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Microtubule-associated STOP protein deletion triggers restricted changes in dopaminergic neurotransmission

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Running title: Accumbic DA system in STOP KO mice

Key words: cytoskeleton, cocaine, dopaminergic transporter and receptors, synaptosomes, schizophrenia, tyrosine hydroxylase

Abbreviations: DA, dopamine; DAergic, dopaminergic; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; KO, knock-out; MAP, microtubule associated protein; PBS, phosphate buffered saline; STOP, stable tubule only polypeptide; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2; WT, wild-type.

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ABSTRACT

The microtubule-associated STOP (Stable Tubule Only Polypeptide) protein plays a key-role in neuron architecture and synaptic plasticity. Recent studies suggest that schizophrenia is associated with alterations in the synaptic connectivity. Mice invalidated for the STOP gene display phenotype reminiscent of some schizophrenic-like symptoms, such as behavioral disturbances, dopamine hyper-reactivity and possible hypoglutamatergia, partly improved by antipsychotic treatment. In the present work, we examined potential alterations in some dopaminergic key proteins and behaviors in STOP knock-out mice.

Whereas the densities of the dopamine transporter, the vesicular monoamine transporter and the D_1 receptor were not modified, the densities of the D_2 and D_3 receptors were decreased in some dopaminergic regions in mutant versus wild-type mice. Endogenous dopamine levels were selectively decreased in dopaminergic terminals areas, although the *in vivo* dopamine synthesis was diminished both in cell bodies and terminal areas. The dopamine uptake was decreased in accumbic synaptosomes, but not significantly altered in striatal synaptosomes. Finally, STOP knock-out mice were hypersensitive to acute and sub-chronic locomotor effects of cocaine, although the drug equally inhibited DA uptake in mutant and wild-type mice.

Altogether, these data showed that deletion of the ubiquitous STOP protein elicited restricted alterations in dopaminergic neurotransmission, preferentially in the meso-limbic pathway.

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INTRODUCTION

Microtubules are key cytoskeletal components in neurons, where they play a role in morphogenesis and protein transport in dendrites and axons. Previous work has indicated a central role for one of the microtubule-associated proteins, called STOP (Stable Tubule Only Polypeptide or MAP1B) in neuron stabilization and synaptic plasticity (Bosc *et al.* 1996; Guillaud *et al.* 1998; van Rossum and Hanisch 1999).

The recently engineered mouse line deficient in the STOP protein (Andrieux *et al.* 2002) exhibits synaptic defects in the hippocampus. They include depleted glutamatergic vesicle pool and marked decrease in long-term potentiation and depression in the CA1 field (Andrieux *et al.* 2002; Zervas *et al.* 2005). Moreover, levels of mRNAs encoding synaptic proteins, such as synaptophysin, growth associated protein-43 and spinophilin, are decreased in some hippocampic sub-regions (Eastwood *et al.* 2006). Moreover, a recent report shows indirect evidence for decreased glutamate release in the CNS of mutant mice (Brenner *et al.* 2007). STOP knock-out (KO) mice show severe behavioral disturbances, namely purposeless and disorganized locomotor activity, impaired social interactions and maternal behavior, deficits in the prepulse inhibition (PPI) of the startle reflex, hypersensitivity to mild stress and to the stimulant locomotor effect of amphetamine (Andrieux *et al.* 2002; Brun *et al.* 2005; Fradley *et al.* 2005). STOP KO mice also display dopamine (DA) hyper-reactivity, preferentially in the limbic dopaminergic system (Brun *et al.* 2005). Whereas basal extracellular DA levels are not modified in the striatum and the nucleus accumbens, electrically evoked DA release is selectively increased in the nucleus accumbens of STOP KO mice. Some of the behavioral and synaptic deficits in STOP KO mice can partly be restored by chronic antipsychotic drug treatment (Andrieux *et al.* 2002; Brun *et al.* 2005). Altogether, the behavioral disturbances, the DA hyper-reactivity in limbic system and the indirect evidence of a decreased glutamatergic neurotransmission suggest that STOP KO mice may represent a pertinent experimental model for some schizophrenic symptoms. Interestingly, we recently report that nicotinic neurotransmission can be deficient in STOP KO mice, despite of increased nicotinic α7 receptor density in some brain areas, and that mutant mice display cognitive deficits improved by the rather selective α7 receptor agonist choline (Bouvrais-Veret *et al.* 2007).

Several lines of evidence suggest that schizophrenia is associated with alterations of neuronal architecture, particularly in the synaptic connectivity (Mirnics *et al.* 2001; Frankle *et al.* 2003; Benitez-King *et al.* 2004; Owen *et al.* 2005b). Recently, the protein DISC1 (Disrupted-In-Schizophrenia 1) has been

demonstrated to bind to microtubules and its truncation in schizophrenic patients contributes to impair intraneuronal transport, neurite cytoarchitecture and/or neuronal migration (Morris *et al.* 2003; Owen *et al.* 2005a; Ishizuka *et al.* 2006). Furthermore, the MAP6 gene, the human homologous of STOP gene, is localized on chromosome 11q14, a region highly associated with schizotypal personality disorders (Lewis *et al.* 2003). Interestingly, an association between single nucleotide polymorphism markers of the MAP6 gene and schizophrenia has been recently published, together with an up-regulation of the isoform 2 of MAP6 transcripts in the frontal cortex of schizophrenic patients (Shimizu *et al.* 2006).

Thus, the STOP KO mice, which represent a pertinent experimental model for some schizophrenic related symptoms, are likely to be informative to understand how possible structural-related changes may translate into dysfunctions of neutransmitter systems, especially DAergic neurotransmission. So, we investigated various DA neuron-related markers: the densities of the DA transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) and the D_1, D_2 and D_3 DAergic receptors in various brain areas in STOP KO compared to wild-type (WT) mice. We also measured the endogenous DA and metabolite levels and the *in vivo* DA synthesis rate in various brain areas containing DAergic cell bodies and terminals. DA uptake and its inhibition by cocaine were measured using striatal and accumbic synaptosomes. Finally, we assessed the effects of acute cocaine on the locomotor activity of WT and STOP KO mice, as well as the cocaine-induced behavioral sensitization.

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MATERIALS AND METHODS

Animals

Homozygous STOP KO mice and their WT littermates were obtained by crossing (F2) heterozygous 50:50 BALBc/129 SvPas-F1. The genotype of mice was determined from tail biopsies as previously described (Andrieux *et al.* 2002). Animals were housed 4-6 per cage by litter, gender and genotype. They were kept under standard conditions, with food and water available *ad libitum*, temperature at 23±2°C, humidity at 55±10% and a 12 h light:12 h dark cycle, with lights on at 07:30 *a.m.*. Experiments were carried out in accordance with the European Communities Council directive (86/809/EEC) and approved by the local ethical committee. Mice were allowed to habituate to the animal holding room for at least one week prior to use. All experiments were conducted with 99 WT and 99 KO mice (about 60%/40% females/males), of the same litters and at 3-4 months of age.

Drugs

(±)-Butaclamol hydrochloride, 1,3-di-*o*-tolylguanidine, dopamine hydrochloride, NSD 1015 (3-hydroxybenzyl-hydrazine) were purchased from Sigma-Aldrich (Saint Quentin-Fallavier, France). Apomorphine hydrochloride was purchased from Tocris (Bristol, UK). Ascorbate oxidase and nomifensine maleate were purchased from RBI (Natik, USA) and cocaine hydrochloride from Cooper (Melun, France). Cocaine and NSD 1015 were dissolved in 0.9% NaCl as free base and were administered intraperitoneally (i.p.) under a volume of 100 µl per 20 g body weight. Polyclonal antibody against VMAT2 was from Phoenix Europe GMBH (Karlsruhe, Germany). [7,8-^{3}H]-Dopamine (1.5-2.2 Tbq/mmol), rabbit [^{125}I]-Ig F (ab')2 fragment antibodies (28-111 TBq/mmol), [^{125}I]-iodosulpride (74 TBq/mmol) and [N-methyl-^{3}H]-SCH 23390 (2.2-3.3 TBq/mmol) were from GE Healthcare (Orsay, France). [^{125}I]-R(±)*trans*-7-hydroxy-2-[N-(3'-iodo-2'-propenyl)amino] tetralin ([^{125}I]-7-OH-PIPAT, 81.4 TBq/mmol) was from Perkin Elmer-NEN (Orsay, France).

Before use, [^{125}I]-IgG antibodies were purified by gel filtration onto a PD10 column (Sephadex G25 M, Pharmacia) in PBS (50 mM NaH_2PO_4/Na_2HPO_4, 154 mM NaCl) containing 0.1% gelatin. The first 0.25 ml eluate was discarded and the following 3.5 ml eluate was mixed with 400 ml PBS supplemented with 3% bovine serum albumin (BSA), 1% goat serum, 1 mM NaI and 0.02% sodium azide. The purified [^{125}I]-IgG batch was kept at 4°C, for 4-6 weeks.

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Quantitative autoradiography

Mice were killed by cervical dislocation between 10:00 *a.m.* and 4:00 *p.m.* and their brains rapidly removed and frozen in isopentane at –30°C. Serial 10 µm coronal sections were cut at –20°C, thaw-mounted on Superfrost Plus® slides and stored at –80°C until use.

Immunoautoradiography of dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2)

Brain sections were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS and then incubated for 1 h at room temperature in PBS containing 3% BSA, 1% goat serum and 1 mM NaI. Sections were subsequently incubated for 2 h at room temperature (or overnight at 4°C) in the presence of polyclonal DAT antiserum (1:20,000 dilution, Martres *et al.* 1998) or polyclonal VMAT2 antiserum (1:8,000 dilution). After extensive washes, slides were incubated for 2 h at room temperature with purified anti-rabbit [^{125}I]-IgG (370-740 MBq/100 ml). Sections were extensively washed with PBS, dried and exposed to Biomax MR film for 1-4 days.

Autoradiographic determination of D_1, D_2 and D_3 DAergic receptor densities

To rule out possible binding of endogenous DA to its receptors, sections were pre-incubated in buffer before addition of radioactive ligands.

Labelling of D_1 receptors was performed according to Fernagut *et al.* (2003). Slides were pre-incubated for 20 min at room temperature in 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Tris-ions buffer). They were then incubated for 90 min at room temperature in a fresh Tris-ions buffer containing 3 nM [^{3}H]-SCH 23390, with or without 10 µM butaclamol to determine non-specific binding. After four washes for 5 min with ice-cold Tris-ions buffer, sections were dipped in ice-cold water, dried and exposed to BAS-TR Fuji Imaging screen for 3 days. The screens were scanned with a Fuji Bioimaging Analyzer BAS-5000.

Labelling of D_2 receptors was done according to Martres *et al.* (1985). Slides were pre-incubated three times for 5 min at room temperature in Tris-ions buffer supplemented with 0.1% BSA and 0.57 mM ascorbic acid. They were then incubated for 60 min at room temperature in Tris-ions buffer in the presence of 0.2 nM [^{125}I]-iodosulpride, with or without 10 µM apomorphine to determine non-specific binding. Sections were washed as described for D_1 receptor labelling and exposed to Biomax MR film (GE Healthcare) for 12-36 h.

Labelling of D_3 receptors was performed according to Stanwood *et al.* (2000), with some modifications: slides were pre-incubated three times for 5 min at room temperature in 50 mM HEPES buffer, pH 7.4, supplemented with 1

mM EDTA, 2.8 mM ascorbic acid, 0.1% BSA, 100 µM GTP (to dissociate D_2 receptors from G proteins) and 25 µM 1,3-di-*o*-tolylguanidine (to prevent the labelling of σ receptors). Then, sections were incubated for 60 min at room temperature in the same buffer in the presence of 0.25 nM [^{125}I]-7-OH-PIPAT, with or without 10 µM dopamine to determine non-specific binding. Sections were washed four times for 15 min each in ice-cold HEPES buffer, dipped in ice-cold water, dried and exposed to Biomax MR film for 1-3 days.

Quantification

Standard radioactive microscales (GE Healthcare) were exposed onto each Imaging screen or autoradiographic film to ensure that labelling densities were in the linear range. The autoradiograms were scanned and densitometry was performed with Multi Gauge software or MCID™ analysis software for [^{3}H]- or [^{125}I]-radioligand, respectively. Specific labelling densities (mean grey values in arbitrary units) of four sections per area were averaged for each mouse.

Determination of tissue levels of DA and its metabolites and of *in vivo* tyrosine hydroxylase activity

The levels of endogenous DA, its precursor L-DOPA and its metabolites DOPAC and HVA were determined as follows: dissected brain areas were homogenized in 5-10 vol (v/w) of ice-cold 0.1 M $HClO_4$ containing 0.05% EDTA and 0.05% $Na_2S_2O_5$. Homogenates were centrifuged at 22,500 g for 20 min at 4 °C. After neutralization with 2 M KH_2PO_4/K_2HPO_4, pH 7.4, containing 0.01 mg/ml ascorbate oxidase, supernatants were further centrifuged at 30,000 g for 10 min. Aliquots (10 µl) of clear supernatants were injected into a HPLC column (Ultrasphere IP, Beckman, Gagny, France; 25x4.6 cm, C18 reversed-phase, particle size 5 µm) protected with a Brownlee precolumn (3 cm, 5 µm). The mobile phase for the elution (flow rate: 1 ml/min) consisted of 70 mM KH_2PO_4, 2.1 mM triethylamine, 0.1 mM EDTA, 1.25 mM octane sulphonate and 16% methanol, adjusted to pH 3.02 with solid citric acid. The electrochemical detection system (ESA 5011, Bedford, USA) comprises an analytical cell with dual coulometric monitoring electrodes (+50 mV and +350 mV). The generated signal was integrated by a computing integrator (Millenium^{32}-Waters, Saint Quentin Fallavier, France). Quantitative determinations were made using appropriate standards.

For the *in vivo* measurement of tyrosine hydroxylase activity, animals were intraperitoneally injected with 100 mg/kg NSD 1015, to block aromatic L-amino acid decarboxylase, 30 min before sacrifice. Accumulated L-DOPA levels were measured by HPLC coupled to electrochemical detection as described above.

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Synaptosomal uptake of [^{3}H]-DA

Pooled striata or nuclei accumbens from 2 mice were homogenized in 15-20 volumes (v/w) of ice-cold 0.32 M sucrose with a glass-Teflon potter (Braun Biotech International; Sartorius, Palaiseau, France). Homogenates were diluted to 40 volumes (v/w) with 0.32 M sucrose, centrifuged at 1,000 *g* for 10 min at 4 °C and the resulting supernatants were centrifuged again at 30,000 *g* for 10 min. The pellets were gently homogenized in 50 volumes of 0.32 M sucrose and 25 µl aliquots were incubated in a final volume of 500 µl of buffer containing 4 mM Tris-HCl, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 $MgSO_4$, 5.6 mM D-glucose and 0.5 mM ascorbic acid, pH 7.4. For saturation experiments, synaptosomes were incubated in the presence of 20 nM [^{3}H]-dopamine plus 0.01-1.0 µM unlabelled DA. For cocaine inhibition experiments, synaptosomes were incubated in the presence of 10 nM [^{3}H]-dopamine with or without 0.03-3.0 µM cocaine. In all cases, non-specific uptake was determined in the presence of 30 µM nomifensine. After 7 min at 37 °C, samples were diluted with 3 ml of ice-cold buffer and rapidly filtered through Whatman GF/B glass fibers presoaked with 0.05% polyethylenimine. After three washes with 3 ml of ice-cold buffer, filters were dried and the entrapped radioactivity counted by liquid scintillation spectrometry. Proteins were quantified according to Lowry *et al.* (1951) with BSA as standard.

Locomotor activity

In both experiments, mice were tested for their locomotor activity between 9:00 and 12:00 *a.m.*.

Acute effect of cocaine

The horizontal locomotion activity was assessed in transparent activity cages (20 x 15 x 25 cm), with automatic monitoring of photocell beam breaks, located at 1.5 cm above the floor (Imetronic, Bordeaux, France). Mice were allowed to habituate to actimeter cages 20 min before i.p. administration of saline or 5-40 mg/kg cocaine. Their locomotor activity was further recorded for 50 min.

Behavioral sensitization to cocaine

Mice were allowed to habituate to actimeter cages during 20 min before administration of the drug. Animals of both genotypes received saline or cocaine (10 mg/kg i.p.) for 5 consecutive days (initiation phase) and their locomotor activity was recorded for 60 min after each administration. Twenty days after the last saline or cocaine administration (day 25), all pre-treated mice received saline and their locomotion was recorded. On day 26 (expression phase), all mice

received cocaine (10 mg/kg i.p.) and their locomotion was further recorded for 60 min.

Statistical analyses

For autoradiographic quantifications, determinations of endogenous DA and metabolite levels and tyrosine hydroxylase activity, data were subjected to factorial one-, two- or three-way analysis of variance (ANOVA), with genotype, sex and area as between-group factors. Significant main effects were further analyzed by post hoc comparisons of means using Fisher's test.

The kinetic parameters of uptake experiments with synaptosomes, i.e. Km, Vmax and IC50, were calculated by non-linear regression using GraphPad Prism 2.01 software (San Diego, USA). Inhibition constants (Ki) were calculated from IC50 values using the following equation: Ki=IC50/(1+L/Km), where L represents the concentration of [^{3}H]-DA and Km the affinity constant of DA. The means ± SEM were then compared using Student's t test.

Behavioral data were subjected to factorial two-, three- or four-way ANOVA, with genotype, sex and treatment as between-group factors and time as within-group factor. Significant main effects were further analyzed by post hoc comparisons of means using Fisher's test. Statistical significance was set at $p<0.05$.

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RESULTS

No statistically significant differences were noted between males and females of each genotype in all experimental conditions (not shown). Accordingly, data for males and females in the studies reported therein were pooled.

DA transporters and receptors in various brain areas of STOP KO versus WT mice

The densities of the DA transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) and the D_1, D_2 and D_3 DAergic receptors were determined in various brain areas of WT and STOP KO mice (Fig. 1 and Tables 1, 2). Analyses of the densities of DAT, VMAT2 and D_1 receptors in the various areas studied showed no significant effect of genotype.

Analysis of the density of D_2 receptors showed a significant effect of genotype and area (genotype: $F_{(1,49)}=16.31$, $p<0.001$; area: $F_{(5,49)}=76.9$, $p<0.001$). The density of D_2 receptors was decreased by 23% in the striatum ($p=0.027$), by 28% in the nucleus accumbens ($p=0.015$), by 28% in the core ($p=0.014$) and by 27% in the shell ($p=0.018$). No differences in D_2 receptor density were noted in the pars compacta of the substantia nigra and in the ventral tegmental area between the two mouse strains (Table 2).

Analysis of the density of D_3 receptors showed a significant effect of genotype and area (genotype: $F_{(1,75)}=29.73$, $p<0.001$; area: $F_{(7,75)}=177.5$, $p<0.001$; genotype x area: $F_{(7,75)}=3.43$, $p=0.003$). The density of D_3 receptors was significantly decreased by 24% in the median pars compacta of the substantia nigra ($p=0.017$), 37% in the ventral tegmental area ($p=0.006$), 18% in the nucleus accumbens ($p=0.050$), by 24% in the core ($p=0.011$) and 21% in the shell ($p=0.027$), while its density was not modified in the other DAergic areas examined. The amplitude of the changes in the D_3 receptor density was significantly larger in the ventral tegmental area ($p=0.019$) and the nucleus accumbens ($p=0.007$) than in the substantia nigra and the striatum, respectively.

Tissue levels of DA and its metabolites DOPAC and HVA

The levels of endogenous DA and its metabolites DOPAC and HVA in some DAergic areas were compared between STOP KO and WT mice (Table 3). Analyses of the data showed a significant effect of genotype and area on DA levels (genotype: $F_{(1,34)}=25.49$, $p<0.001$; area: $F_{(3,34)}=170.6$, $p<0.001$; genotype x area: $F_{(3,34)}=8.87$, $p<0.001$), DOPAC levels (genotype: $F_{(1,37)}=19.38$, $p<0.001$; area: $F_{(3,37)}=61.44$, $p<0.001$; genotype x area: $F_{(3,37)}=3.99$, $p=0.015$) and HVA levels (genotype: $F_{(1,38)}=21.73$, $p<0.001$; area: $F_{(3,38)}=108.1$, $p<0.001$; genotype x area: $F_{(3,38)}=3.62$, $p=0.022$).

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No significant differences in DA levels were found between mice of the two genotypes in the substantia nigra plus the ventral tegmental area and in the frontal cortex. In contrast, DA levels were significantly lower in the striatum (-29%, $p<0.001$) and in the nucleus accumbens (-63%, $p<0.001$). The levels of DOPAC were not significantly modified in the substantia nigra plus the ventral tegmental area and the frontal cortex, but they were decreased by 23% in the striatum ($p=0.011$) and by 53% in the nucleus accumbens ($p<0.001$). The level of HVA was not modified in the substantia nigra plus the ventral tegmental area and the frontal cortex, but it was decreased by 24% in the striatum ($p=0.002$) and by 50% in the nucleus accumbens ($p<0.001$). In the latter two areas, the DOPAC and HVA levels were decreased to the same extent as DA levels in STOP KO mice, so that the ratio of metabolites over DA was not modified as compared to that in WT mice (see Fig. 2).

Interestingly, the amplitudes of the STOP KO-associated decreases were significantly two-fold higher in the nucleus accumbens than in the striatum for DA ($p=0.050$), DOPAC ($p=0.025$) and HVA levels ($p=0.010$).

***In vivo* tyrosine hydroxylase activity in various brain areas**

The *in vivo* activity of tyrosine hydroxylase (TH) was determined by measuring L-DOPA accumulation after inhibition of aromatic L-amino acid decarboxylase activity by systemic administration of NSD 1015 (Table 4).

Statistical analysis of data showed a significant effect of genotype and area (genotype: $F_{(1,83)}=20.39$, $p<0.001$; area: $F_{(3,83)}=64.48$, $p<0.001$; genotype x area: $F_{(3,83)}=2.69$, $p=0.051$). In STOP KO mice, the accumulated L-DOPA levels were significantly reduced in the substantia nigra plus the ventral tegmental area (-31%, $p=0.004$), the striatum (-19%, $p=0.009$) and the nucleus accumbens (-29%, $p=0.001$). Interestingly, the amplitude of the STOP KO-associated decrease in in vivo TH activity was not significantly different in these three areas. In contrast, L-DOPA accumulation in the frontal cortex did not differ between STP KO and WT mice.

In order to compare the extent of decrease in DA, metabolites tissue levels and TH activity, values in STOP KO mice were expressed as percentages of the respective values in WT mice (Fig. 2). A significant difference was seen between the change in DA levels and TH activity in the substantia nigra plus the ventral tegmental area (101±17% versus 69±4%, respectively, $p=0.037$) and the nucleus accumbens (37±4% versus 71±6%, respectively, $p=0.012$).

DA uptake in synaptosomes from the striatum and the nucleus accumbens

The kinetic parameters of [^{3}H]-DA uptake were determined using synaptosomes from the striatum and the nucleus accumbens of mice of both genotypes (Fig. 3).

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In striatal synaptosomes, uptake of [^{3}H]-DA, at all concentrations tested, tended to be higher in STOP KO mice than in WT mice, with a nearly significant increase at 100 nM [^{3}H]-DA (+32%, (p=0.083). However, the maximal uptake velocity Vmax and the Michaelis's constant Km were not significantly different in the two genotypes, i.e. Vmax=19.5±2.4 and 24.5±2.1 pmol/mg prot/min, Km=50.4±5.7 and 73.4±7.5 nM, in WT and STOP KO mice, respectively.

In contrast, uptake of [^{3}H]-DA, at all concentrations tested in nucleus accumbens synaptosomes, was regularly lower in STOP KO than in WT mice, with a significant decrease at 500 nM [^{3}H]-DA (-26%, p=0.049). The maximal uptake velocity was significantly decreased by 24% in STOP KO mice compared to WT mice, without any modification of DA affinity, i.e. Vmax=18.2±1.3 and 14.1±0.6 pmol/mg prot/min (p=0.024) and Km=191.3±5.2 and 185.0±2.5 nM, in WT and STOP KO mice, respectively. Interestingly, the Km value for DA was significantly 3-4-fold higher (p<0.001) in the nucleus accumbens than in the striatum, in both WT and STOP KO mice.

Effects of cocaine on the locomotor activity of STOP KO versus WT mice

In order to reveal a potential functional consequence of the decreased Vmax of [^{3}H]-DA uptake in nucleus accumbens synaptosomes of mutant mice, we compared the effects of acute cocaine at increasing doses on the locomotor activity of WT versus STOP KO mice (Fig. 4).

During the 20-min habituation phase in the actimeter cages (not shown), analysis of data showed a significant effect of genotype on the locomotor activity (genotype: $F_{(1,228)}$=4.14, p=0.045), which was increased by 29% in STOP KO mice, as previously reported (Brun *et al.* 2005).

Statistical analysis of the cocaine effects on locomotor activity showed a significant effect of genotype, dose and time (genotype: $F_{(1,594)}$=15.65, p<0.001; dose: $F_{(4,594)}$=17.55, p<0.001; time: $F_{(9,594)}$=18.93, p<0.001; genotype x dose: $F_{(4,594)}$=3.49, p=0.012; genotype x time: $F_{(9,594)}$=4.66, p<0.001; dose x time: $F_{(36,594)}$=4.71, p<0.001; genotype x dose x time: $F_{(36,594)}$=3.31, p<0.001). STOP KO mice displayed a marked hypersensitivity to the stimulant effect of cocaine. Whereas cocaine at 10 mg/kg had no effect on the activity of WT mice, it increased by 842% (p<0.001) the locomotor activity of mutant mice. Moreover, the increase induced by cocaine at 25 mg/kg was significantly higher in STOP KO mice than WT mice (p=0.002, Fig. 4B).

Cocaine inhibition of [^{3}H]-DA uptake in synaptosomes from the striatum and the nucleus accumbens

The differences in the locomotor effects of cocaine in mutant versus WT mice led us to investigate whether [^{3}H]-DA uptake inhibition by cocaine

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exhibited peculiarities in STOP KO mice (Fig. 5). In fact, the concentration of cocaine which inhibited the [^{3}H]-DA uptake by 50% (IC50) was similar in mice of both genotypes, i.e. IC50=99±27 and 102±33 nM in striatal and 193±24 and 205±23 nM in accumbic synaptosomes of WT and STOP KO mice, respectively. Corresponding Ki values were 83±22 and 90±29 nM for the striatum and 183±22 and 195±22 nM for the nucleus accumbens of WT and STOP KO mice, respectively. Finally, the Ki value of cocaine was 2.2-fold higher in the nucleus accumbens than in the striatum in both WT and mutant mice (p=0.037).

Behavioral sensitization to cocaine in STOP KO versus WT mice

To assess the sensitivity of STOP KO mice towards sub-chronic cocaine treatment, we measured the effects of repeated administration of cocaine in a paradigm of behavioral sensitization (Fig. 6). We chose the lowest active dose of cocaine in STOP KO mice (10 mg/kg, see Fig. 4). Statistical analyses of data during the initiation phase (days 1-5) and at the expression phase (day 26) showed a significant effect of genotype, treatment and time (genotype: $F_{(1,205)}$=6.25, p=0.017; treatment: $F_{(1,205)}$=18.40, p<0.001; time: $F_{(5,205)}$=19.26, p<0.001; genotype x treatment: $F_{(1,205)}$=4.78, p=0.035; genotype x time: $F_{(5,205)}$=2.19, p=0.056; treatment x time: $F_{(5,205)}$=2.98, p=0.013).

In WT mice, during the initiation phase, cocaine 10 mg/kg had no effect at day 1 (Figs. 4 and 6), but significantly increased the locomotor activity at day 5 (230% versus saline, p=0.010; 140% versus cocaine at day 1, p=0.034). After a 21-day withdrawal, during the expression phase, the effect of cocaine was similar in saline- and cocaine-pretreated mice, suggesting an environmental sensitization. Finally, the effect of cocaine on cocaine-treated WT mice was significantly higher at day 26 than at day 1 (230%, p=0.001).

In STOP KO mice, during the initiation phase, 10 mg/kg cocaine significantly enhanced the locomotor activity by 350% at day 1 (p=0.015; Figs. 4 and 6) and by 600% at day 5 (p<0.001) versus saline, indicating a 70% increase (p=0.037) in the response from day 1 to day 5. After a 21-day withdrawal, the effect of cocaine at day 26 in cocaine-treated mice was significantly higher versus saline-treated mice (70%, p=0.005) and versus cocaine at day 1 (120%, p<0.001).

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DISCUSSION

Altogether, our data show that the suppression of the relatively ubiquitous microtubule-associated STOP protein (Andrieux *et al.* 2002) elicited discrete changes in DAergic neurotransmission systems, affecting mainly the meso-limbic pathway. Indeed, STOP KOP mice exhibited DAergic hyper-reactivity as decreased DAergic D_2 receptor level, decreased DA uptake by accumbic synaptosomes and hypersensitivity to cocaine.

Whereas the densities of the DA transporter and the vesicular monoamine transporter 2 were not altered in any brain area examined in STOP KO compared to WT mice, the density of DAergic D_2 receptors was decreased in DAergic terminal areas, but not in DAergic cell bodies regions in mutant mice. In contrast, the density of D_3 receptors was decreased in both DAergic cell bodies and terminal areas, particularly in the meso-accumbic pathway. Evidence for higher DAergic neurotransmission in STOP KO mice has been clearly demonstrated in previous studies (Brun et al., 2005). The decreased density of D_2 receptors, restricted to DAergic terminal areas, might well correspond to a compensatory mechanism to elevated extracellular DA concentrations in STOP KO mice, as previously reported in the constitutively hyperDAergic DAT KO mice (Giros *et al.* 1996; Jones *et al.* 1998; Weiss *et al.* 2007), which are also considered a pertinent model for some symptoms of schizophrenia (Gainetdinov *et al.* 2001). In contrast, these changes in D_3 receptor density could not be related to some decrease in extracellular DA levels, as expected from the paradoxical adaptive downregulation of D_3 receptors in rats with decreased DAergic tone (Levesque *et al.* 1995). Finally, in contrast with data in DAT KO mice (Giros *et al.* 1996; Jones *et al.* 1998; Weiss *et al.* 2007), the density of D_1 receptors was not modified in any area examined in STOP KO mice. This could mean that alterations of D_2 receptor density in STOP KO mice not only depend on the DAergic area, but also on the nature of the neurons expressing D_2 versus D_1 receptors.

Interestingly, our data showing the absence of alteration of D_2 autoreceptor density in DA cell bodies areas did not match the previous finding of Brun *et al.* (2005). Using an amperometric method, the authors reported an enhanced autoinhibition of evoked DA release in the nucleus accumbens in STOP KO mice. Determination of the ratio of D_2 and D_3 autoreceptors over postsynaptic receptors in the nucleus accumbens of mutant mice and of their respective coupling efficiency and functional characteristics should help to elucidate these apparent discrepancies.

The occurrence of STOP KO-associated changes restricted to some DAergic areas was confirmed by measurements of endogenous DA and

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metabolite levels. Whereas DA, DOPAC and HVA levels were not modified in the substantia nigra and the ventral tegmental area containing DAergic cell bodies of STOP KO mice, they were significantly decreased in DAergic terminal areas, i.e. striatum and nucleus accumbens. Interestingly, the amplitude of the drop in endogenous DA and metabolite levels was twice as high in the nucleus accumbens as in the striatum. The decreased DA levels in STOP KO mice are reminiscent of that reported in the striatum of DAT KO mice (Jones et al. 1998) and might be due to an impaired transport of tyrosine hydroxylase protein from DAergic cell bodies to terminals. However, measurement of L-DOPA accumulation after aromatic L-amino acid decarboxylase inhibition showed that the rate of DA synthesis was decreased to the same extent in both DAergic cell bodies and terminals in mutant mice. This reduction might reflect alterations in the expression and/or catalytic activity of tyrosine hydroxylase. The similar decrease in DA synthesis in both DAergic cell bodies and terminals areas in STOP KO mice does not explain why the decrease of endogenous DA levels was restricted to DAergic terminals areas and the reason why the latter decrease was twice as large in the nucleus accumbens as in the striatum. Relevant investigations have to be performed to elucidate the actual underlying mechanisms.

Furthermore, in the three DAergic areas examined, the decrease of endogenous DA levels did not always parallel that of *in vivo* TH activity. The difference is particularly striking in the substantia nigra plus the ventral tegmental area where endogenous DA levels were not modified, despite a 31% decrease of *in vivo* TH activity. This could be due to a diminished DA catabolism, as indicated by the tendency of DOPAC and HVA levels to be less (-20%, ns) in mutant mice. In the striatum, the parallel decrease of DA and metabolites levels and of TH activity suggests that DA turn-over was not altered beyond TH step in STOP KO mice. In the nucleus accumbens, the 69% decrease of DA levels could not be explained by the 29% decrease of TH activity. However, the difference could not be due to enhanced DA catabolism, because DOPAC and HVA levels decreased to the same extent as DA levels in mutant mice. Interestingly, previous analysis of the striatal DA turn-over in DAT KO mice provided evidence that the reserve pools of DA are more dependent on recycled extracellular DA than on the newly synthetized amine (Jones *et al.* 1998). Thus, an increased DA release (Brun *et al.* 2005) associated with decreased DA reuptake (present study) could partly explain the large drop of endogenous DA levels in the nucleus accumbens of STOP KO mice. Another non exclusive hypothesis which remains to be tested could be a deficit of vesicular storage of newly synthetized DA in this particular region.

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Finally, it is difficult to reconcile a decreased in vivo DA synthesis with the hypothesis that the reduced density of postsynaptic D2 receptors represents a homeostatic adaptation to higher extracellular DA level. This discrepancy probably means that, in spite of decreased synthesis, the DA releasable pool could be larger and/or exocytotic processus could be more efficient in STOP KO mice. Although this hypothesis is compatible with the hypersensitivity of mutant mice to amphetamine (Brun *et al.* 2005), further experiments aimed at assessing VMAT2 functional status have to be performed in STOP KO compared to WT mice.

Whereas DAT density was not modified in any DAergic area, DAT activity in synaptosomes was increased in the striatum (by 30%, ns) and decreased in the nucleus accumbens (by 25%, $p<0.05$) in STOP KO compared with WT mice. Such opposite changes in DA uptake in the striatum versus the nucleus accumbens, not observed at low DA concentrations, paralleled previous data reported by Brun *et al.* (2005) on extracellular DA levels. These authors found that electrically-evoked DA release is decreased in the striatum, although not significantly, and, in contrast, significantly increased in the nucleus accumbens of mutant compared to WT mice. Further investigations are necessary to explain how STOP protein deficiency can affect DAT activity differently in the two regions.

Another interesting feature of this study lays in the lower affinity (higher Km value) of DAT for DA in the nucleus accumbens compared with the striatum, in mice of both genotypes. To our knowledge, the kinetic parameters of DA uptake by synaptosomes from the mouse nucleus accumbens have never been reported. Such regional difference in DA affinity may be underlain by the association of DAT with chaperone proteins which may vary in an area-dependent manner, in agreement with previous studies showing a 10-fold lower affinity of DA for DAT in cultured cells than in striatal synaptosomes (Giros *et al.* 1992; Giros and Caron, 1993).

To assess potential functional consequences of altered DA reuptake in STOP KO mice, we tested the response of mutant mice to the acute locomotor effect of the DAT inhibitor cocaine. STOP KO mice displayed a clear-cut hypersensitive response to the stimulatory effect of cocaine on locomotion, as previously reported for amphetamine (Brun *et al.* 2005). Indeed, the cocaine dose-response curve showed a 10-fold leftward shift in mutant compared with WT mice. In addition, we showed that cocaine-induced behavioral sensitization persisted much longer in STOP KO mutants (up to at least 21 days) than in WT mice. The hypersensitivity of mutant mice was not related to some alterations in DA uptake inhibition by cocaine, because the latter drug was equally potent in mice of both genotypes. Hypersensitivity of STOP KO mice might rather be due to hyper-reactivity of postsynaptic DA receptors, at the level of G protein

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coupling and/or downstream signaling pathways. Another non exclusive explanation could be that cocaine partly mediates its effects via another monoamine transporter, such as the serotonin transporter for which it displays a high affinity (Giros *et al.* 1992). Indeed, serotonin neurotransmission is probably altered in STOP KO mice, in line with previous data demonstrating marked anxiety-like behavior in these mice (Andrieux *et al.* 2002). In addition, the implication of the NA transporter cannot be ruled out because cocaine also displays some affinity for this transporter (Giros *et al.* 1992) and because its affinity for DAT was two-fold lower in accumbic than in striatal synaptosomes from mice of both genotypes.

Altogether our study shows that genetic deletion of the microtubule-regulating STOP protein can induce significant adaptations of dopaminergic neurotransmission, further supporting the idea that STOP KO mice are a relevant model for the study of some symptoms of schizophrenia. In agreement with previous results (Brun *et al.* 2005), we showed that mutant mice displayed DAergic hyper-reactivity preferentially in the meso-limbic pathway. This suggests a probable hyperDAergia as in schizophrenia, even if no alterations of D_2 and D_3 receptor density, endogenous DA level and in vivo DA synthesis have been reported in schizophrenic brain (Gurevich *et al.* 1997, Seeman and Kapur 2000). Interestingly, STOP KO mice were also hyper-responsive to psychostimulants, in agreement with co-morbidity of schizophrenia and drug abuse (Batel 2000; Winklbaur *et al.* 2006). To conclude, our study and previous works clearly indicate that the deletion of a relatively ubiquitous structural protein (Couegnas *et al.* 2007) can translate into specific and restricted alterations of glutamatergic (Andrieux *et al.* 2002; Brenner *et al.* 2007), dopaminergic (Brun *et al.* 2005 and this study) and nicotinic neurotransmission (Bouvrais-Veret *et al.* 2007). This is what happens in humans, where deletion/mutation of single genes broadly distributed can actually be responsible for specific symptoms (Jamain *et al.* 2003; Ishizuka *et al.* 2006).

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Table 1: Density of DA transporters in various brain areas of STOP KO versus WT mice

Transporter	Area	WT	KO	KO/WT
DAT	SNc	(6) 353 ± 11	(5) 370 ± 19	+5% ns
	VTA	(5) 355 ± 13	(5) 387 ± 21	+9% ns
	Striatum	(6) 359 ± 8	(6) 360 ± 12	0%
	N Acc	(5) 314 ± 7	(5) 326 ± 13	+4% ns
	Core	(5) 350 ± 11	(6) 362 ± 13	+3% ns
	Shell	(5) 279 ± 9	(6) 291 ± 19	+4% ns
VMAT2	SN	(5) 571 ± 16	(4) 581 ± 28	+2% ns
	VTA	(4) 265 ± 27	(4) 241 ± 27	-9% ns
	Striatum	(5) 436± 25	(5) 482 ± 44	+11% ns
	N Acc	(5) 494 ± 30	(5) 519 ± 24	+5% ns
	Core	(5) 454 ± 42	(5) 499 ± 37	+10% ns
	Shell	(5) 533 ± 32	(5) 538 ± 25	+1% ns

Densities are the means ± SEM of specific autoradiographic labellings in arbitrary mean grey values. The number of mice is indicated in parentheses. Core: nucleus accumbens, core part; N Acc, nucleus accumbens; Shell: nucleus accumbens, shell part; SN, substantia nigra; SNc, substantia nigra, pars compacta; VTA, ventral tegmental area.

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Table 2: Density of DA receptors in various brain areas of STOP KO versus WT mice

Receptor	Area	WT	KO	KO/WT
D_1	SNr	(6) 131 ± 4	(6) 132 ± 7	0%
	Striatum	(5) 494 ± 10	(5) 486 ± 21	-2% ns
	N Acc	(5) 338 ± 8	(5) 340 ± 16	+1% ns
	Core	(5) 356 ± 7	(5) 332 ± 16	-7% ns
	Shell	(5) 324 ± 12	(5) 358 ± 14	+11% ns
D_2	SNc	(5) 264 ± 33	(5) 231 ± 11	-12% ns
	VTA	(5) 237 ± 18	(4) 211 ± 11	-11% ns
	Striatum	(6) 774 ± 70	(6) 596 ± 50	-23% *
	N Acc	(5) 280 ± 28	(5) 202 ± 11	-28% *
	Core	(5) 257 ± 23	(5) 185 ± 14	-28% *
	Shell	(5) 240 ± 22	(5) 175 ± 15	-27% *
D_3	SN	(6) 21.3 ± 1.1	(5) 18.8± 2.2	-12% ns
	SNmc	(6) 46.9 ± 2.0	(5) 35.7 ±4.0	-24% *
	SNr	(6) 14.8 ±0.7	(5) 13.9 ± 1.0	-6% ns
	VTA	(6) 14.3 ± 0.8	(4) 8.9 ± 1.9	-37% *#
	Striatum	(6) 16.7 ±1.1	(6) 18.0 ±1.6	+8% ns
	N Acc	(6) 68.9 ±2.3	(6) 56.7 ± 3.9	-18% *##
	Core	(6) 70.3 ± 4.8	(6) 53.3 ± 4.8	-24% *
	Shell	(6) 103.2 ± 4.6	(6) 81.6 ± 5.3	-21% *

Densities are the means ± SEM of specific autoradiographic labellings in arbitrary mean grey values. The number of mice is indicated in parentheses. Core: nucleus accumbens, core part; N Acc, nucleus accumbens; Shell: nucleus accumbens, shell part; SN, substantia nigra; SNc, substantia nigra, pars compacta; SNmc, substantia nigra, median pars compacta; SNr, substantia nigra, pars reticulata; VTA, ventral tegmental area. Post hoc Fisher's test: *$p<0.05$, comparison between genotypes; #$p<0.05$, comparison with SN in STOP KO mice, ##$p<0.05$, comparison with striatum in STOP KO mice.

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Table 3: DA, DOPAC and HVA levels in various brain areas of WT and STOP KO mice

Area	Genotype	DA	DOPAC	HVA
SN+VTA	WT	(6) 0.77±0.04	(6) 0.41±0.04	(6) 0.47±0.02
	KO	(5) 0.78±0.13	(6) 0.32±0.05	(5) 0.38±0.05
		+1% ns	-23% ns	-19% ns
Striatum	WT	(5) 11.27±0.97	(5) 1.11±0.16	(6) 1.72±0.12
	KO	(6) 7.95±0.31	(6) 0.85±0.04	(6) 1.30±0.07
		-29% **	-23% ns	-24% **
N Acc	WT	(5) 5.45±0.86	(5) 0.88±0.11	(6) 0.87±0.07
	KO	(6) 2.01±0.24	(6) 0.42±0.04	(6) 0.43±0.04
		-63% $^{**\#}$	-53% $^{**\#}$	-50% $^{***\#}$
Fr Cx	WT	(4) 0.21±0.02	(6) 0.11±0.01	(6) 0.43±0.04
	KO	(5) 0.26±0.06	(5) 0.08±0.004	(5) 0.38±0.05
		+25% ns	-26% *	-12% ns

Levels (µg/g tissue) of DA and its metabolites HVA and DOPAC are expressed as means ± SEM. The number of mice is indicated in parentheses. Fr Cx, frontal cortex; N Acc, nucleus accumbens; SN+VTA, substantia nigra + ventral tegmental area.

Post hoc Fisher's test: *$p<0.05$; **$p<0.01$; ***$p<0.001$, comparison between genotypes; $^{\#}p<0.001$, comparison with the decrease in the striatum in STOP KO mice.

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Table 4: In vivo activity of tyrosine hydroxylase in various brain areas of WT and STOP KO mice

Area	L-DOPA (ng/g)		
	WT	KO	KO/WT
SN+VTA	(12) 861±55	(12) 590±38	-31% ***
Striatum	(10) 1405±83	(12) 1145±63	-19% **
N Acc	(11) 1188±108	(11) 846±76	-29% ***
Fr Cx	(11) 382±32	(12) 395±58	+3% ns

Mice received 100 mg/kg NSD 1015 30 minutes before sacrifice. Respective basal endogenous L-DOPA levels were subtracted from L-DOPA accumulated after NSD 1015 administration. L-DOPA levels (ng/g tissue) are expressed as means ± SEM. The number of mice is indicated in parentheses. Fr Cx, frontal cortex; N Acc, nucleus accumbens; SN+VTA, substantia nigra + ventral tegmental area.
Post hoc Fisher's test: **p<0.01; ***p<0.001, comparison between genotypes.

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LEGENDS TO FIGURES

Fig. 1 Representative autoradiographic labelling of plasmic DA (DAT) and vesicular monoamine (VMAT2) transporters and of D_1, D_2 and D_3 DAergic receptors in brain areas of WT and STOP KO mice. N Acc, nucleus accumbens, SNc, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulata; VTA, ventral tegmental area.

Fig. 2 Comparison of the variations of tissue DA and metabolite levels with tyrosine hydroxylase activity in DAergic areas of STOP KO mice.
Values of endogenous DA and metabolite (MET) levels and of TH activity are expressed as means ± SEM of percentages of respective values in WT mice. Fr Cx, frontal cortex; N Acc, nucleus accumbens; SN+VTA, substantia nigra + ventral tegmental area; Str, striatum.
Post hoc Fisher's test: $*$ $p < 0.05$, $**$ $p < 0.01$, comparison between DA and TH;
# $p < 0.05$, comparison with WT (100%).

Fig. 3 DA uptake in synaptosomes from the striatum and the nucleus accumbens of STOP KO versus WT mice.
Values represent means ± SEM in pmol [^{3}H]DA taken up /min /mg prot from 3 (striatum) and 6 (nucleus accumbens) independent experiments, each on pooled areas from two WT or KO mice.

Fig. 4 Effect of acute administration of cocaine on the locomotor activity of STOP KO versus WT mice.
A: Time course of the locomotor activity of WT and STOP KO mice after administration of cocaine at increasing doses. Means ± SEM of photocell counts per 5 min period. B: Dose-response effects of cocaine administration on the locomotor activity of WT and STOP KO mice. Data represent means ± SEM of photocell counts over a 50 min period for 9 WT and 10 KO at cocaine 0, 8 WT and 9 KO at cocaine 5, 8 WT and 7 KO at cocaine 10 and 7 WT and 7 KO at cocaine 25 and 40 mg/kg.
Post hoc Fisher's test: $*$ $p<0.05$, $***$ $p<0.001$, comparison with the saline group of the same genotype; ## $p<0.01$, ### $p<0.001$, comparison between genotypes.

Fig. 5 Inhibition of [^{3}H]DA uptake by cocaine in synaptosomes from the striatum and the nucleus accumbens of STOP KO versus WT mice.
Uptake of 10 nM [^{3}H]DA in the presence of cocaine at increasing concentrations. Values are expressed as % of specific DA uptake in the absence of cocaine.

Means ± SEM of 3 independent experiments, each on pooled areas from two WT or KO mice.

Fig. 6 Behavioral sensitization to cocaine of WT and STOP KO mice.
Top: Horizontal locomotor activity of WT and STOP KO mice after administration of saline (Sal) or cocaine 10 mg/kg (Coc), once daily for 5 days. After a treatment-free period of 20 days, all mice received saline at day 25 (25 Sal) and cocaine 10 mg/kg at day 26 (26 Coc). Means ± SEM of counts over 60 min from 12 WT Sal, 11 WT Coc, 12 KO Sal and 10 KO Coc. Bottom: Cumulated counts over 60 min period, after cocaine administration, at day 1 (acute administration), day 5 (end of the acquisition period) and at day 26 (expression period) for saline-pretreated or cocaine-pretreated mice.
Post hoc Fisher's test: * $p<0.05$, ** $p<0.01$, *** $p<0.01$, comparison with the saline group; # $p<0.05$, ### $p<0.001$, comparison of cocaine effect between day.

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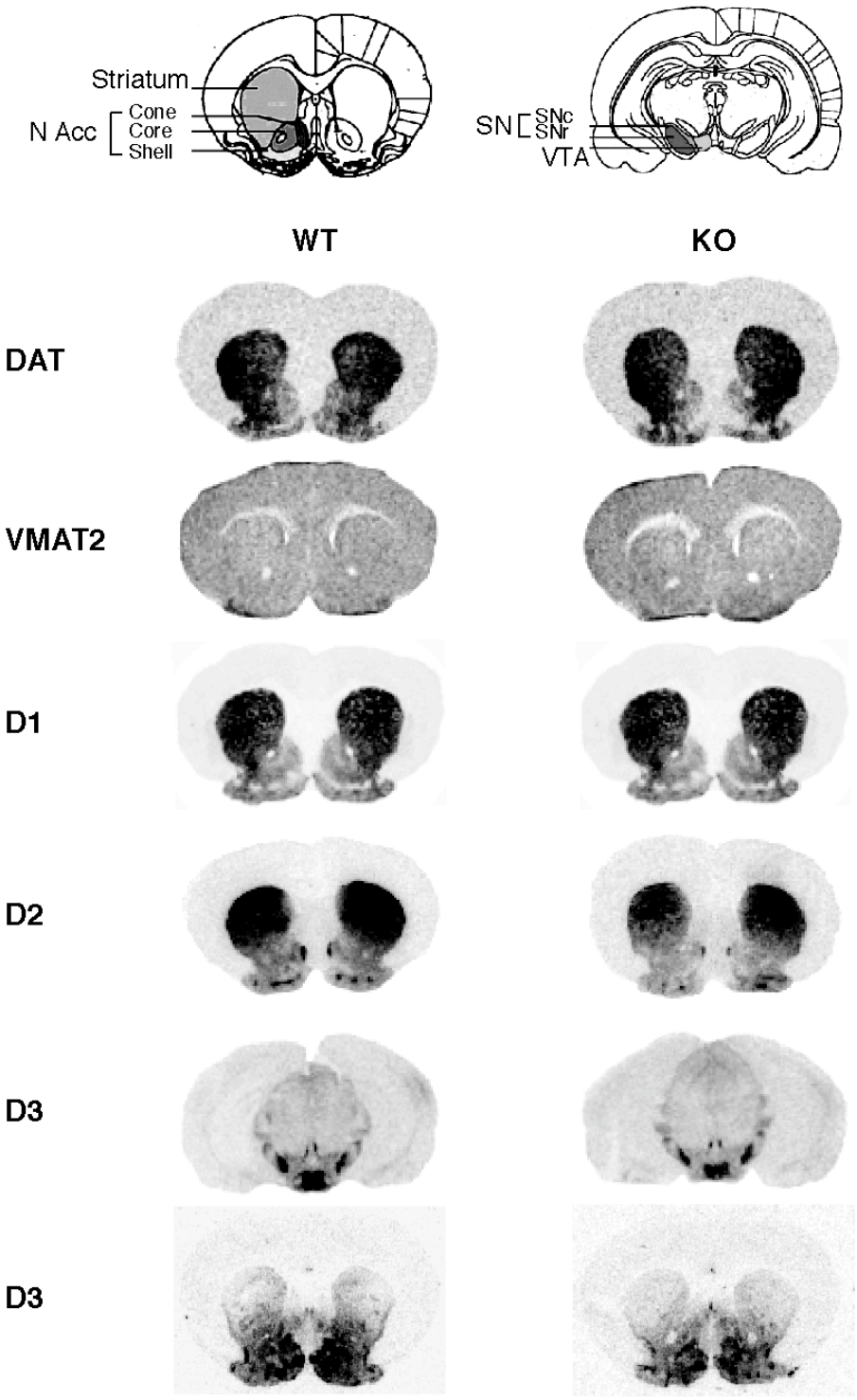


Figure 1

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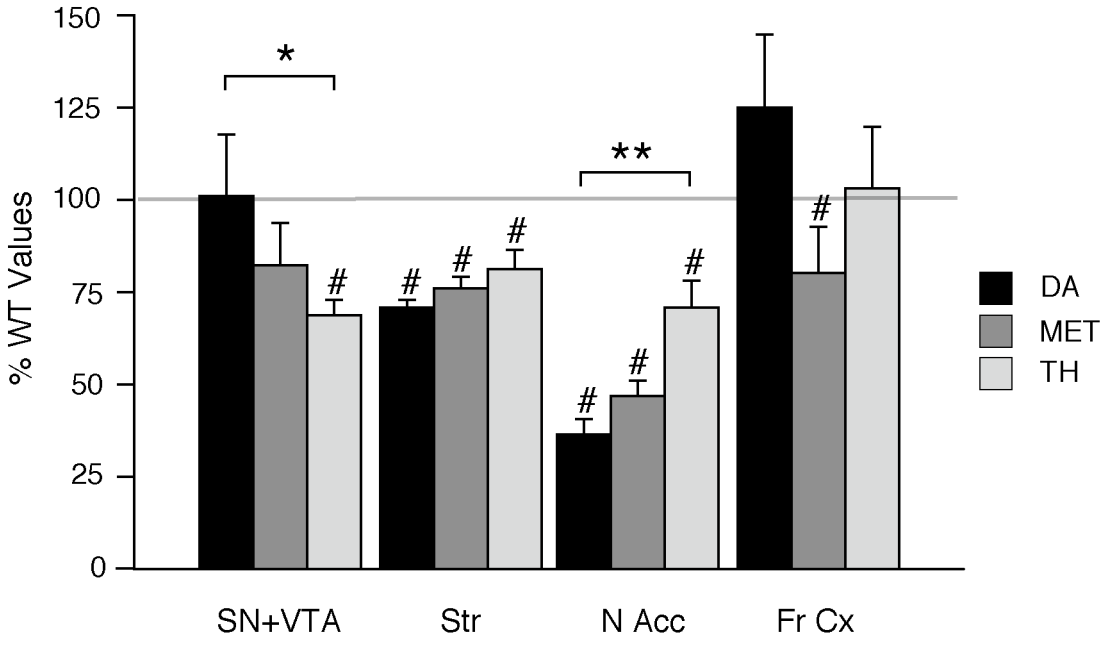


Figure 2

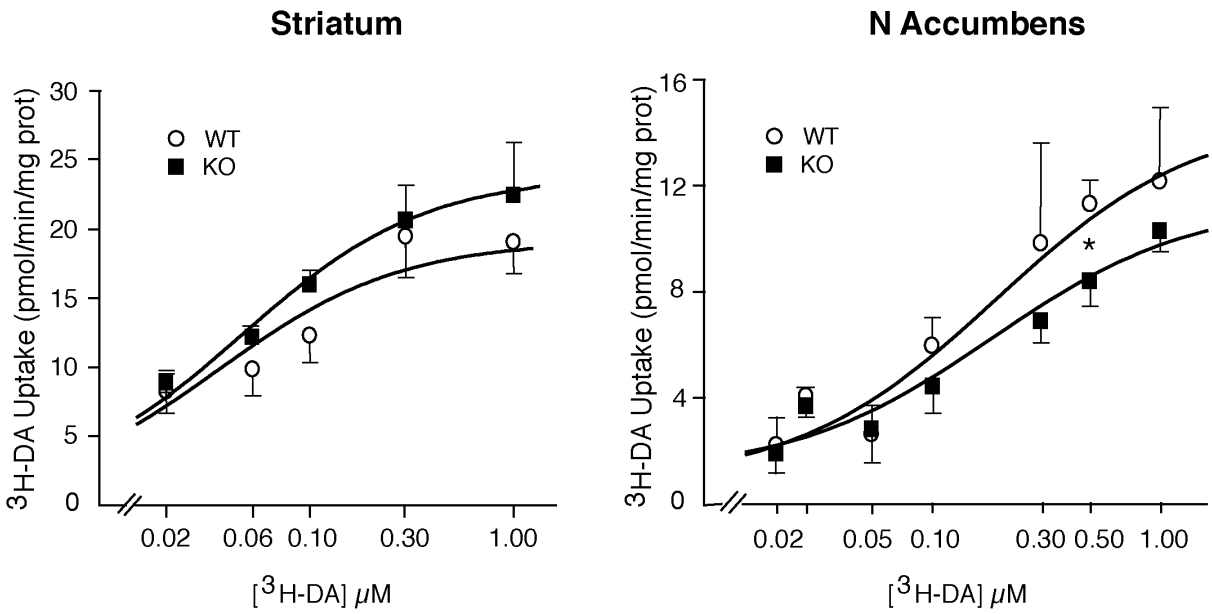


Figure 3

inserm-00375628, version 2 - 29 Apr 2009

inserm-00375628, version 2 - 29 Apr 2009

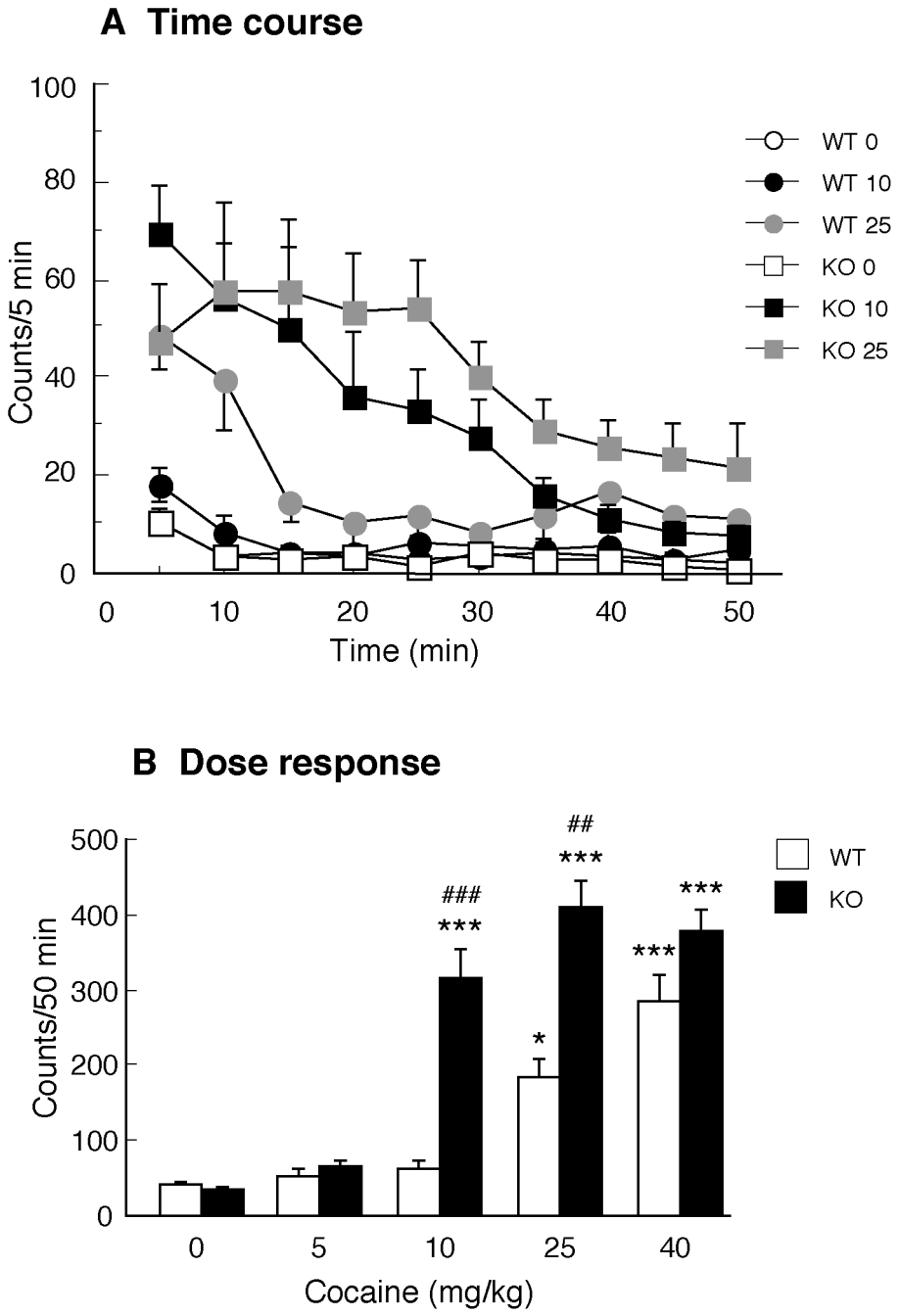


Figure 4

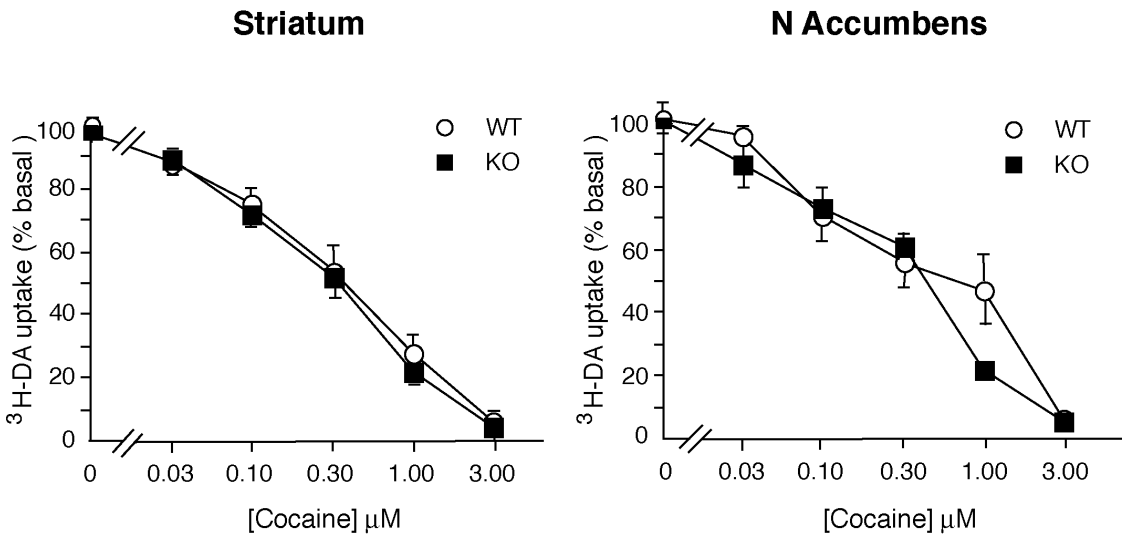


Figure 5

inserm-00375628, version 2 - 29 Apr 2009

inserm-00375628, version 2 - 29 Apr 2009

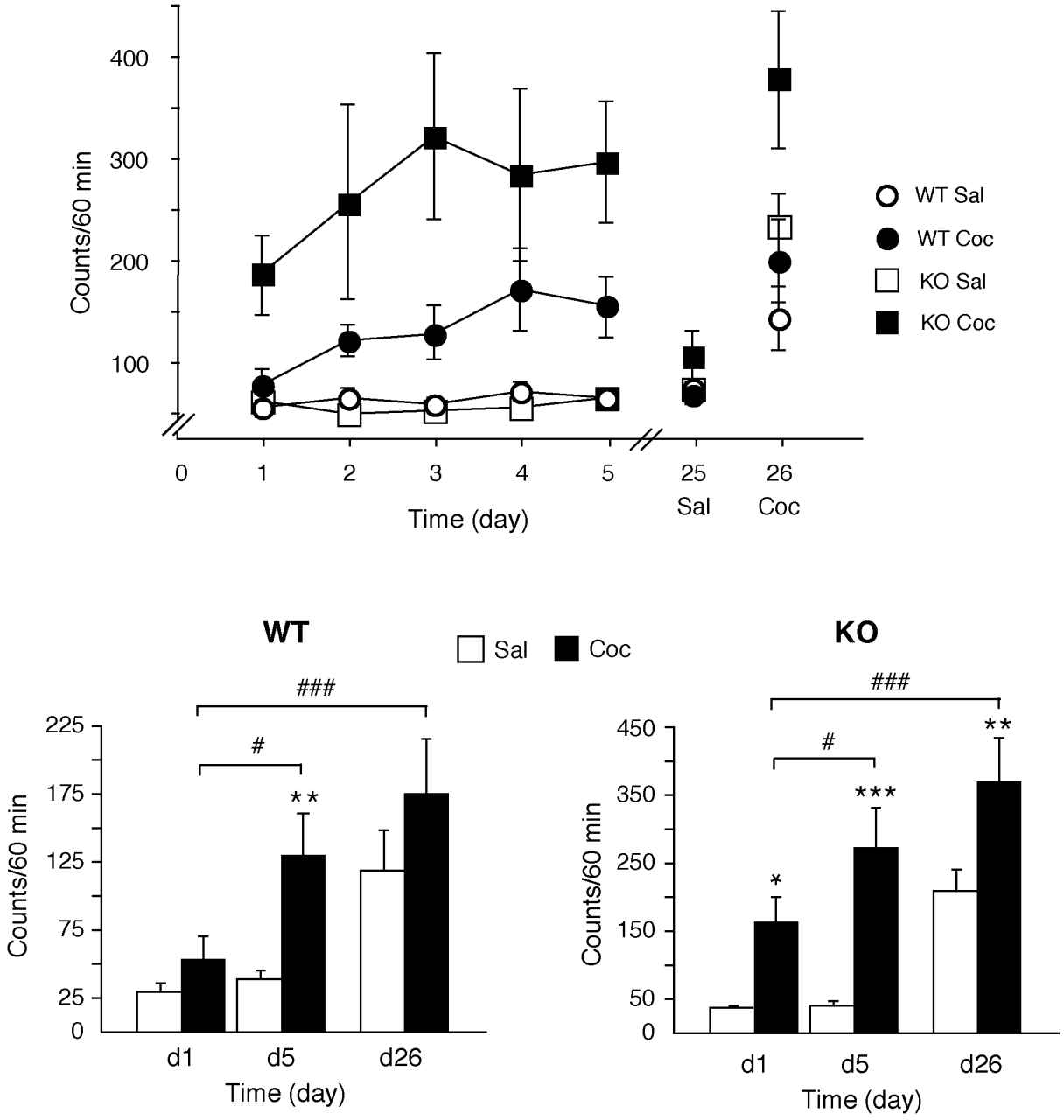


Figure 6