al., 1999).

Despite normal expression of DAT, COMT knockout mice displayed attenuated GBR 12909- and cocaine-induced locomotion, but only in male mice. Importantly, attenuation of cocaine-mediated behaviors was observed in COMT knockout mice with both C57BL/6 and 129/Sv genetic background, confirming a robust effect of COMT deficiency in male mice. This unexpected finding may be a reflection of the sexual dimorphism seen in our previous studies (Gogos et al., 1998; Huotari et al., 2002). Attenuated locomotor response induced by a high dose of GBR 12909 may be explained by enhanced stereotypical behavior that was not registered by our motility meter. Also estrus cycle can have some effects on dopamine levels and its metabolism (Jori et al., 1976). In our study, for practical reasons, the phase of estrus cycle was not determined. We assume that different phases of estrus cycle were randomly distributed among female mice and were unlikely to cause any systematic error.

Although, there were some gender-related differences in brain tissue catecholamine levels, they do not explain unexpected motoric divergence between sexes. Also there were no gender-related differences in striatal extracellular fluid. Therefore, the neurochemical mechanism underlying attenuation of locomotor responses in male COMT knockout mice remains to be determined. One reasonable explanation would be that COMT inactivation attenuates response to psychostimulants by interfering indirectly with a presynaptic dopaminergic function. However, determination of the effects of COMT deficiency on the expression of striatal DAT using autoradiography or Western blot failed to reveal differences in ligand binding between wild-type and COMT knockout mice (Fig. 5), suggesting that the modulatory effects of COMT on the behavioral and neurochemical effects upon DAT inhibition are not mediated through compensatory changes in DAT expression levels. Alternatively, lack of COMT may have some postsynaptic effects that may account for the attenuation of motoric response to DAT inhibitors. DAT inhibition stimulates motor activity through concomitant and synergistic activation of dopamine receptors (Segal and Kuczenski, 1994; Self and Nestler, 1995). However, in striatum the number and properties of D_1 and D_2 dopamine

receptors are not changed between genotypes (J. García and P. T. Männistö, unpublished data). Whether COMT knockout mice demonstrate alterations in signal transduction, or in behavioral response to D_1 and D_2 agonists, is under investigation. Nonetheless, current data do not preclude a COMT-mediated alteration in D_1 and D_2 signal transduction (for example by postreceptor events involving G protein-coupling and intracellular signaling pathways).

Also, dopamine metabolites have been studied for their possible effects on motoric activity and dopamine neurochemistry. High-dose DOPAC, administered to lateral ventricle, has been shown to cause modest increase of behavioral activity and stereotypy in rats (Nakazato and Akiyama, 2002). Even though COMT deficiency increases DOPAC levels severalfold in our mice, there were no signs of increased locomotor activity (Fig. 3, A and D). Furthermore, neither intracerebroventricularly administered HVA in rats (Nakazato and Akiyama, 2002) nor complete lack of HVA in our COMT-deficient mice has induced any behavioral changes. Moreover, in terms of the recently identified trace amine TA_1 receptor, it seems that the *meta-O*-methyl metabolites of dopamine noradrenaline and adrenaline have even higher potency and efficacy to activate the TA_1 receptor that the catecholamines themselves (Borowsky et al., 2001; Bunzow et al., 2001). These metabolites generated by COMT could well be lacking in our COMT-deficient mice. However, the physiological importance of function mediated via trace amine receptors has remained obscure.

Regardless of the profound changes in catecholamine metabolism in the COMT-deficient mice, we have not yet detected any compensatory changes in the protein levels of other catecholamine-metabolizing (MAO-A/B, phenylsulfotransferase) or -synthesizing (tyrosine hydroxylase, dopa decarboxylase, and dopamine-β-hydroxylase) enzymes tested (Huotari et al., 2002). It should be noted that the absence of any striking compensatory change in the brains of COMT-deficient mice is in sharp contrast with the DAT-deficient mice, where a number of compensatory changes occurred in dopamine synthesis, release, clearance, metabolism, and dopamine receptor functions (Giros et al., 1996; Jones et al., 1998, 1999; Fauchey et al., 2000; Ralph et al., 2001). A more detailed analysis of compensatory changes in COMT-deficient mice is currently underway using gene microarray expression assays.

In conclusion, in mice lacking COMT, the inhibition of DAT results in higher total tissue dopamine levels in striatum and hypothalamus compared with control mice. However, such a dopamine potentiation in COMT disrupted mice was lacking in the striatal extracellular fluid. Unexpectedly, hyperlocomotor effect of high doses of DAT inhibitors was attenuated in COMT knockout male mice, i.e., they have an inability to sustain the hyperactivity induced by DAT inhibition in that sex. Despite some hints of interactions between DAT and extraneuronal uptake (plus subsequently COMT) the role of COMT in dopamine elimination seems to be minimal in conditions when is DAT inhibited.

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