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Nur77 Gene Knockout Alters Dopamine Neuron Biochemical Activity and Dopamine Turnover

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Abstract

Background—Transcription factors of the Nur family (*Nurr1*, *Nur77*, and *Nor-1*) are orphan nuclear receptors closely associated with dopamine neurotransmission in the central nervous system. *Nur77* expression is strongly modulated by antipsychotic and ant-parkinsonian drugs in dopaminoceptive brain areas. However, the role of *Nur77* in dopamine neuron activity and turnover remains elusive.

Methods—We compared various behavioral and biochemical parameters between *Nur77* knockout –/– and wild-type +/+ mice in basal and haloperidol-challenged conditions.

Results—We report here that *Nur77*-deficient mice display enhanced spontaneous locomotor activity, greater sensitivity to a small dose of the dopamine D_2 receptor agonist quinpirole acting mainly at autoreceptor sites, and higher levels of the dopamine metabolite DOPAC relative to wild-type mice. Dopamine turnover disturbances are also found after acute challenge with haloperidol, a dopamine D_2 receptor antagonist. These alterations are associated with increased tyrosine hydroxylase expression and activity, and reduced catechol-O-methyltransferase expression.

Conclusion—Taken together, these results are consistent with the involvement of *Nur77* in dopamine neuron biochemical activity and dopamine turnover.

Keywords

NGFI-B; antipsychotic drug; tyrosine hydroxylase (TH); catechol-O-methyltransferase (COMT); dopamine D_2 autoreceptor; dopamine metabolism; *Nurr1*

Transcription factors represent a vast family of genes that encode regulatory factors, which modulate the expression of target genes. They play an important role during central nervous system (CNS) development and actively participate in adaptive responses after changes in the environment of neuronal cells, such as after ischemia, lesion, or denervation and after exposure to drugs that affect neurotransmitter systems in a mature CNS (Evans 2004;

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Gronemeyer et al 2004; Olson et al 1998). Recent evidence suggests that orphan receptor members of the nuclear receptor family of transcription factors, namely the *Nur* family (*Nurr1*, *Nur77* and *Nor*-1), are closely associated with dopamine (DA) neurotransmission. For example, in the absence of *Nurr1* (NR4A2), DA midbrain precursors adopt normal localization and neuronal phenotype, but fail to differentiate into DA neuron phenotype, as demonstrated by the lack of tyrosine hydroxylase (TH) expression (Zetterström et al 1997). *Nurr1* can activate the transcription of DA biosynthetic enzymes, such as TH and L-aromatic amino-acid decarboxylase (AADC) in cultured cell lines (Hermanson et al 2003; Iwawaki et al 2000; Sakurada et al 1999). In addition, *Nurr1* can modulate the expression of the DA transporter (DAT) and vesicular monoamine transporter (VMAT) (Hermanson et al 2003; Sacchetti et al 2001). Responsive elements sensitive to *Nurr1* present in those gene promoters can also represent putative targets for other *Nur* members (Maira et al 1999; Perlmann and Jansson 1995). However, *Nur77* (NR4A1) and *Nor*-1 (NR4A3) messenger RNA (mRNA) levels are extremely low in the substantia nigra (SN) and ventral tegmental area (VTA) in basal conditions (Zetterström et al 1996). On the other hand, *Nur77* and *Nor*-1 are highly expressed in target areas of DA neurons, such as the striatal complex and prefrontal cortex (Beaudry et al 2000; Werme et al 2000a; Zetterström et al 1996). In these areas, strong modulation of *Nur77* and *Nor*-1 has been observed after manipulation of DA neurotransmission with DA receptor antagonists (neuroleptics) or psychostimulants, or after DA denervation (Beaudry et al 2000; Ethier et al 2004a; St-Hilaire et al 2003a; 2003b; Werme et al 2000a; 2000b). In addition, we have shown that *Nur77*-deficient mice had an altered response after treatment with antipsychotic drugs (Ethier et al 2004a; 2004b). The cataleptic response after conventional antipsychotic drug administration, such as haloperidol, was strongly attenuated, while orofacial dyskinesias (vacuous chewing movements), which developed after prolonged treatment, were exacerbated (Ethier et al 2004a; 2004b). *Nur77* is selectively induced by antipsychotic drug treatment in striatal enkephalin-containing cells (bearing D_2 receptors) (Beaudry et al 2000). Thus, alteration of these behavioral responses after haloperidol suggests an involvement of *Nur77* in intracellular signaling events associated with the blockade of the DA D_2 receptor in a medium spiny (postsynaptic to DA cells) cell population of the striatum (Ethier et al 2004a). Interestingly, we recently observed that *Nur77* could be strongly induced in the SN/VTA complex after acute treatment with numerous antipsychotic drugs (Maheux et al 2005). This observation suggests that *Nur77* may influence DA neuron biochemical activity under specific circumstances. Taken together, these observations indicate that *Nur77* is involved in biochemical and behavioral effects of antipsychotic drugs.

To further investigate the role of *Nur77* in DA neurotransmission, we conducted a series of experiments aimed at exploring the role of *Nur77* in DA turnover and DA neuron biochemical activity. In the present study, we report that genetic ablation of *Nur77* alters multiple components related to DA neuron activity, including DA metabolites, TH and *Nurr1* levels, and TH activity, as well as catechol-O-methyltransferase (COMT) expression and autoreceptor-related locomotor behavior. In addition, basal and haloperidol-induced DA turnover are strongly altered in *Nur77*-deficient mice, suggesting a role of *Nur77* in DA clearance.

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Methods and Materials

Animals and Treatments

All procedures, including means to minimize discomfort, were reviewed and approved by the Laval University Animal Care Committee. Male wild-type C57BL/6 mice (*Nur77* +/+) were purchased from Charles River Laboratories, St-Constant, Quebec, Canada. *Nur77* knockout (*Nur77* –/–) mice were developed and graciously provided by Dr. Jeff Milbrandt from the University of Washington in St. Louis, Missouri (Lee et al 1995). These mice were healthy and reproduced normally. They were produced in a mixed background and have been backcrossed into the C57BL/6 strain for at least 10 generations to reduce genetic background heterogeneity (Jeff Milbrandt, personal communication, 2002). We maintain a *Nur77* –/– mouse colony at the animal care facility of our research center. Young adults *Nur77* +/+ and *Nur77* –/– male mice weighing 20–25 g were used for the present experiments. A group of C57BL/6 mice were purchased from Harlan (Teckland, Indianapolis, Indiana) to evaluate the contribution of the difference in the genetic background of the C57BL/6 mouse strain from Charles River, Canada, and the C57BL/6 mouse strain that has been used to backcross *Nur77* –/– mice.

Two series of *Nur77* +/+ and –/– mice were used for locomotor evaluation in basal condition and after saline or quinpirole administration. Two other groups of *Nur77* +/+ and *Nur77* –/– mice were treated with vehicle (saline) or haloperidol (.5 mg/kg intraperitoneal [IP]). One group of both strains was sacrificed 1 hour after haloperidol challenge to assess *Nur77* and *Nurr1* mRNA levels. The other groups were sacrificed 5 hours after haloperidol administration in order to evaluate the levels of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin (5-HT) and its metabolite 5-hydroxyindolacetic acid (5-HIAA), as well as TH mRNA, catechol-O-methyltransferase (COMT) mRNA and DAT-binding capacity.

For catecholamine measurements, brains were rapidly removed from the skull and placed onto a mouse brain stainless steel matrix (Plastic One Inc., Roanoke, Virginia) and sliced at three levels to isolate the frontal cortex (Bregma 2.70 mm), striatal complex (Bregma –1.20 mm) and substantia nigra/ventral tegmental area (SN/VTA) regions (from Bregma –4.50 to –6.20 mm). Note that the frontal region section mainly includes the prefrontal cortex. Then, regions of interest were dissected out from these brain pieces and immediately homogenized in .1 N perchloric acid ($HClO_4$) and frozen at –80°C until assayed.

For in situ hybridization and autoradiographic techniques, brains were rapidly removed from the skull and immediately frozen in dry ice–cooled isopentane (–40°C) and stored at –80°C until used. For immunohistochemistry, mice were deeply anesthetized with a ketamine/xylazine solution (80 and 10 mg/kg, respectively, IP) and perfused intracardially with 150 mL of saline solution (.9% NaCl), followed by 250 mL of 4% paraformaldhehyde (PFA) in .1 mol/L borax buffer (pH 9.5 at 4°C). After perfusion, the brains were removed and postfixed 90 min in 4% PFA. After postfixation, the brains were cryoprotected by an overnight immersion in 20% sucrose diluted in the fixative solution, and then frozen and stored until further processing. Frozen brains were sectioned at 30-μm with the use of a

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sliding microtome (Leica Microsystem SM200R, Richmond Hill, Ontario, Canada) in the coronal plane. Sections were collected in cold cryoprotectant solution and stored at −20°C.

Detection of DA and Its Metabolites

The concentration of DA and its metabolites, DOPAC and HVA, were evaluated by high-performance liquid chromatography (HPLC) with electrochemical detection according to previously published procedures with slight modifications (Di Paolo et al 1986; Morissette and Di Paolo 1996). Striatum extracts were homogenized at 0 to 4°C in 250 μL of .1 N $HClO_4$ and centrifuged at 10,000*g* for 10 min to precipitate proteins; SN/VTA and frontal cortex extracts were homogenized in 100 μL of .1 N $HClO_4$. The pellets were dissolved in 100 μL of .1 mol/L NaOH for the determination of protein content (Lowry et al 1951). The supernatant of striatal, SN/VTA, and frontal cortex tissues, as well as standards, were injected into a HPLC system consisting of an autosampler automatic injector (Waters 717+, Waters Ltd, Mississauga, Ontario, Canada), a pump (Waters 515) equipped with a C-18 column (Waters, Nova-Pak C_{18}, 3 μm, 3.9 × 150 mm), an electrochemical detector (LC-4C, BASi, West Lafayette, Indiana), and a glassy carbon electrode. The mobile phase consisted of .025 mol/L citric acid, 1.7 mmol/L 1-heptane-sulfonic acid, and 10% methanol, in filtered distilled water, delivered at a flow rate of .8 mL/min. The final pH of 3.85 was obtained with the addition of NaOH. The electrochemical potential was set at .8 V with respect to an Ag/AgCl reference electrode. All HPLC reagents were of analytical grade.

L-DOPA Measurement

Levels of L-DOPA were measured by HPLC with electrochemical detection. Supernatants of striatal, frontal cortex, or SN/VTA tissue were directly injected into the HPLC system, as described for DA and 5-HT measurements. The mobile phase consisted of 50 mmol/L KH_2PO_4, .2 mmol/L 1-heptane-sulfonic acid, .1 mmol/L EDTA, and 10% methanol, in filtered distilled water, delivered at a flow rate of .8 mL/min. The final pH of 2.70–2.80 was obtained by addition of phosphoric acid. The electrochemical potential was set at .8 V with respect to an Ag/AgCl reference electrode.

In Situ Hybridization Procedures

Cryostat coronal brain sections (12 μm) were mounted onto Snowcoat X-tra slides (Surgipath; Winnipeg, Manitoba, Canada) and stored at −80°C until used. Brain sections were fixed in 4% PFA at 4°C for 20 min. For single in situ hybridization, specific [^{35}S]UTP-radiolabeled complementary RNA (cRNA) probes were used. The production and synthesis of *Nur77*, *Nurr1,* and TH probes were previously described in detail (Beaudry et al 2000; Cossette et al 2004; Ethier et al 2004a). The cRNA probe for COMT was generated from a 565-bp fragment (nucleotides 155–720) from the mouse sequence (Gogos et al 1998), subcloned into PCRII-TOPO plasmid, and linearized with *Apa*I. In situ hybridization of the riboprobes with tissue sections were done at 55°C–58°C, overnight, in a standard hybridization buffer containing 50% formamide (Beaudry et al 2000; Ethier et al 2004a). Tissue sections were then apposed against BiomaxMR (Kodak; New Haven, Connecticut) radioactive sensitive films for 1–5 days. Quantification of autoradiograms was performed by means of computerized analysis (ImageJ 1.32i software, Wayne Rasband, National Institutes of Health, Bethesda, Maryland). Optical gray densities were transformed into nCi/g or μCi/g

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of tissue equivalent by using standard curves generated with ^{14}C-microscales (ARC 146A-^{14}C standards; American Radiolabeled Chemicals Inc., St. Louis, Missouri). Average levels of labelling for each area were calculated from four adjacent brain sections of the same animals. Background intensities were subtracted from every measurement.

The double in situ hybridization procedure was performed as previously described (Beaudry et al 2000; Cossette et al 2004). Briefly, the proportion of *Nur77* colocalization with TH mRNA in vehicle- and haloperidol-treated animals was evaluated by means of double in situ hybridization with a [^{35}S]UTP-labeled *Nur77* probe and a nonradioactive digoxygenin-labeled TH probe. Single- or double-labeled cells were visualized and manually counted under bright-field illumination with a Zeiss photomicroscope (Zeiss, Toronto, Ontario, Canada) at a magnification of ×400. Cell counts were performed on four different sections obtained from a total of five animals per group investigated.

Immunohistochemistry of the COMT Protein

Free-floating sections from wild-type and *Nur77* −/− mice were washed three times in phosphate-buffered saline (PBS) .1 mol/L for 10 min before a 30-min incubation in H_2O_2 1%. Sections were washed again three times in PBS .1 mol/L and then preincubated with a solution of PBS (pH 7.4) containing 5% normal horse serum and .1% Triton X-100. Sections were incubated overnight at 4°C in the solution containing the mouse monoclonal anti-COMT antibody (1:2000; BD Transduction Laboratories, Missisauga, Ontario, Canada) in PBS. Tissues were rinsed several times in PBS and then incubated for 1 hour at room temperature with biotinylated horse immunoglobulin G. Then, the sections were incubated for 1 hour in 2% avidin-biotin complex (ABC; Vector Laboratories, Burlingame, California). Finally, after the sections were washed three times in PBS, bound peroxidase was revealed by placing the sections in a solution containing .05% 3,3-diaminobenzidine tetrahydrochloride (Sigma) and .0018% H_2O_2 (30%) in PBS at room temperature. After less than 10 min, the reaction was stopped with three rinses in PBS, The sections were mounted, dehydrated, and coverslipped with DPX mounting medium for microscopy (Electron Microscopy Science, Washington, Pennsylvania). To determine the specificity of the signal, sections were treated as described above, but the primary antibody was omitted in the incubation solution.

Dopamine Transporter Binding Capacity

Labeling of the DAT was performed with the [^{125}I]RTI-121 ligand (20 pmol/L) (Boja et al 1995). Specific binding was obtained by subtraction of the nonspecific binding obtained in the presence of 5 μmol/L mazindol. Incubation buffer was composed of Na_2HPO_4 10 mmol/L, KH_2PO_4 1.8 mmol/L, NaCl 137 mmol/L, and KCl 2 mmol/L at pH 7.5. Slides were preincubated in the phosphate buffer for 30 min. Then, the buffer containing the ligand was placed on slides and incubated for 1 hour at room temperature. The slides were rinsed in the same buffer at 4°C, two times for 20 min. Finally, the slides were immersed for 5 sec in water to remove salts. After drying, the slides were apposed against Kodak film for 24 hours.

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Behavioral Testing

The DA D_2 receptor is present at both pre and postsynaptic location. At the presynaptic site, it serves as an autoreceptor and is involved in the control of DA synthesis and release, and DA neuron firing. We have used a low dosage of quinpirole (.025 mg/kg IP), which is thought to act on D_2 autoreceptors located at axon terminals and somatodendritic area (Egan et al 1996a; Eilam and Szechtman 1989). Locomotor activity was evaluated with the use of a Flex Field photobeam activity system (San Diego Instruments Inc., San Diego, California). For basal locomotor activity, *Nur77* +/+ and –/– mice were placed in the Flex apparatus, and horizontal locomotor activity was monitored for a period of 2 hours. The number of beams interrupted for the first and the second hour of monitoring was summarized to represent the total horizontal locomotor activity of the mice for this time interval. For the effect of quinpirole on locomotor activity, *Nur77* +/+ and –/– mice were placed in the Flex chamber for a 30-min adaptation. Then, the animals were injected with the drug (in .25 mL IP) or its vehicle (saline) and immediately put back in the Flex apparatus for an additional 90 min. Photobeam crossing counts were computed every 2 min.

Statistical Analysis

All data were expressed as group mean ± SEM, and statistical analysis was performed with the "StatView" (SAS Institute, Cary, North Carolina) program. Statistical analysis was performed using a Student *t* test when *Nur77* +/+ and –/– mouse genotypes were compared. When a treatment was introduced, a two-factor (2 × 2) analysis of variance (ANOVA) (genotype [*Nur77* +/+ vs. –/–] × treatment [vehicle vs. haloperidol or benserazide]) was performed. For post hoc analysis, a least significant difference Fisher's test was used to allow multiple comparisons of means when a significant ANOVA analysis was reported ($p < .05$).

Results

Nur77 Knockout –/– Mice Are Spontaneously Hyperactive

Nur77 –/– mice displayed an apparent normal phenotype. They were healthy and reproduced normally (Ethier et al 2004a; 2004b; Lee et al 1995). However, quantitative assessment of locomotor activity indicated that *Nur77* –/– mice had a higher locomotor activity when placed in a novel environment relative to *Nur77* +/+ mice (Figure 1). *Nur77* –/– mice displayed about two- to threefold higher locomotor activity during the first hour in the open field apparatus. This difference was maintained during the second hour of measurement (Figure 1). Both *Nur77* +/+ and –/– mice showed a similar reduction of their locomotor activity during the second hour of evaluation that could be attributed to environmental adaptation. Since *Nur77* –/– mice had been backcrossed into a C57BL/6 mouse strain from a United States supplier (Harlan), whereas the C57 strain used here as controls were obtained from Charles River, Canada, we compared locomotor activity of C57 mice from both origins to evaluate the potential effect of the two different genetic backgrounds. We measured total locomotor activity of mice from both origins for 2 hours. For the first hour, total activity counts (number of beam interrupted) represented 3912 ± 174 and 4953 ± 454 for the Charles River and Harlan mice, respectively ($N = 10$, $p = .7363$, *ns*). For comparison, *Nur77* –/– mice displayed an activity count of 9130 ± 961 (Figure 1) for

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the same period. This suggests that putative genetic background differences of these two strains of C57 mice had no significant impact on the locomotor phenotype displayed by the *Nur77* –/– mice backcrossed into C57 genetic background.

A Presynaptic Mechanism Contributes to Enhanced Locomotion of *Nur77* –/– Mice

To test if DA neuron activity is associated with the locomotor hyperactivity displayed by *Nur77* –/– mice, we performed a behavioral assessment of DA autoreceptors with the use of a low dose of a DA D_2 agonist. A low dose of quinpirole has been shown to selectively activate autoreceptor functions (Eilam and Szechtman 1989; Schmitz et al 2003). The selective activation of D_2 autoreceptors reduced spontaneous locomotor activity, as opposed to stimulation when postsynaptic D_2 receptors are targeted (Eilam and Szechtman 1989; Schmitz et al 2003). As previously reported, administration of quinpirole (.025 mg/kg) reduced locomotor activity in both *Nur77* +/+ and –/– mice (Figure 2). The effect of this low dose of quinpirole had a short duration of action (Eilam and Szechtman 1989). Thus, a 30-min interval after administration of the drug was selected for further analysis. Quinpirole effect was about twofold stronger in *Nur77* –/– mice relative to +/+ mice (Figure 2, inset). During this interval, quinpirole reduced *Nur77* +/+ mice locomotor activity by 27% (from 49.5 ± 7.8 to 23.6 ± 3.4% on average; Figure 2, inset), whereas the same treatment reduced locomotor activity by 55% in *Nur77* –/– mice (from 78.7 ± 11.4 to 22.1 ± 2.8%; Figure 2, inset). This finding indicated that *Nur77* –/– mice are more sensitive to a low dose of quinpirole. However, D_2 receptor densities measured with [^{125}I]iodosulpride binding in the SN and VTA were similar in *Nur77* +/+ and –/– mice (SN: *Nur77* +/+ = .34 ± .03 and *Nur77* –/– = .37 ± .04 optical density [OD]; VTA: *Nur77* +/+ = .32 ± .03 and *Nur77* –/– = .34 ± .03 OD), as opposed to our previously reported levels for postsynaptic D_2 receptor binding in the dorsolateral striatum (Ethier et al., 2004b). However, we cannot rule out the possibility that D_2 autoreceptor activity might have been altered.

Tyrosine Hydroxylase and *Nurr1* mRNA Levels Are Upregulated in *Nur77* –/– Mice

The previous result suggests that DA neuron activity may be altered in *Nur77* –/– mice. To further characterize the role of *Nur77* on DA neuron activity, we measured TH and *Nurr1* mRNA levels in *Nur77* +/+ and *Nur77* –/– mice in the substantia nigra pars compacta (SNpc) and VTA (Figure 3). Although the magnitude of the effect was low, *Nur77* –/– mice showed a significant increase of TH mRNA levels in the SNpc, whereas they remained unchanged in the VTA (Figure 3A). Since *Nurr1* is an important transcription factor related to the DA neuron phenotype, the levels of *Nurr1* mRNA were compared in both mouse strains. Interestingly, *Nurr1* mRNA levels were significantly higher in both SNpc and VTA in *Nur77* –/– mice, compared with +/+ mice (Figure 3C).

Since the DAT represents a putative target of *Nur*-dependent transcriptional activity (Hermanson et al 2003; Sacchetti et al 2001), we also compared DAT binding capacity in *Nur77* +/+ and –/– mice. The density of DAT binding sites was evaluated in the SNpc, VTA, and striatal sections. The binding levels of DAT were assessed with the [^{125}I]RTI-121 ligand (Boja et al 1995). High levels of DAT were found in the striatum, whereas lower DAT levels were measured in DA neuron areas (SNpc and VTA) (Figure 3B). Specific [^{125}I]RTI binding

indicated no significant modulation of DAT binding capacity in the striatum, SNpc, and VTA in *Nur77* −/−, compared with +/+ mice.

TH Activity Is Elevated in *Nur77* −/− Mice

We compared TH activity between *Nur77* +/+ and −/− mice by measuring L-DOPA accumulation in the presence of an L-aromatic amino-acid decarboxylase (AADC) inhibitor, benserazide. Although benserazide is considered to be a peripheral AADC inhibitor, it can significantly reduce striatal DA levels at high concentrations (Jonkers et al 2001). However, in the present paradigm which uses HPLC coupled with electrochemical detection of L-DOPA, systemic administration of benserazide (50 mg/kg) did not significantly alter striatal DA levels (data not shown). However, it reduced DA levels in the frontal cortex of *Nur77* +/+ (47% reduction compared with vehicle-treated mice) and strongly increased L-DOPA levels in all the brain areas investigated (Figure 4). Interestingly, benserazide administration induced significantly higher L-DOPA accumulation in striatal and frontal cortex tissue homogenates in *Nur77* −/− relative to +/+ mice (Figure 4). These results suggest that the TH activity is elevated in the *Nur77* −/− relative to +/+ mice.

DA Metabolite DOPAC, But Not HVA, Is Elevated in *Nur77* −/− Mice

We evaluated the level of DA and its main metabolites DOPAC and HVA in the striatum, SN/VTA, and frontal cortex regions in *Nur77* +/+ and −/− mice using a highly sensitive HPLC method combined with electrochemical detection. The levels of DA were similar in *Nur77* +/+ and −/− mice in all the brain regions investigated (Table 1). However, *Nur77* −/− mice had higher levels (two- to threefold) of the DA metabolite DOPAC in the SN/VTA and frontal cortex relative to *Nur77* +/+ mice, whereas HVA levels remained similar (Table 1). The DA levels were 10–20 times higher in the striatum than in the SN/VTA area and frontal cortex, whereas relative proportions of DA metabolites DOPAC and HVA were much higher in these latter structures (Table 1). Thus, the ratios of DA metabolite levels over DA contents (DOPAC/DA and HVA/DA), which are indications of DA turnover (Sharman 1985), were about five times higher in the SN/VTA area and frontal cortex, compared with the striatum (Table 1). This observation indicates that DA turnover was higher in those brain regions. Interestingly, the DOPAC/DA ratio was elevated and HVA/DA ratio was reduced in SN/VTA, whereas the DOPAC/DA ratio was increased in the frontal cortex of *Nur77* −/− mice. The ratios DOPAC/DA and HVA/DA were not altered in the striatum o f *Nur77* −/− mice (Table 1). Overall, these results suggested that DA turnover was increased in *Nur77* −/− mice. However, this increased metabolism was not reflected at HVA levels, suggesting that conversion of DOPAC into HVA was somehow disrupted.

COMT Expression Is Reduced in *Nur77* −/− Mice

Since DOPAC conversion into HVA seemed to be altered in *Nur77* −/− mice, we investigated the enzymatic pathway involved. The enzyme responsible for the conversion of DOPAC into HVA is COMT. Two distinct promoters that generate a short mRNA coding for a soluble isoform and a long mRNA yielding both soluble and membrane-bound isoforms control the expression of the COMT gene (Mannisto and Kaakkola 1999). The soluble isoform is mainly expressed by cell-surrounding neurons such as astrocytes, and the membrane isoform is expressed in postsynaptic neurons to DA cells (Matsumoto et al 2003).

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The COMT mRNA probe we have designed in the present study detected both mRNA species. Thus, total COMT mRNA levels were compared between both mouse strains. *Nur77* –/– mice displayed reduced COMT mRNA levels in all areas investigated (Figure 5A and 5B). We confirmed the modulation of the COMT expression using immunohistochemistry labeling of the COMT protein with a specific antibody (Figure 5C). We observed a clear reduction of COMT immunostaining in the *Nur77* –/– mice relative to *Nur77* +/+ mice in all the regions investigated (prefrontal cortex, striatum, and nucleus accumbens).

In addition, the 3-methoxy-4-hydroxy-phenylethylamine (3-MT) metabolite level that is produced from the direct activity of the COMT enzyme on DA at postsynaptic sites (membrane-bound isoform) was reduced in striatal tissues of *Nur77* –/– mice, compared with *Nur77* +/+ mice (*Nur77* +/+: 5.05 ± 1.23 ng/mg protein; *Nur77* –/–: 3.44 ± 1.37 ng/mg protein, $N = 5$, $p < .05$). However, we were not able to evaluate the levels of 3-MT in other areas (SN/VTA and frontal cortex), because of resolution problems (unknown peak detection interferences) in our HPLC detection conditions and because we did not treat the mice with pargyline to inhibit the rapid catabolism of 3-MT (Egan et al 1996a).

The Effect of Haloperidol on DA Turnover Is Altered in *Nur77* –/– Mice

We have previously shown that haloperidol, a typical antipsychotic drug, strongly increases *Nur77* expression in both the striatal complex and prefrontal cortex (Beaudry et al 2000; Ethier et al 2004a). It has previously been observed that acute haloperidol administration alters DA neuron functions (Egan et al 1996b). Thus, haloperidol, a DA D_2 receptor antagonist, was used to acutely challenge the DA system to compare adaptive capacities of *Nur77* +/+ and –/– mice. Acute blockade of D_2 autoreceptors by haloperidol caused a transient increase of the DA turnover (Figure 6). In the present paradigm, DA turnover was evaluated 5 hours after haloperidol administration. In this time frame and the method used for measurements (HPLC on tissue homogenates), DA levels reflect total DA contents (intra- and extracellular) and modulations of DA levels would therefore reflect modulation of DA synthesis instead of DA release. Indeed, the absolute level of DA in the striatum was not elevated after this acute haloperidol challenge in *Nur77* +/+ mice. In fact, DA levels were reduced after haloperidol administration (Figure 6, left panels). Interestingly, this reduction was not observed in *Nur77* –/– mice. Haloperidol administration increased DOPAC and HVA levels in the striatum of *Nur77* +/+ mice, indicating that haloperidol increased DA turnover. However, a stronger effect of haloperidol was observed on striatal DOPAC levels in *Nur77* –/– mice relative to +/+ mice, whereas haloperidol-induced HVA levels were similar in both strains (Figure 6, left panels). Consequently, the DOPAC/DA ratio was higher and HVA/DA ratio was lower after haloperidol challenge in *Nur77* –/– mice relative to +/+ mice.

In the SN/VTA area, we did not detect any variation of DA levels after haloperidol treatment (Figure 6, middle panels). However, the elevation of DOPAC levels seen after haloperidol administration was higher in *Nur77* –/– mice (Figure 6, middle panels), as we observed in the striatum. On the other hand, the effect of haloperidol on HVA levels was reduced in the SN/VTA area of *Nur77* –/– mice (Figure 6, middle panels). Consequently, the effect of haloperidol on DOPAC/DA and HVA/DA ratios were strongly altered in SN/VTA areas of

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Nur77 −/− mice. This discrepancy in the effect of haloperidol on DOPAC and HVA levels is consistent with the results obtained in basal conditions (Table 1).

In frontal cortex tissues, a significant elevation of DA levels was detected after haloperidol administration in *Nur77* +/+ mice (Figure 6, right panels). In *Nur77* −/− mice, this effect was strongly attenuated (Figure 6, right panels). The effects of haloperidol on DOPAC and HVA levels were also reduced in the frontal cortex of *Nur77* −/− relative to +/+ mice. However, the lower effect of haloperidol on DA metabolite levels may be related to a reduced effect of the drug on DA contents observed in the frontal cortex of *Nur77* −/− mice. Elevated DOPAC/DA and HVA/DA ratios in the frontal cortex after haloperidol indicated that the drug challenge increased DA turnover in *Nur77* +/+ mice (Figure 6, right panels). However, in *Nur77* −/− mice, DA turnover was on the contrary reduced, as suggested by the lower HVA/DA ratio (Figure 6, right panels). Again, this indicates a discrepancy between respective DOPAC/DA and HVA/DA ratios, suggesting an altered DOPAC to HVA conversion in the frontal cortex of *Nur77* −/− mice.

Contents of 5-HT and its metabolite 5-HIAA were similar in *Nur77* −/− mice relative to +/+ mice in the three brain areas investigated (see Supplement 1). Levels of 5-HT and its metabolite 5-HIAA were not significantly modified by haloperidol in the striatum and SN/VTA area. However, haloperidol administration increased 5-HT and 5-HIAA contents in the frontal cortex of *Nur77* +/+ mice. But, this effect was not observed in *Nur77* −/− mice (see Supplement 1).

Blockade of D_2 Receptors Induces *Nur77* mRNA Expression in Midbrain DA Cells

The results gathered so far indicate that *Nur77* deficiency or overexpression (after haloperidol) modulated DA turnover in target regions, such as the striatum and frontal cortex. *Nur77* modulations also altered DA neuron activity and turnover in the SN/VTA. However, under basal conditions, *Nur77* is barely detectable in DA neurons (Maheux et al 2005), suggesting that these effects in the SN/VTA may be driven by a feedback mechanism from DA targets areas, where *Nur77* is normally expressed. We recently showed that *Nur77* was induced in SN and VTA by numerous typical and atypical antipsychotic drugs (Maheux et al 2005). However, we did not examine colocalization with TH-expressing cells. As previously shown (Maheux et al 2005), haloperidol induced *Nur77* mRNA expression in SN/VTA areas (Figure 7A and 7B). Double in situ hybridization with *Nur77* and TH probes revealed that haloperidol increased both the number of cells expressing *Nur77* in the SN and VTA (from 0 to about 50 cells/.25 mm^2; Figure 7C and 7D), and the percentage of colocalization of *Nur77* and TH transcripts (nearly 100%) (Figure 7E). Interestingly, haloperidol-induced upregulation of *Nur77* mRNA levels was almost exclusively observed in TH-expressing cells of the SNpc and VTA (Figure 7C, 7D, and 7E). Thus, after blockade of DA receptors by haloperidol (1 hour after drug administration), activation or derepression of a signaling cascade leads to increased expression of *Nur77* in DA neurons. This observation indicates that *Nur77* may directly alter DA neuron biochemical activity in certain circumstances.

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Discussion

Taken together, the present results indicate that genetic ablation of *Nur77* alters DA neuron biochemical activity and DA turnover. This phenotype may be generated, at least in part, by a compensatory response resulting from the missing signaling events associated with *Nur77* expression in the striatal complex and frontal cortex. *Nur77* −/− mice displayed higher locomotor activity, compared with *Nur77* +/+ mice, and are more sensitive to a small dose of a DA agonist selective for DA autoreceptors. In addition, *Nur77* −/− mice expressed higher TH and *Nurr1* mRNA levels, enhanced DOPAC metabolite contents in SN/VTA and frontal cortex, and elevated L-DOPA accumulation after administration of an AADC inhibitor. Taken together, these observations strongly suggest that DA neuron biochemical activity (TH) is enhanced in *Nur77* −/− mice. Moreover, *Nur77* can be induced in TH-containing cells of the SN and VTA after haloperidol administration in wild-type mice, which suggests that *Nur77*-dependent transcriptional activity may occur, along with *Nurr1*, in DA neurons and may influence their activity under specific conditions. The present study also demonstrates that *Nur77* is involved in the control of DA clearance, especially in areas where DA reuptake is less important, such as the SN and prefrontal cortex, by modulating the expression of the catabolic enzyme COMT.

Because *Nur77* is normally expressed at postsynaptic sites in a basal condition, one of the most unexpected observations of the present study was the alteration of DA turnover in *Nur77* −/− mice. Both basal and haloperidol-induced DOPAC levels were higher in *Nur77* −/− relative to wild-type mice; however, the discrepancy between DOPAC and HVA levels (both in basal and haloperidol-challenged conditions) was especially intriguing. Normally, high levels of DOPAC should have generated higher HVA contents (Nissbrandt et al 1989; Zetterström et al 1984), which suggests that the conversion of DOPAC into HVA might be altered in *Nur77* −/− mice. This observation is reminiscent of the effect of COMT gene knockout, which induces DOPAC accumulation from the activity of monoamine oxydase, whereas no HVA is produced (Huotari et al 2002). In accordance with these observations, reduced COMT mRNA and immunostaining levels were observed in *Nur77* −/− mice, suggesting that blunted HVA production may be related to a reduced expression of the COMT enzyme. However, this reduction of COMT expression was found throughout the brain, suggesting that both neuronal (membrane-bound isoform) and nonneuronal (soluble isoform) COMT expression might have been altered by the knockout. Indeed, COMT is expressed in both neurons and type 2 astrocytes (Karhunen et al 1995), and *Nur77* can be induced in type 2 astrocytes by various stimuli (Hung et al 2000). Reduced 3-MT contents in the striatal complex also reinforces the possibility that the membrane-bound isoform of the enzyme is altered in *Nur77* −/− mice. There is no apparent *Nur* responsive element in the COMT promoter. However, COMT expression can be modulated by glucocorticoids (Lindley et al 2005). Since *Nur77* can interfere with glucocorticoid-dependent transcriptional activity (Philips et al 1997), it suggests that the modulation of COMT expression in *Nur77* −/− mice could be driven from an alteration of the *Nur77*-glucocorticoid receptor complex activity.

On the other hand, haloperidol increased DA levels in the frontal cortex of *Nur77* +/+ mice as previously reported (Li et al 2005), but this effect is blunted in *Nur77* −/− mice. Since

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COMT is mandatory for DA clearance in the frontal cortex (Bilder et al 2004), one might expect that reduced COMT levels would have instead exacerbated haloperidol effects on DA levels in *Nur77* −/− mice. This discrepancy might be explained by the presence of an additional alteration at monoamine synthesis that alters DA content in the frontal cortex of *Nur77* −/− mice. Indeed, increased TH and *Nurr1* mRNA levels and TH activity, in addition to increased DA turnover in SN/VTA area, suggest that DA neuron activity might be enhanced in *Nur77* −/− mice. Thus, it is possible that this effect may prevent further modulation of DA levels, as normally produced by haloperidol administration. Another possibility might have been a modulation of the norepinephrine transporter (NET) level in the prefrontal cortex, because NET can uptake DA in some circumstances (Carboni and Silvagni 2004). However, this effect is unlikely, because *Nur77* −/− showed similar NET binding capacity in the prefrontal cortex relative to *Nur77* +/+ mice (see Supplement 1). Taken together, these results suggest that the mesocortical DA pathway is particularly affected by *Nur77* gene ablation, which may be related to the distinct regulation of DA neurotransmission found in the mesocortical pathway relative to the nigrostriatal pathway (Schmitz et al 2003). Indeed, in the striatum, DA levels are under the control of DA autoreceptors located on both axon terminals and somatodendritic areas. In addition, synaptic DA contents are tightly regulated by the DAT activity in the striatal complex. At the level of the mesocortical pathway, axon terminals do not control DA activity, and DA synaptic clearance is not driven by the DAT. The present results suggest that DA neurotransmission is not properly regulated in *Nur77* −/− mice. This property mainly affects DA levels in the frontal cortex, because *Nur77* mainly alters enzymatically driven DA turnover instead of DAT expression.

The biochemical activity of DA cells seems to be enhanced in *Nur77* −/− mice, as illustrated by increased *Nurr1* and TH mRNA levels, and enhanced TH activity and elevated DA turnover, as estimated by the DOPAC/DA ratio. Tyrosine hydroxylase is the rate-limiting enzyme that produces DA, and *Nurr1* is essential for the development of TH-positive cells of the SN (Zetterström et al 1997). We observed a concomitant increase of *Nurr1* and TH mRNA levels, suggesting that modulation of DA neuron activity observed in *Nur77* −/− mice may be an indirect consequence resulting from the modulation of *Nurr1* expression. On the other hand, no modulation of DAT levels, which is also a putative target for *Nurr1* transcriptional activity (Sacchetti et al 2001), is observed in *Nur77* −/−.

Another interesting observation is the induction of *Nur77* into TH-positive cells of the SN/VTA. These cells already express high basal levels of *Nurr1* (Zetterström et al 1996). In fact, a single dose of haloperidol or other antipsychotic (Maheux et al 2005) induced very strong *Nur77* mRNA levels that are about tenfold higher relative to *Nurr1* mRNA levels. This observation suggests the possibility that *Nur77* may alter *Nurr1*-dependent transcriptional activity in DA cells. The molecular and structural organization of *Nur77* is very close to *Nurr1,* and it has been reported that both can interact with the same responsive elements (Maira et al 1999; Paulsen et al 1995; Perlmann and Jansson 1995). Thus, we might expect that induction of *Nur77* would enhance *Nur*-dependent transcriptional activity in DA cells. However, further identification of targeted genes of *Nur*-dependent transcriptional activity in DA cells in vivo will be required to test this hypothesis.

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The present data raise another important issue regarding antipsychotic drug actions. Indeed, *Nur77* −/− mice displayed an enhanced autoreceptor-driven locomotor activity accompanied by an altered DA metabolites response after haloperidol treatment. Furthermore, this antipsychotic also induced *Nur77* expression in the SN/VTA (present data and (Maheux et al 2005). This observation suggests that some alterations previously observed with *Nur77* −/− mice after antipsychotic drug treatment might have been driven, at least in part, by alteration of DA neuron biochemical activity. Such responses included a blunted cataleptic and exacerbated dyskinetic responses after haloperidol treatment (Ethier et al 2004a; 2004b). Interestingly, it has been previously proposed that dyskinesias might be related to the activity of DA neurons (Garris et al 2003).

In summary, the present study strongly suggests that *Nur77* gene knockout alters DA neuron biochemical activity and DA turnover. Given the importance of COMT for the control of DA clearance in the prefrontal cortex, *Nur77* may then represent an important component of the regulation of DA neurotransmission in this brain region. Indeed, many aspects of the phenotype displayed by *Nur77* −/− mice, such as spontaneous locomotor hyperactivity, enhanced DA neuron activity and perturbations of DA turnover in the prefrontal cortex are reminiscent of alterations associated with psychotic symptoms. Indeed, we have previously shown that *Nur77* expression is reduced in the prefrontal cortex of adult rats bearing a neonatal ventral hippocampus lesion, a model that reproduces some abnormal behaviors associated with schizophrenia (Bhardwaj et al 2003). These observations stress for further examinations of the transcriptional activity of *Nur77* associated with DA neurotransmission and reinforce the relationship that we, and others, previously illustrated between the *Nur* family of transcription factors and DA systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparison of the total cumulative horizontal locomotor activity in wild-type (*Nur77* +/+) and *Nur77*-deficient (*Nur77* −/−) mice. *Nur77* +/+ and −/− mice were placed individually in plexiglas boxes connected to a FlexField monitoring system, and locomotor activity was measured for a total of 2 hours. Histogram bars represent means ± SEM of total cumulative horizontal locomotor activity expressed in the number of beams interrupted for the first- and second-hour periods (***$p < .0001$ vs. *Nur77* +/+ mice [$N = 6$]).

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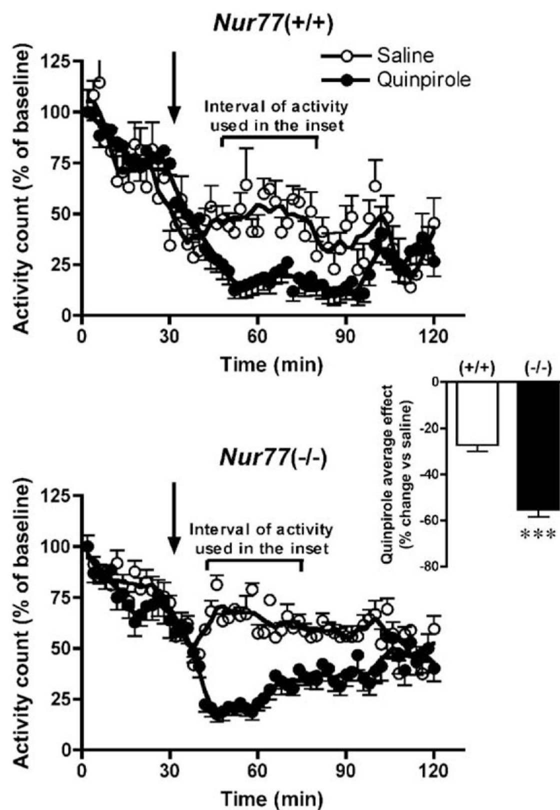

Figure 2.

Comparison of the effect of a low dose of quinpirole (.025 mg/kg) on locomotor activity between wild-type (*Nur77* +/+) (top panel) and *Nur77*-deficient (*Nur77* –/–) mice (bottom panel). Activity counts were measured and averaged at every 2-min interval for a total duration of 120 min. All 2-min activity counts were then averaged from 6 animals in *Nur77* +/+ and 10 animals for *Nur77* –/– mice. Activity counts were then transformed into percent of the baseline activity of the first 2-min interval (baseline activities were: *Nur77* +/+ = 303.4 ± 17.2 activity counts; *Nur77* –/– = 398.2 ± 41.8 activity counts, $p < .05$) and curves were smoothed by successively averaging nine neighbor values. The arrows indicate the time of saline or quinpirole (.025 mg/kg) injection. (Inset) Comparison of the average quinpirole effect for a 30-min interval in both mouse strains. A 30-min interval (as indicated in the figure) after saline or quinpirole administration was selected to evaluate the effect of the DA D_2 agonist on locomotor activity. *Nur77* –/– mice displayed a significant stronger locomotor activity reduction after quinpirole administration (***$p < .001$ vs. *Nur77* +/+ mice).

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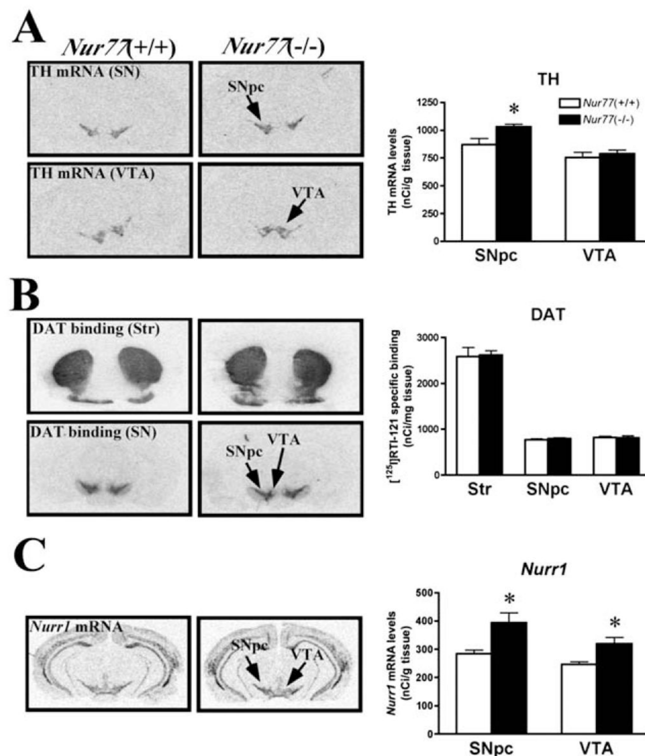

Figure 3.

Comparison of dopamine (DA) neuron markers in *Nur77* +/+ and −/− mice. Autoradiograms were generated with a specific [^{35}S]UTP-labeled probe for tyrosine hydroxylase (TH) mRNA **(A)**, DA transporter (DAT) binding capacity using [^{125}I]RTI-121 specific binding **(B)**, and *Nurr1* mRNA **(C)** after in situ hybridization in mice brain sections from wild-type (*Nur77* +/+) and *Nur77*-deficient (*Nur77* −/−) mice. We performed the analysis in the substantia pars compacta (SNpc)and ventral tegmental area (VTA) for TH and *Nurr1* mRNA, and in the SNpc, VTA, and striatum for the DAT. Left panels show representative examples of autoradiograms generated from respective DA neuron markers; right panels show quantification of the signals. Each histogram bar represents a mean ± SEM from 8–10 animals. Values are expressed in nanocuries per gram of tissue for TH and *Nurr1* mRNA levels, and in nanocuries per milligram of tissue for DAT binding (*$p < .05$ vs. *Nur77* +/+ respective group).

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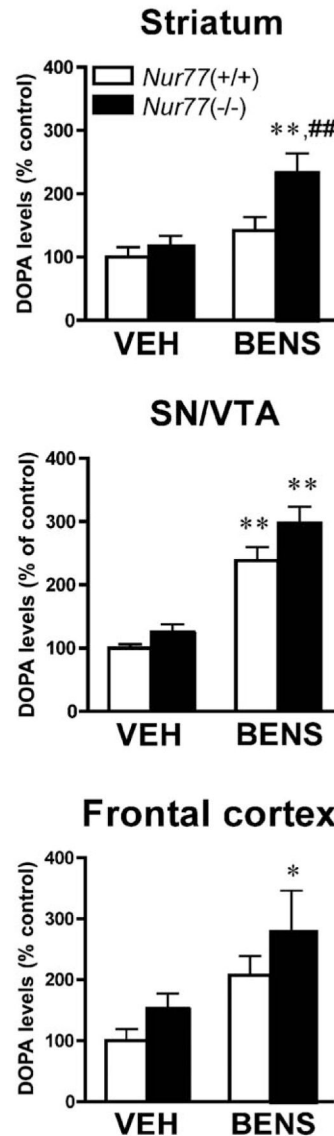

Figure 4.

Comparison of levels of L-DOPA accumulation in the presence of benserazide, a L-aromatic amino-acid decarboxylase (AADC) inhibitor, and in *Nur77* +/+ and −/− mice. Levels of L-DOPAs were measured by using HPLC with electrochemical detection in striatal (upper panel), substantia nigra/ventral tegmental area (SN/VTA, middle panel), and frontal cortex (lower panel) tissue homogenates form *Nur77* +/+ and −/− mice treated with the AADC inhibitor benserazide (BENS) (50 mg/kg) or its vehicle (VEH). Histogram bars represent mean ± SEM ($N = 5$) (*$p < .05$; **$p < .01$ vs. respective VEH group; ##$p < .01$ vs. *Nur77* −/− plus BENS group). Values are expressed in percent (%) of control. Absolute L-DOPA levels of *Nur77* +/+ control groups are striatum = .670 ± .105 ng/mg of protein, SN/VTA = .511 ± .070 ng/mg of protein, and frontal cortex = .794 ± .152 ng/mg of protein.

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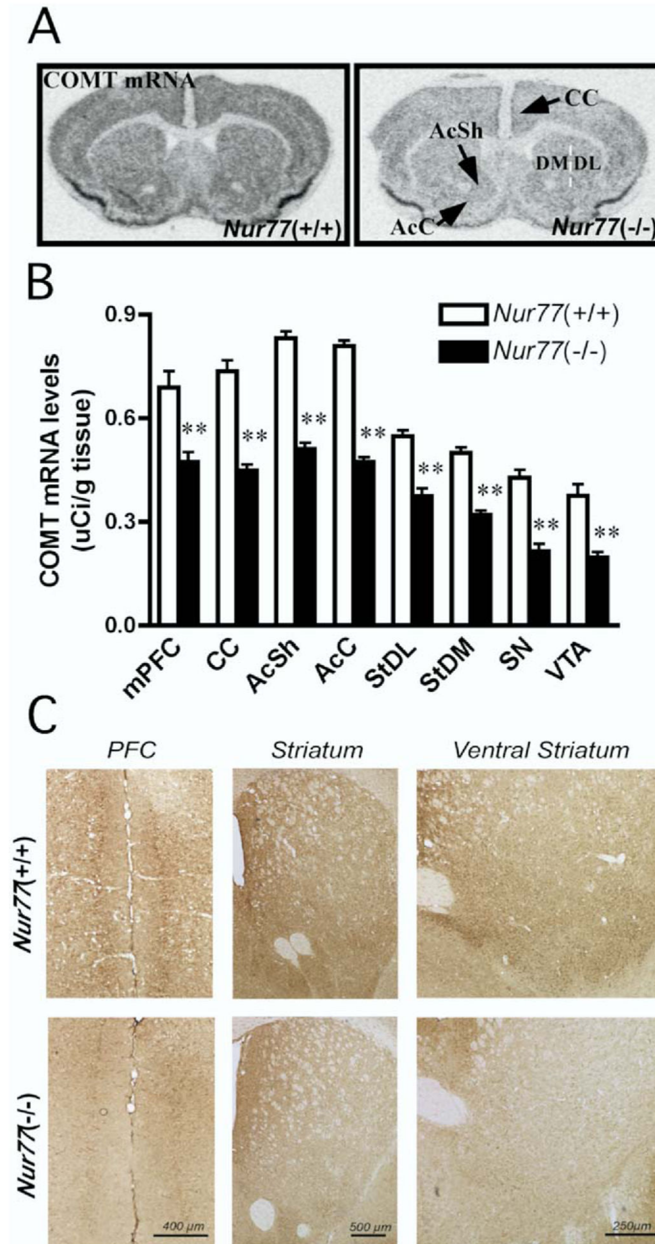

Figure 5.

Comparison of the catechol-O-methyltransferase (COMT) expression in various brain areas between wild-type (*Nur77* +/+) and *Nur77*-deficient (*Nur77* −/−) mice. **(A)** Representative autoradiograms showing in situ hybridization signals for the COMT mRNA probe at the level of the striatum. **(B)** Quantification of COMT mRNA levels in *Nur77* +/+ and −/− mice. The COMT levels were evaluated in the nucleus accumbens shell (AcSh) and core (AcC), dorsolateral (StDL) and dorsomedial (StDM) portions of the striatum, medial prefrontal cortex (PFC), cingulate cortex (CC), substantia nigra (SN), and ventral tegmental area (VTA). Histograms represent mean ± SEM from 10 animals. Messenger RNA levels are expressed in microcuries per gram (μCi/g) of tissue (**$p < .01$ vs. respective *Nur77* +/+

mice group). **(C)** Representative images of immunostaining signals for COMT using a specific antibody at the levels of prefrontal cortex (PFC, left panels), striatum (low magnification, middle panels), and nucleus accumbens (ventral striatum, right panels) in *Nur77* +/+ (top panels) and *Nur77* −/− (bottom panels) mice.

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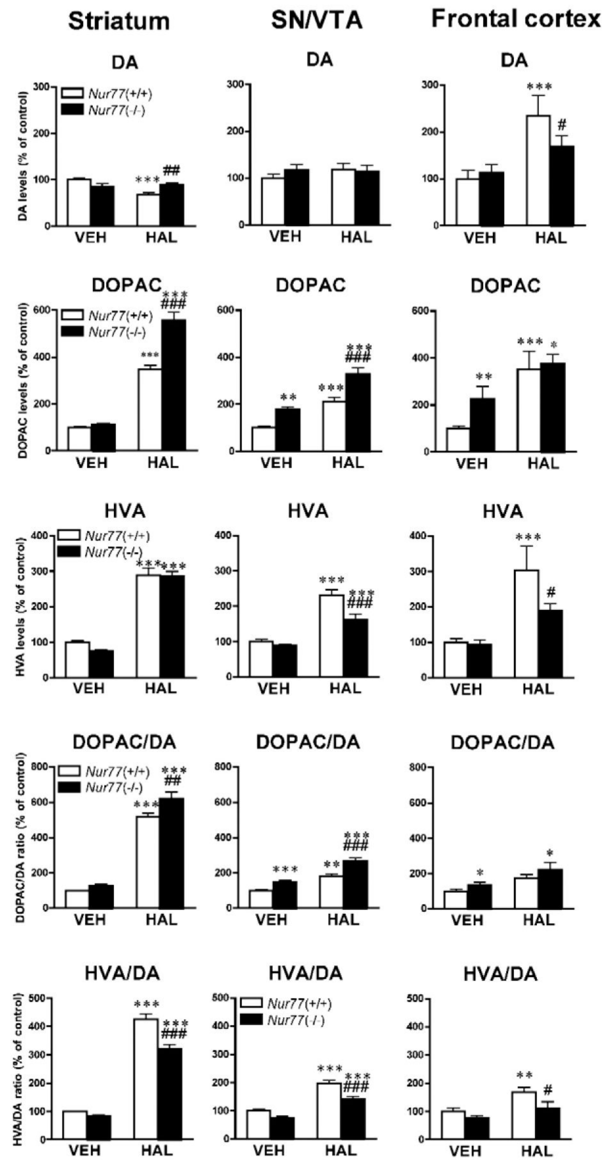

Figure 6.

Comparison of the effect of a haloperidol (.5 mg/kg) challenge on dopamine (DA) turnover in wild-type (*Nur77* +/+) and *Nur77*-deficient (*Nur77* −/−) mice. The DA and metabolite contents were evaluated in tissue homogenates from the striatum (left panels), substantia nigra and ventral tegmental area regions (SN/VTA) (middle panels), and frontal cortex (mainly including the prefrontal cortex area) (right panels) by using HPLC with electrochemical detection. Dopamine, metabolites (3,4-dihydroxyphenylacetic acid [DOPAC], and homovanillic acid [HVA]), and DOPAC/DA and HVA/DA ratios are expressed in percent of control (*Nur77* +/+ mice treated with the vehicle). *$p < .05$, **$p < .01$, ***$p < .001$ vs. *Nur77* +/+ treated with the vehicle (VEH) and #$p < .05$, ##$p < .01$, and ###$p < .001$ vs. *Nur77* +/+ mice treated with haloperidol (HAL). Basal (control) values expressed in nanogram per milligram of protein for *Nur77* +/+ and −/− mice can be found in Table 1.

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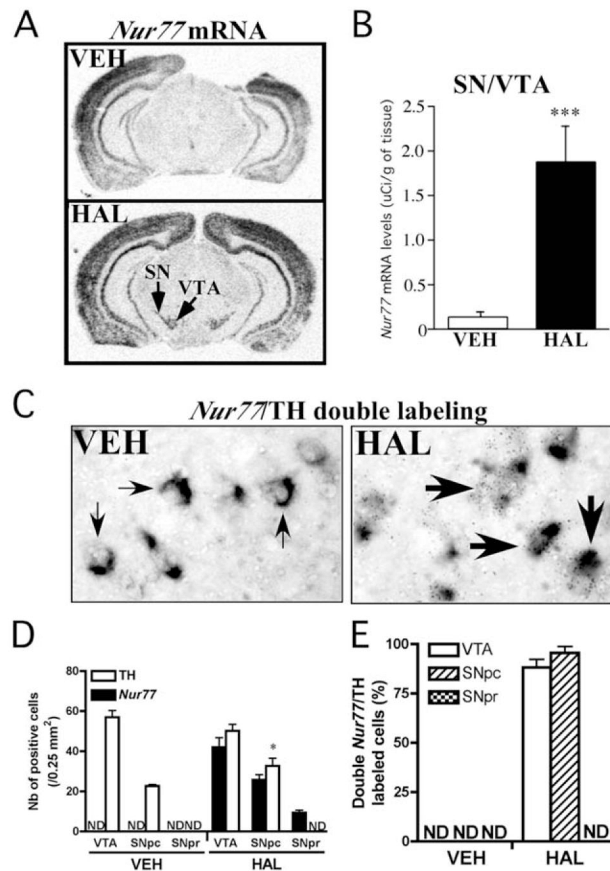

Figure 7.

Induction of *Nur77* in dopamine (DA) neurons after haloperidol (HAL) treatment in *Nur77* +/+ mice. **(A)** Representative autoradiograms of *Nur77* mRNA levels in the substantia nigra (SN) and ventral tegmental area (VTA) in *Nur77* +/+ mice treated with a single injection of the vehicle (VEH) or haloperidol (.5 mg/kg IP). **(B)** Quantification of the effect of HAL on *Nur77* mRNA levels in the SN/VTA area. Histograms represent mean ± SEM *Nur77* mRNA levels from five animals. **(C)** Representative photomicrographs of the effect of HAL on double in situ hybridization labeling of *Nur77* (silver grain) and tyrosine hydroxylase (TH; dark depot) mRNA. Thin arrows indicate single-labeled TH-positive cells in the vehicle (VEH) condition and bold arrows indicate double-labeled *Nur77*/TH cells in haloperidol (HAL)-treated animals. **(D)** Quantitative analysis of the effect of HAL on the number of *Nur77*- and TH-positive cells (per .25 mm^2 surface) in the substantia nigra pars compacta (SNpc), substantia nigra pars reticulata (SNpr), and ventral tegmental area (VTA). Each histogram bar represents mean ± SEM from five animals (*$p < .05$ vs. VEH-treated group). **(E)** Quantitative analysis of the proportion (in percent) of double *Nur77*/TH-labeled cells in the substantia nigra (SNpc and SNpr) and VTA. Almost 100% of double-labeled cells were observed in SNpc and VTA, whereas no double-labeled cell was detected in the SNpr. Each histogram bar represents mean ± SEM from five animals. ND, not detected.

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Table 1

Comparison of DA and Its Metabolite Levels in *Nur77*(+/+) and (−/−) Mice

	Nur77(+/+)	***Nur77***(−/−)
Striatum		
DA	74.5 ± 7.0	74.9 ± 9.9
DOPAC	4.63 ± .42	6.65 ± 1.43
HVA	8.89 ± 1.17	7.57 ± 1.16
DOPAC/DA	.062 ± .001	.091 ± .018
HVA/DA	.131 ± .007	.105 ± .004
SN/VTA		
DA	5.14 ± .52	7.03 ± 1.20
DOPAC	1.63 ± .13	3.60 ± .46[a]
HVA	2.67 ± .31	2.48 ± .27
DOPAC/DA	.307 ± .007	.413 ± .018[b]
HVA/DA	.584 ± .022	.354 ± .029[b]
Frontal Cortex		
DA	2.37 ± .31	2.67 ± .25
DOPAC	.76 ± .08	2.07 ± .45[a]
HVA	1.90 ± .13	1.11 ± .13
DOPAC/DA	.350 ± .035	.764 ± .195[b]
HVA/DA	.471 ± .066	.403 ± .039

DOPAC/DA and HVA/DA ratios were calculated from DA, DOPAC, and HVA levels in respective tissue homogenates collected from the striatum, substantia nigra/ventral tegmental area (SN/VTA), and frontal cortex. Values are expressed in nanogram per milligram (ng/mg) of protein in tissue homogenates and represent means ± SEM from 8–10 animals per group.

DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.

[a] $p < .01$ vs. wild-type mice (*Nur77*+/+).

[b] $p < .05$ vs. wild-type mice (*Nur77*+/+).