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Neurotensin gene expression and behavioral responses following administration of psychostimulants and antipsychotic drugs in dopamine D₃ receptor deficient mice

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Abbreviated running title: Role of D₃ receptors in neurotensin gene expression

Abstract

Exposure to psychostimulants and antipsychotics increases neurotensin (NT) gene expression in the striatum and nucleus accumbens. To investigate the contribution of D₃ receptors to these effects we used mice with targeted disruption of the D₃ receptor gene. Basal NT mRNA expression was similar in D₃ receptor mutant mice and wild-type animals. Acute administration of haloperidol increased NT gene expression in the striatum in D₃^{+/+}, D₃^{+/-} and D₃^{-/-} mice. Similarly, acute cocaine and amphetamine induced NT mRNA expression in the nucleus accumbens shell and olfactory tubercle to a comparable extent in D₃ mutants and wild-type mice. Daily injection of cocaine for seven days increased NT mRNA in a restricted population of neurons in the dorsomedial caudal striatum of D₃^{+/+} mice, but not in D₃^{-/-} and D₃^{+/-} animals. No differences were observed between D₃ receptor mutant mice and wild-type littermates in the locomotor activity and stereotyped behaviors induced by repeated cocaine administration. These findings demonstrate that dopamine D₃ receptors are not necessary for the acute NT mRNA response to drugs of abuse and antipsychotics but appear to play a role in the regulation of NT gene induction in striatal neurons after repeated cocaine. In addition, our results indicate that the acute locomotor response to cocaine and development of psychostimulant-induced behavioral sensitization do not require functional D₃ receptors.

Key words: dopamine D₃ receptor, neurotensin, cocaine, amphetamine, haloperidol, *in situ* hybridization, behavioral sensitization

INTRODUCTION

The administration of psychostimulants such as cocaine or amphetamine, and antipsychotics like haloperidol, promotes the expression of the neuropeptide neurotensin (NT) in the rat striatum and nucleus accumbens (Merchant et al. 1992, 1994a, 1994b; Castel et al. 1994; Betancur et al. 1997). These observations, together with numerous anatomical, behavioral, and neurochemical studies indicating that NT modulates dopamine transmission in the nigrostriatal and mesolimbic pathways (Kasckow and Nemeroff 1991), suggest that alterations in neurotensinergic transmission may be involved in the mechanism of action of neuroleptics and psychostimulant drugs.

Several lines of evidence implicate NT in the etiology of schizophrenia and in the mechanism of action of antipsychotic drugs. NT concentrations in the cerebrospinal fluid are reduced in drug-free schizophrenic patients and increase with clinically effective antipsychotic treatment (Widerlöv et al. 1982; Garver et al. 1991; Sharma et al. 1997). The increases in NT mRNA expression and NT content in the nucleus accumbens and striatum after administration of antipsychotic drugs are predictive of clinical efficacy and extrapyramidal side effects, respectively (Merchant and Dorsa, 1993; Kinkead et al. 1999). In rodents, centrally

administered NT produces behavioral effects similar to those induced by neuroleptics. Thus, when injected into the nucleus accumbens or the cerebral ventricles, NT blocks psychostimulant-induced locomotor hyperactivity (Ervin et al. 1981; Robledo et al. 1993) and induces a cataleptic response in mice (Snijders et al. 1982). Based on these findings, it has been postulated that NT plays a role as an endogenous neuroleptic (Nemeroff 1980). However, NT has also been implicated in the mechanism of action of psychostimulant drugs, as some of the effects of the neuropeptide resemble those induced by psychostimulants. For instance, injection of NT into the ventral tegmental area increases locomotor activity and dopamine release in the nucleus accumbens (Kalivas et al. 1983; Kalivas and Duffy 1990). Like cocaine, NT possesses rewarding properties: rats self-administer NT into the ventral tegmental area (Glimcher et al. 1987) and injection of NT in the same region induces conditioned place preference (Glimcher et al. 1984). In addition, endogenous NT has been implicated in the locomotor activating properties of cocaine (Betancur et al. 1998) and in the development of psychostimulant-induced behavioral sensitization in rats (Horger et al. 1994).

Previous pharmacological studies have demonstrated that stimulation of D₁ receptors or blockade of D₂ receptors both result in increased expression of NT mRNA in the rat striatum. The effects of cocaine and amphetamine on NT concentration and gene transcription are blocked with selective D₁ antagonists (Merchant et al. 1988; Hanson et al. 1989; Castel et al. 1993), whereas the effects of haloperidol appear to involve blockade of D₂-like receptors, *i.e.*, D₂, D₃ and D₄ receptors (Merchant et al. 1989, Augood et al. 1991). Little is known about the role of dopamine D₃ receptors in these effects. D₃ receptors are primarily expressed in mesolimbic areas associated with cognitive functions and motivated behavior and have been implicated in the antipsychotic effects of neuroleptics as well as in the rewarding effects of drugs of abuse (Sokoloff et al. 1990; Caine and Koob 1993; Pilla et al. 1999; see Shafer and Levant 1998 for a review). Two studies suggest the involvement of D₃ receptors in the regulation of NT gene expression. First, administration of D₃ receptor antisense oligonucleotides to rats has been reported to reduce NT mRNA expression in the nucleus accumbens shell (Tremblay et al. 1997). Second, blockade of D₂-like receptors by haloperidol increases NT mRNA expression in the septal pole of the nucleus accumbens, which contains a high level of D₂ receptors, but decreases it in the D₃ receptor-rich ventromedial shell, suggesting that D₃ receptors exert a tonic stimulation of NT expression, as opposed to the inhibitory influence of D₂ receptors (Diaz et al. 1994). However, the functional significance of D₃ receptors in the modulation of NT expression has been difficult to establish due to the lack of selective ligands.

To investigate the selective contribution of D₃ receptors to psychostimulant and antipsychotic drug-induced NT mRNA expression we used mice with targeted disruption of the D₃ receptor gene (Accili et al. 1996). D₃ mutant mice show no overt behavioral abnormalities or major compensatory changes within the dopaminergic system (Accili et al. 1996; Xu et al. 1997). We first studied the acute effects of two indirect dopamine receptor agonists, cocaine

and amphetamine, and the D₂-like receptor antagonist haloperidol. We also examined the effects of repeated cocaine administration on NT gene expression. Quantitative *in situ* hybridization histochemistry with [³⁵S]ATP-labeled oligonucleotide probes was used to assess NT mRNA expression in the nucleus accumbens and the striatum.

In addition, to further characterize the behavior of D₃ mutant mice, we evaluated basal and cocaine-induced locomotor activity, after acute and daily administration of the drug, to examine the role of D₃ receptors in the development of behavioral sensitization. The mesolimbic system plays a critical role in the induction and expression of behavioral sensitization to psychostimulants (Pierce and Kalivas 1997). The restricted distribution of the D₃ receptor in the projection areas of the mesolimbic system suggests that activation of the D₃ receptor may play a role in the locomotor activating properties of psychostimulants. Sensitization requires activation of D₁-like (D₁ and D₅ receptors) and D₂-like dopamine receptors (Wise 1995; Henry et al. 1998), but the contribution of D₃ receptors to this phenomenon is not known.

MATERIALS AND METHODS

Mice

Dopamine D₃ receptor mutant mice were obtained by homologous recombination (Accili et al. 1996). To establish the colony at the INSERM U.339 (Hôpital Saint-Antoine, Paris), a C57BL/6J x 129Sv D₃ receptor deficient (D₃^{-/-}) male mouse was backcrossed once with C57BL/6J females. Subsequent generations were derived from heterozygous intermatings. The genotype at the D₃ receptor locus was determined by reverse transcription-polymerase chain reaction of tail RNA. Homozygous (D₃^{-/-}) and heterozygous (D₃^{+/-}) D₃ receptor mutant mice and their wild-type (D₃^{+/+}) littermates were used for the experiments. Animals were maintained under a 12 h light-dark cycle (lights on at 8:00), with food and water available *ad libitum*, except during behavioral testing. They were housed in same-sex groups of 4-6 per cage and used at 10–12 weeks of age. All experiments were performed in accordance with the European Communities Council Directive for the care and use of laboratory animals.

Drug treatments

In the first experiment, mice received a single, acute intraperitoneal (i.p.) injection of cocaine hydrochloride, 30 mg/kg, calculated as the free base (Coopération Pharmaceutique Française, Melun, France); *d*-amphetamine sulfate, 10 mg/kg (Sigma); or haloperidol, 1 mg/kg (injectable solution, Janssen-Cilag). Control mice were injected with saline in a volume of 1 ml/100 g body weight. After evaluation of the acute behavioral response to psychostimulants and haloperidol, mice were killed by decapitation 3 h after the injection; the brains were rapidly removed, frozen on dry ice and stored at –80°C. In the second experiment, mice were injected

with cocaine (20 mg/kg i.p.) or saline once a day for seven days, their behavioral response was evaluated on days 1 and 7 and they were killed 3 h after the last injection.

Behavioral testing

In the first experiment, locomotor activity was measured in Plexiglas cages (41 x 28 x 18 cm) placed inside frames equipped with infrared photobeam cells, 2 cm above the floor (Opto-varimex mini, Columbus Instruments, Columbus, Ohio, USA). The number of beam breaks was recorded automatically by a computer with Opto-varimex software. Activity boxes were placed in a quiet room and $D_3^{+/+}$, $D_3^{+/-}$ and $D_3^{-/-}$ mice were analyzed at the same time. Basal activity levels were evaluated during 3 h sessions during the light (10:00–13:00) and dark (21:00–24:00) phases of the circadian cycle in separate groups of mice. The motor-stimulating effects of acute cocaine were tested one week later during the light phase. Mice were placed in the activity cages for a 2-h habituation period, then injected with cocaine (30 mg/kg i.p.) or saline, and replaced in the cages for an additional 3 h.

Catalepsy testing was conducted 1 h following injection with haloperidol (1 mg/kg i.p.). The degree of catalepsy was measured by placing both forepaws on a horizontal wooden bar (0.7 cm in diameter), 4 cm above the tabletop. The time in seconds until the mouse removed both forepaws from the bar was recorded, with a maximum cut-off time of 5 min.

In the second experiment, mice were tested after the first and the last of seven daily injections of cocaine (20 mg/kg i.p.) or saline, to evaluate the development of behavioral sensitization. On days 2–6, mice received daily injections of cocaine or saline in the home cage. On the days of behavioral testing, mice were placed in Plexiglas testing boxes (area: 30 x 20 cm) for 1 h of habituation. They were then injected with cocaine or saline and their behavior was recorded for 2 h, using a videocamera attached to the ceiling. Horizontal activity (distance moved expressed in m) was measured automatically from the videotapes with a motion analysis system, Ethovision (Noldus Information Technology, Wageningen, the Netherlands), and expressed as total values for each 10 min interval.

Stereotyped behaviors were assessed with a behavioral scale described by McLennan and Maier (1983), after the first and the last of seven daily cocaine injections. The behavioral rating was performed by an investigator unaware of the genotype and drug treatment of the animals. Mice were rated for 30-sec periods starting 5 min after cocaine injection, and continuing every 5 min for 1 h using the following scale: 0, inactive; 1, intermittent activity; 2, continuous activity; 3, rearing; 4, intermittent stereotypic sniffing, repetitive head movements, or both, with periods of nonstereotypic behavior longer than 2 sec; 5, intermittent stereotypic sniffing, repetitive head movements, or both, with periods of nonstereotypic behavior shorter than 2 sec; 6, continuous stereotypic sniffing; repetitive head movements, or both; and 7, continuous and restricted stereotypic sniffing, repetitive head movements, or both.

In situ hybridization histochemistry

Coronal brain sections (20 μ m) were cut on a cryostat, thaw-mounted on Superfrost plus slides (Menzel-Glaser) and stored at -20°C until used. The level of NT mRNA was assessed by *in situ* hybridization using ^{35}S -labeled oligonucleotide probes. To increase the intensity of the hybridization signal, we used a mixture of two oligonucleotide probes, complementary to nucleotides 272–319 and 338–372 of the rat NT gene (Kislauskis et al. 1988). In addition, we used two probes complementary to the mouse NT gene, from a partial clone published in the GenBank EST Division database (accession no. AA184803). The rat and mouse oligonucleotides yielded identical patterns of hybridization signal. Oligonucleotides were synthesized by Life Technologies. The probe was labeled at the 3' end with ^{35}S -ATP (specific activity: $>1000\text{ Ci/mmol}$; Amersham) using terminal transferase (Boehringer-Manheim).

The sections were warmed at room temperature, fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, acetylated in a solution of 4 x standard saline citrate (SSC; 1 x SSC: 0.15 M NaCl and 0.015 M sodium citrate) containing 0.25% acetic anhydride and 0.1 M triethanolamine, pH 8, dehydrated in ascending concentrations of ethanol, delipidated in chloroform, rehydrated in 95% ethanol and allowed to dry. The hybridization medium (50% formamide, 4 x SSC, 1% sarcosyl, 0.1 M potassium phosphate pH 7.4, 250 $\mu\text{g/ml}$ yeast tRNA, 250 $\mu\text{g/ml}$ herring sperm DNA, 10% dextran sulfate, 1 x Denhardt's solution, 50 $\mu\text{g/ml}$ polyA, and 10 mM dithiothreitol), containing 0.5 pmol/ml of each oligonucleotide probe ($1\text{--}1.2 \times 10^7$ cpm/ml), was applied onto each slide and sealed under a coverslip. Hybridization was allowed to proceed overnight at 42°C in humidified boxes. Coverslips were removed in 4 x SSC and the sections were rinsed at 42°C in decreasing concentrations of SSC for 30 min each time (once in 4 x SSC with 0.02% sodium dodecyl sulfate, twice in 1 x SSC and twice in 0.1 x SSC), dehydrated and allowed to dry. Film autoradiograms were obtained by apposition of radiolabeled sections to Hyperfilm β max (Amersham) for one month at room temperature. The films were developed in Kodak D-19 developer and fixed in Kodak Unifix.

Analysis of hybridization signal

Quantitative optical density measurements were carried out using a computer-based image analysis system, HISTO-RAG (Biocom, Les Ulis, France). The optical density of the background (measured in the parietal cortex, a region that does not contain NT mRNA) was determined in each tissue section and subtracted from all values. Optical density values were expressed as nCi/mg tissue using ^{14}C standards, exposed together with the hybridized tissue. Brain structures were identified according to the atlas of Franklin and Paxinos (1997). Hybridization signal was analyzed at two striatal levels, shown in Fig. 1: rostral (0.98 mm anterior to bregma) and caudal (-0.10 mm posterior to bregma). The following regions were analyzed at the rostral level: nucleus accumbens shell and core, olfactory tubercle, lateral septum and dorsolateral, dorsomedial and ventrolateral striatum. At the caudal level, measurements were performed in the dorsolateral, dorsomedial, and ventrolateral striatum. The analysis was performed bilaterally on at least six brain sections per animal at each level.

Statistics

In situ hybridization results from each brain region were evaluated with analyses of variance (ANOVA), with two between subjects factors (treatment and genotype), followed when indicated by a Tukey test for multiple comparisons to establish significant differences between the means. Behavioral data were analyzed using a two-way ANOVA for repeated measures over time.

RESULTS

Basal expression of NT mRNA

The distribution of NT mRNA expression in vehicle-injected mice was restricted rostrally to the nucleus accumbens shell, the olfactory tubercle and the lateral septum (Fig. 2), in agreement with studies in the rat (Alexander et al. 1989; Merchant and Dorsa 1993; Betancur et al. 1997). Labeling in the nucleus accumbens shell was irregular, with intensely labeled cell clusters scattered within the region. The signal was more prominent in the lateral part of the shell than in the medial region. The caudate putamen expressed very low levels of NT mRNA, except in the dorsomedial region of the caudal striatum, where a strong labeling was observed in the periventricular area, beneath the corpus callosum. At the caudal level, an intense hybridization signal was present in the medial preoptic area. No significant differences in basal levels of NT mRNA expression were observed among $D_3^{-/-}$, $D_3^{+/-}$ and $D_3^{+/+}$ mice in any of the brain regions studied (Table 1).

Effect of acute administration of haloperidol on NT mRNA expression

Analysis of film autoradiograms showed that acute administration of haloperidol induced a marked increase in NT gene expression in the dorsolateral, dorsomedial and ventrolateral striatum in wild-type mice and $D_3^{-/-}$ and $D_3^{+/-}$ mutants, both at rostral and caudal levels, compared to vehicle-injected animals (Figs. 2 and 3). This up-regulation was stronger in the dorsolateral and ventrolateral striatum (~800%) than in the dorsomedial area (120%). The magnitude of the increases in NT mRNA expression did not differ among the genotypes. Acute administration of haloperidol did not increase NT gene transcription in the shell of the nucleus accumbens in any of the three genotypes (Fig. 3).

Effects of acute administration of cocaine and amphetamine on NT mRNA expression

As shown in Fig. 3, acute administration of cocaine or amphetamine increased NT mRNA levels in the shell of the nucleus accumbens and in the olfactory tubercle. Cocaine also enhanced NT gene expression in the dorsomedial region of the striatum, both at rostral and caudal levels. However, the magnitude of these increases (30-40% in the nucleus accumbens and olfactory tubercle, and 35-55% in the striatum) was lower than that observed in the caudate putamen after haloperidol. Thus, the effects of cocaine and amphetamine on NT mRNA expression were significant only as a treatment effect (ANOVA, cocaine or

amphetamine vs. saline-treated mice, irrespective of genotype), but not when mice of each genotype were analyzed separately.

Effect of repeated administration of cocaine on NT mRNA expression

Three hours after the last of seven daily injections of cocaine, a marked increase in NT mRNA expression was observed in a restricted population of neurons in the dorsomedial striatum at the caudal level in wild-type animals (Figs. 4 and 5A). In contrast, the same repeated administration of cocaine failed to increase NT mRNA levels in the caudate putamen of $D_3^{-/-}$ and $D_3+/-$ mice. Repeated cocaine did not modify NT gene transcription in the rostral striatum (Fig. 5B), nucleus accumbens shell and core (Fig. 5C), olfactory tubercle (Fig. 5C) or lateral septum (data not shown) of wild-type or D_3 receptor mutant mice.

Behavioral analysis

Contradictory results have been reported concerning the presence of a transient increase in spontaneous locomotor activity during the initial (5 or 15 min) exploration of a novel environment in D_3 receptor deficient mice (Accili et al. 1996; Xu et al. 1997; Boulay et al. 1999). Our results indicate that basal activity levels in wild-type and mutant mice were similar during the light period of the light/dark cycle (Fig. 6A). However, $D_3^{-/-}$ mice were markedly hyperactive during the dark period (Fig. 6A), when the dopaminergic system is physiologically activated in nocturnal animals such as rodents. This hyperactivity was observed from the beginning of the test session and persisted for the whole testing period (3 h). The locomotor activity of $D_3^{-/-}$ and $D_3+/-$ mice was significantly enhanced during the night cycle compared to the day cycle, whereas the increase during the dark cycle observed in wild-type animals was not statistically significant (Fig. 6A). As shown in Fig. 6B, acute cocaine (30 mg/kg) stimulated locomotor activity to a similar extent in the three groups of mice. Moreover, no differences were observed among $D_3^{-/-}$, $D_3+/-$ and $D_3+/+$ mice in haloperidol-induced catalepsy (Fig. 6C).

In the second experiment, mice received seven daily cocaine injections (20 mg/kg) and their behavioral response was evaluated on the first and the last cocaine challenges to assess the development of behavioral sensitization. On day 1, cocaine significantly enhanced locomotor activity in the three groups of mice, although the response of $D_3+/-$ and $D_3^{-/-}$ mice was lower than that of wild-type mice (Fig. 7A). The locomotor response to cocaine on day 7 was similar in the three genotypes. Wild-type and D_3 receptor deficient mice failed to show an increment in cocaine-induced locomotor hyperactivity after daily injections of the psychostimulant compared to day 1 (Fig. 7A). However, as shown in Fig. 7B, repeated exposure to cocaine induced an increase in stereotypies such as repetitive sniffing and head bobbing in wild-type and mutant mice, indicating the development of behavioral sensitization. Stereotypies after repeated cocaine were quantitatively and qualitatively similar in $D_3^{-/-}$, $D_3+/-$ and $D_3+/+$ mice.

DISCUSSION

Mice lacking functional dopamine D₃ receptors were used to examine the role of D₃ receptors in the transcription of the NT gene using *in situ* hybridization. There was no modification in the expression pattern of NT mRNA in D₃ receptor deficient mice under basal conditions. Acute treatment with haloperidol dramatically up-regulated NT mRNA levels in the striatum of wild-type and mutant mice. Acute administration of cocaine or amphetamine resulted in smaller increases in NT mRNA expression in the nucleus accumbens shell and olfactory tubercle, which were observed in mice regardless of their genotype. In contrast, repeated administration of cocaine enhanced NT gene expression in neurons confined to the dorsomedial striatum in wild-type mice, but failed to affect NT mRNA levels in D₃^{-/-} and D₃^{+/-} mice. These findings suggest that D₃ receptors are not necessary for the induction of NT mRNA by acute administration of psychostimulants or haloperidol, but appear to be implicated in striatal NT gene regulation after repeated cocaine. The behavioral analysis demonstrated that D₃ mutant mice exhibited increased locomotor activity only during the dark phase of the circadian cycle, but did not differ from wild-type littermates in the locomotor response to acute cocaine and the development of behavioral sensitization.

D₃ receptors do not regulate basal NT mRNA expression

Our results showed normal basal expression of NT mRNA in the forebrain of D₃ receptor deficient mice, suggesting that D₃ receptors do not exert a regulatory control on the transcription of the gene encoding NT. These data contradict a recent report in the rat showing that antisense oligodeoxynucleotides directed against D₃ receptors decrease basal NT mRNA expression in the shell of the nucleus accumbens (Tremblay et al. 1997). There may be several reasons for this discrepancy, including species-related differences or compensatory mechanisms in D₃^{-/-} mice during development. D₃ mutant mice show normal number and affinity of D₂ receptors (Accili et al. 1996; Xu et al. 1997; Jung et al. 1999), arguing against a compensatory role of D₂ receptors. Although D₃ deficient mice exhibit apparently normal dopaminergic systems, with normal levels of tyrosine hydroxylase, dopamine transporter, and D₁ receptors (Accili et al. 1996; Xu et al. 1997), possible adaptive alterations at the second messenger level can not be excluded.

Koeltzow et al. (1998) reported that D₃^{-/-} mice exhibit increased basal dopamine release in the nucleus accumbens. Although basal NT gene expression has been shown to be under tonic control by endogenous dopamine (Merchant et al. 1989), our results indicate that the increase in dopamine release in D₃^{-/-} mice is not associated with altered NT mRNA expression, again suggesting the development of compensatory effects to counterbalance enhanced dopamine neurotransmission. In agreement with this hypothesis, previous studies in the rat have shown that repeated administration of amphetamine results in blunted NT mRNA responses in the nucleus accumbens shell to subsequent stimulation (Feldpausch et al. 1998).

Haloperidol-induced NT mRNA expression and catalepsy are unaltered in D₃ receptor mutant mice

In these experiments, D₃ receptor deficient mice were studied to examine the contribution of D₃ receptors to the molecular (NT mRNA) and behavioral (catalepsy) effects produced by the typical antipsychotic agent haloperidol. Results showed that both the regional pattern of induction of NT mRNA by haloperidol, and the magnitude of the effects in the striatum were identical in D₃^{-/-} and wild-type mice. Similarly, haloperidol remained effective in inducing catalepsy in D₃^{-/-} mice, a behavioral response that has been correlated with the extrapyramidal motor side effects seen in humans following antipsychotic drug administration (Hoffman and Donovan 1995). These findings indicate that haloperidol-induced increase in NT mRNA expression and catalepsy can take place in the complete absence of D₃ receptors. Haloperidol-induced NT gene expression is likely to be mediated by other members of the D₂-class receptor family, most probably the D₂ receptor itself. Several lines of evidence suggest the involvement of D₂ receptors in the effects of haloperidol on NT gene transcription. Basal release of dopamine exerts a tonic inhibitory control over NT systems by acting through D₂ receptors. Specifically, blockade of D₂ receptors by antipsychotic drugs or depletion of dopamine after 6-OHDA lesion of the nigrostriatal pathway or treatment with reserpine, increase NT immunoreactivity in the striatum and nucleus accumbens (Merchant et al. 1989). Furthermore, administration of raclopride (Augood et al. 1991) and eticlopride (Sirinathsinghji et al. 1994), two D₂ receptor antagonists, induce a regional pattern of NT mRNA expression that is indistinguishable from that produced by haloperidol. The majority of haloperidol-sensitive NT mRNA-expressing neurons in the dorsal striatum also express enkephalin mRNA (Augood et al. 1997), and immunocytochemical (Yung et al. 1995) and *in situ* hybridization (Le Moine et al. 1995) studies have shown that enkephalin-containing cells express preferentially D₂ receptors as opposed to D₁ receptors, thus indicating that haloperidol-sensitive NT neurons express D₂ receptors.

Blockade of D₂-like receptors by haloperidol has been suggested to exert opposing roles in NT mRNA expression in the nucleus accumbens by acting through D₃ or D₂ receptors (Diaz et al. 1994; Lévesque et al. 1995). D₃ receptor mRNA is abundantly expressed in the ventromedial subdivision of the shell, where it is colocalized with NT mRNA in a large number of neurons (Diaz et al. 1994; 1995). According to these authors, blockade of D₃ receptors by haloperidol decreases NT gene expression in this region, whereas blockade of D₂ receptors increases NT mRNA expression in the D₂ receptor-rich dorsal area of the medial shell (cone). Subregional analysis of NT mRNA expression in the accumbens shell in haloperidol-treated mice (1 mg/kg) showed no evidence of decreased NT gene transcripts in the ventromedial shell (not shown). Similarly, Merchant et al. (1996) failed to observe reduced NT mRNA levels in this region after administration of haloperidol to rats. This discrepancy could be due to the high doses of haloperidol employed in the studies by Diaz et al. (1994) and Lévesque et al. (1995), 20 and 5 mg/kg, respectively, whereas we and Merchant et al. (1996) used 1 mg/kg.

Acute and daily cocaine treatments differentially regulate NT mRNA levels in striatal neurons in D₃ receptor deficient mice

Following acute treatment with cocaine or amphetamine, significant increases in NT gene expression occurred in the shell of the nucleus accumbens and olfactory tubercle. This effect did not differ between wild-type and D₃ receptor null mice, suggesting that D₃ receptors are not involved in NT gene induction in the ventral striatum by these indirect dopamine agonists. However, exposure to daily cocaine led to a significant increase in NT mRNA expression in the dorsomedial division of the caudal striatum in wild-type mice but did not alter NT gene expression in D₃^{-/-} and D₃^{+/-} mice. Interestingly, the highest amounts of D₃ receptor binding sites and mRNA in the striatum in the rat and mouse have been detected in the dorsomedial region (Sokoloff et al. 1990; Levant et al. 1993; Bordet et al. 1997; Levant 1998). Taken together, these findings suggest that D₃ receptors in dorsomedial striatal neurons could be involved in the regulation of NT gene expression after repeated psychostimulant administration. Alternatively, the increase in NT gene transcription by cocaine in this region could be modulated via D₃ receptors located in a distant site. One possibility may be the substantia nigra, where expression of D₃ receptors in dopamine neurons has been reported (Diaz et al. 1995).

The effects of cocaine and amphetamine on NT concentration and gene expression in striatal and accumbal systems involve activation of D₁ receptors, since these effects are blocked by co-administration of the D₁-selective antagonist, SCH 23390 (Merchant et al. 1988; Hanson et al. 1989; Castel et al. 1993). In the ventromedial shell subdivision of the nucleus accumbens, 60% of NT neurons express D₃ receptor mRNA (Diaz et al. 1995), and D₁ and D₃ receptors in this region are coexpressed to a significant extent (Ridray et al. 1998). In spite of this colocalization, D₃ receptors do not appear to participate in the D₁-mediated regulation of NT mRNA by psychostimulants. Similarly, D₁ receptor agonist activation of *c-fos* expression in the ventromedial shell was not affected by D₃ receptor activation (Ridray et al. 1998). However, a recent study using D₃ receptor deficient mice showed that D₃ receptors play a facilitatory role in the regulation of *c-fos* responses to D₁ agonist stimulation (Jung and Schmauss 1999). Thus, functional interactions between D₃ and D₁ receptors appear to be complex and vary depending on the brain region and the gene studied.

D₃ receptor mutant mice exhibit normal cocaine-induced behavioral sensitization

Cocaine produces a rapid, dose-dependent increase in extracellular concentrations of dopamine in the nucleus accumbens and caudate-putamen, regions that appear to be involved in locomotion and stereotypy, respectively. Investigation of the behavioral effects of acute cocaine (30 mg/kg) in D₃ receptor deficient mice revealed that the drug stimulated locomotor activity to a similar extent in D₃^{-/-} and wild-type mice. This result is consistent with a previous study in D₃ mutant mice (Xu et al. 1997) showing increased behavioral sensitivity to the effects of cocaine only after a low dose of the drug (5 mg/kg), whereas no differences were observed at higher doses (10-40 mg/kg). Our results show that after repeated

administration of cocaine, wild-type and D₃ mutant mice exhibited a similar behavioral sensitization to the effects of the drug. The three groups of mice showed increased stereotypies such as sniffing and repetitive head movements on the last cocaine challenge. Repeated exposure to cocaine failed to induce an increment in locomotor hyperactivity in wild-type and D₃ receptor deficient mice, which could be explained by the appearance of stereotyped behaviors. Our results using D₃ receptor mutant mice suggest that D₃ receptors are not required for the expression of psychostimulant-induced behavioral sensitization.

The mesolimbic system, and the nucleus accumbens in particular, is considered the main neural substrate for the rewarding and locomotor activating properties of psychostimulants (Self and Nestler 1995; Koob 1992). Thus, the limbic distribution of D₃ receptors suggests that they may play an important role in drug abuse. In support of this hypothesis, the number of D₃ receptors has been reported to be up-regulated in the nucleus accumbens of cocaine-overdose victims (Staley and Mash 1996; Segal et al. 1997). Moreover, D₃ receptors have been implicated in the development of psychostimulant-induced behavioral sensitization. Repeated cocaine has been reported to induce a significant dose-dependent increase in striatal D₃ receptors in behaviorally sensitized rats, whereas D₃ receptors in the nucleus accumbens are reduced (Wallace et al. 1996). Similarly, increased expression of striatal D₃ receptors has been proposed as the mechanism responsible for the development of sensitization to repeated administration of levodopa (Bordet et al. 1997). However, a similar increase in D₃ receptors was not observed during cocaine-induced sensitization in the present study. Indeed, in D₃^{+/+} mice, repeated exposure to cocaine did not result in increased expression of D₃ receptors in the nucleus accumbens or caudate putamen, as assessed by [³H]-7-OH-DPAT autoradiography (data not shown).

CONCLUSIONS

In summary, the data shown here demonstrate that acute haloperidol administration increased NT mRNA levels in the striatum of D₃ receptor deficient mice, indicating that expression of this neuropeptide can be regulated by D₃ receptor-independent processes. Similarly, deletion of the D₃ dopamine receptor did not alter the ability of cocaine and amphetamine to stimulate neurons in the nucleus accumbens to express NT. However, the absence of D₃ receptors altered dorsal striatal NT responses to repeated cocaine, suggesting the participation of D₃ receptors in dopamine-regulated NT gene expression after long-term administration of psychostimulant drugs. D₃ receptor deficient mice showed typical locomotor responses after acute administration of psychostimulants as well as normal behavioral sensitization to cocaine. These findings demonstrate that D₁ and D₂ dopamine receptors can function without D₃ receptors in the control of NT gene expression and motor behavior by psychomotor stimulants and antipsychotic drugs.

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Table 1. Basal NT mRNA expression in D₃^{+/+}, D₃^{+/-} and D₃^{-/-} mice.

Brain regions	D ₃ ^{+/+}	D ₃ ^{+/-}	D ₃ ^{-/-}
Nucleus accumbens, core	1.44 ± 0.26	1.74 ± 0.35	1.27 ± 0.19
Nucleus accumbens, medial shell	5.66 ± 0.62	6.39 ± 0.73	7.71 ± 0.60
Nucleus accumbens, lateral shell	18.87 ± 3.23	16.22 ± 1.30	18.88 ± 3.37
Olfactory tubercle	13.47 ± 1.90	10.00 ± 1.16	12.08 ± 1.32
Lateral septum	19.63 ± 1.62	22.49 ± 2.20	21.39 ± 1.95
Dorsomedial striatum, rostral	1.76 ± 0.30	2.11 ± 0.34	1.87 ± 0.24
Dorsomedial striatum, caudal	6.58 ± 2.61	6.73 ± 1.95	8.62 ± 2.76
Dorsolateral striatum, rostral	1.75 ± 0.16	1.86 ± 0.30	1.49 ± 0.16
Dorsolateral striatum, caudal	0.52 ± 0.18	0.69 ± 0.13	0.64 ± 0.11
Ventrolateral striatum, rostral	1.96 ± 0.42	1.79 ± 0.37	1.38 ± 0.22
Ventrolateral striatum, caudal	1.36 ± 0.25	1.33 ± 0.20	1.09 ± 0.16

In situ hybridization autoradiograms from vehicle-treated mice from the first experiment (shown in Figs. 2 and 3) were quantified with a computer-assisted image analysis system at rostral (bregma 0.98 mm) and caudal (bregma -0.10 mm) levels. Results are expressed as nCi/mg tissue (mean ± SEM); n = 4-7 mice per group. There were no significant differences in basal NT mRNA expression among D₃^{-/-}, D₃^{+/-} and wild-type mice in any of the regions studied.

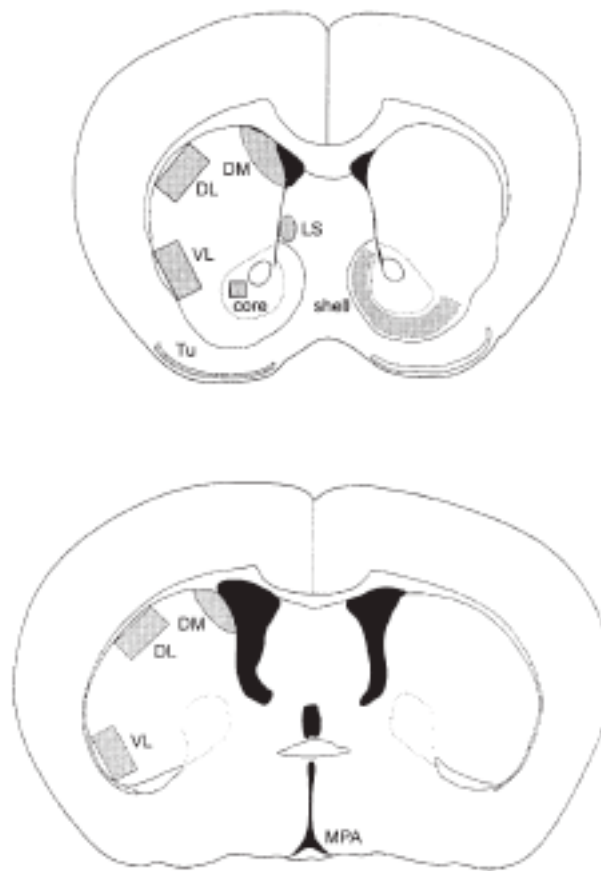


Figure 1. Schematic representation of the forebrain regions where NT mRNA hybridization signal was measured at the rostral (bregma 0.98 mm, top) and caudal (bregma -0.10 mm, bottom) striatal level of the mouse brain. DL, dorsolateral striatum; DM, dorsomedial striatum; MPA, medial preoptic area; VL, ventrolateral striatum; LS, lateral septum; Tu, olfactory tubercle.

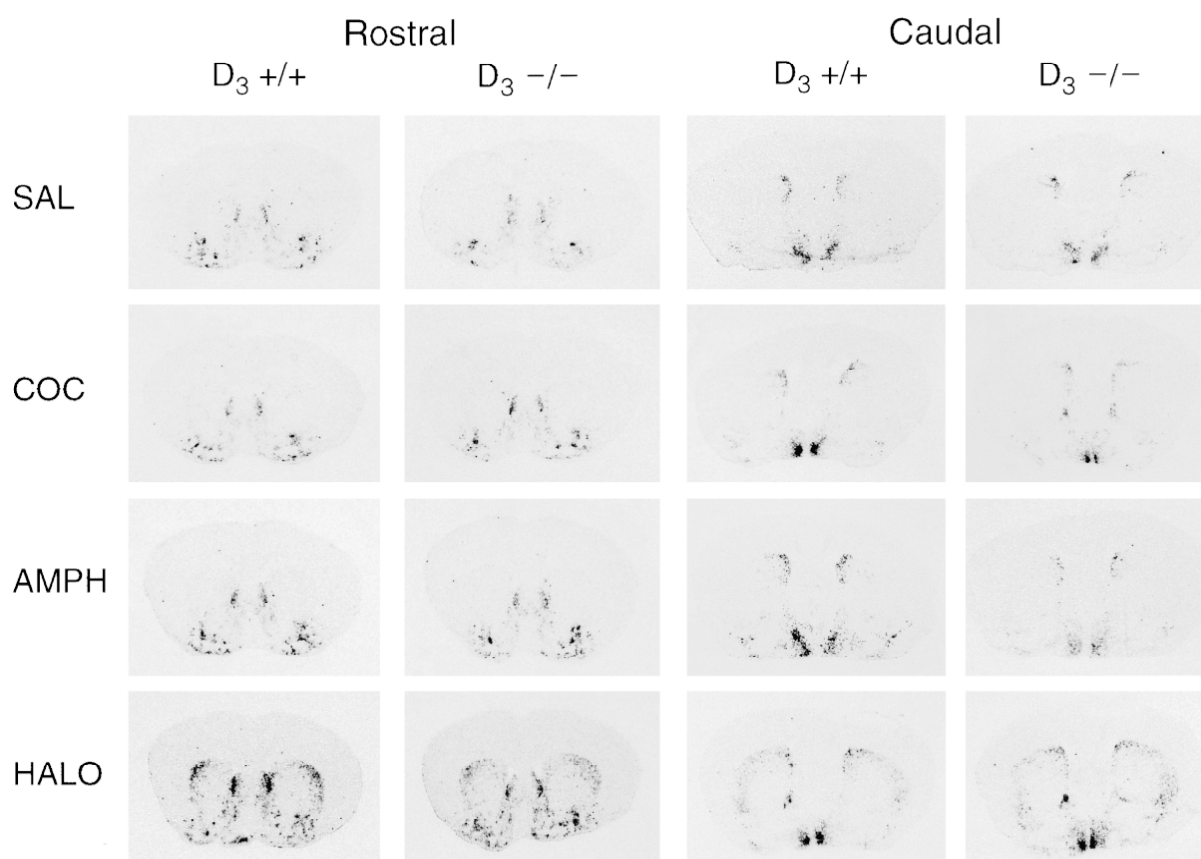


Figure 2. *In situ* hybridization of NT mRNA after an acute injection of saline, cocaine, amphetamine, or haloperidol in $D_3^{+/+}$ and $D_3^{-/-}$ mice. Mice were injected i.p. with a single dose of saline (SAL), cocaine (COC, 30 mg/kg), amphetamine (AMPH, 10 mg/kg), or haloperidol (HALO, 1 mg/kg), and killed 3 h after. Film autoradiograms show NT mRNA expression in coronal brain sections at the rostral and caudal striatal level.

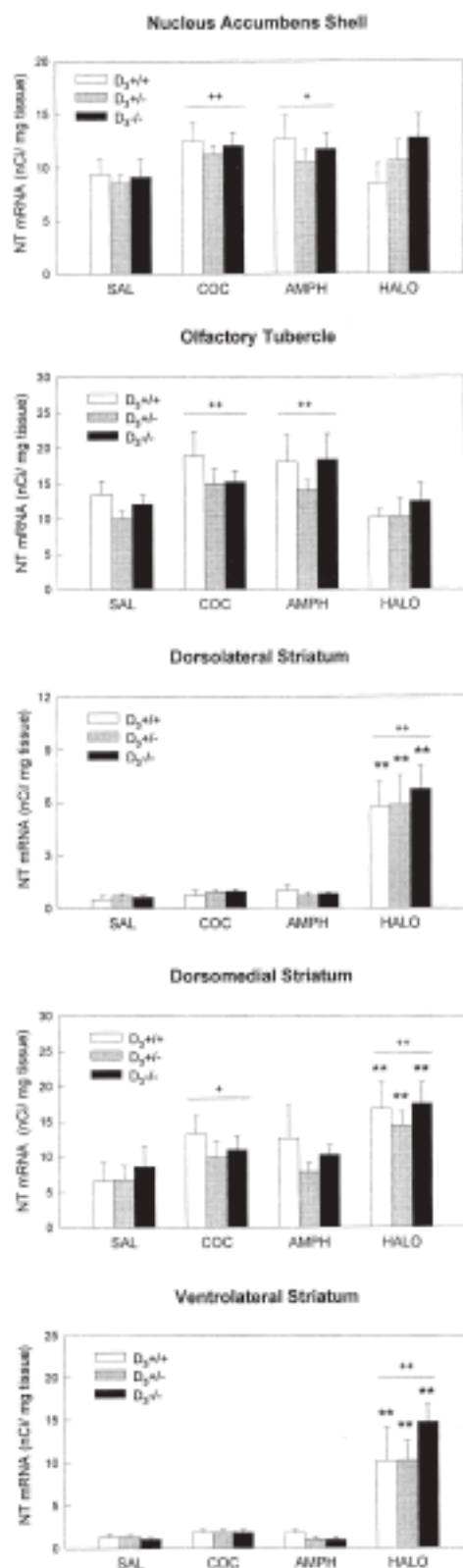


Figure 3. Quantification of NT mRNA *in situ* hybridization autoradiograms after an acute injection of saline, cocaine, amphetamine, or haloperidol in $D_3^{+/+}$, $D_3^{+/-}$ and $D_3^{-/-}$ mice. Films were analyzed with a computer-assisted image analysis system and results expressed as nCi/mg tissue; bars represent mean ± SEM (n = 4-7 mice per group). NT gene expression in the striatum is shown here at the caudal level. Results were analyzed by ANOVA followed by Tukey test. Significant main effects of treatment (vs. saline-treated mice) are indicated by + (p < 0.05), and ++ (p < 0.01). **p < 0.01, vs. saline-treated mice of the same genotype.

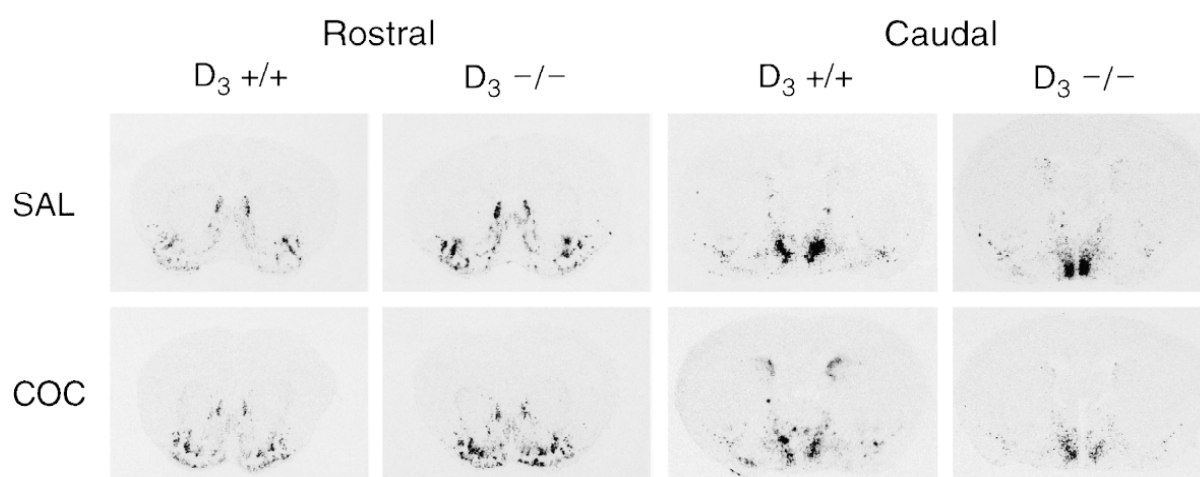


Figure 4. *In situ* hybridization of NT mRNA after repeated cocaine administration in D_3 $+/+$ and D_3 $-/-$ mice. Mice were injected once a day for 7 days with saline (SAL) or cocaine (COC, 20 mg/kg i.p.) and killed 3 h after the last injection. Typical film autoradiograms from saline- and cocaine-injected mice are shown at the rostral and caudal striatal level.

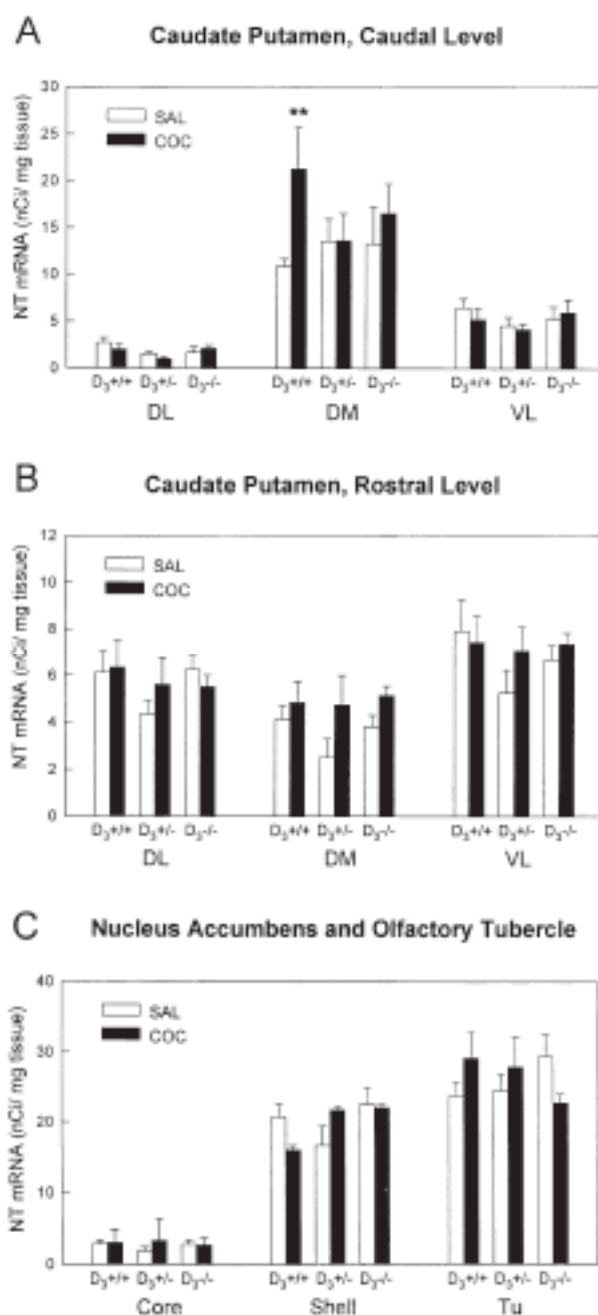


Figure 5. Quantification of NT mRNA *in situ* hybridization autoradiograms after repeated cocaine in D₃^{+/+}, D₃^{+/-} and D₃^{-/-} mice. Results are expressed as nCi/mg tissue; bars represent mean+SEM (n = 5-7 mice per group). **p<0.01, vs. saline-treated wild-type mice, ANOVA followed by Tukey test. DL, dorsolateral striatum; DM, dorsomedial striatum; VL, ventrolateral striatum; Tu, olfactory tubercle.

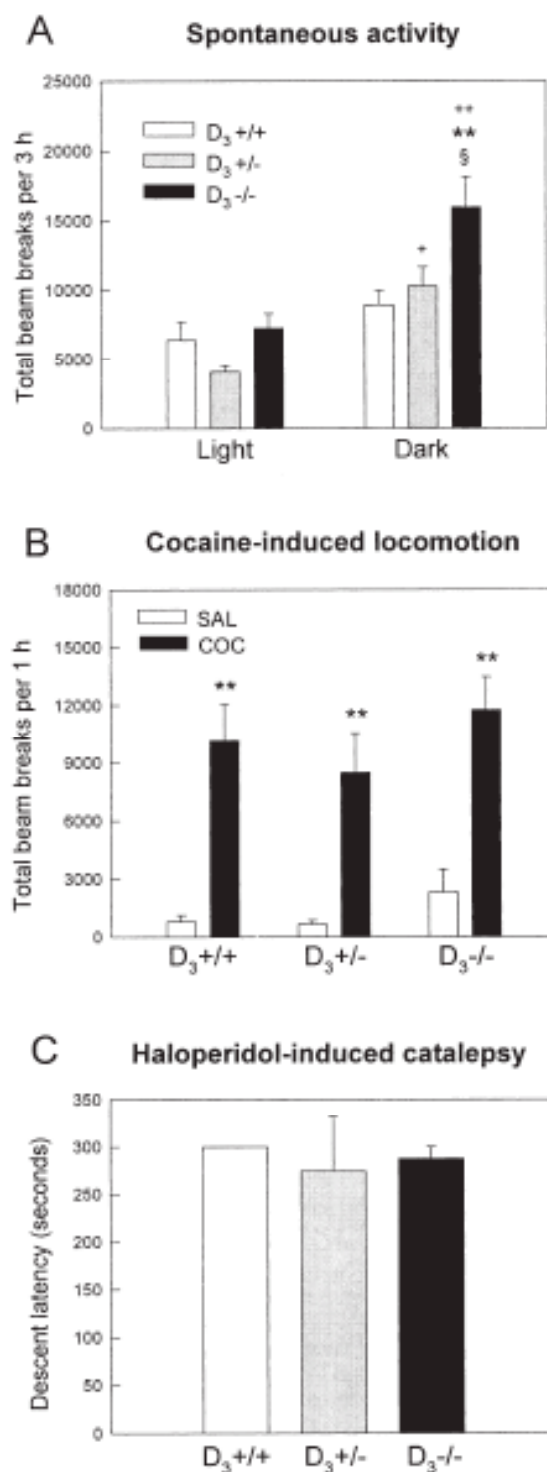


Figure 6. A. Spontaneous locomotor activity of naive D₃^{+/+}, D₃^{+/-} and D₃^{-/-} mice during the light and dark periods of the circadian cycle. Locomotor activity was recorded for a period of 3 h during the light (10:00-13:00) or the dark (21:00-24:00) phase of the light-dark cycle. Values represent mean + SEM photocell beam interruptions for 3 h test sessions (n = 11-15 mice per group). D₃^{-/-} mice were hyperactive during the dark phase compared to D₃^{+/+} (**p<0.01, ANOVA followed by Tukey test) and D₃^{+/-} mice (§p<0.05). The locomotor activity of the D₃^{-/-} and D₃^{+/-} mice was significantly higher during the night cycle compared to the day cycle (+p<0.05, ++p<0.01). B. Effect of acute cocaine on the locomotor activity of wild-type and D₃ receptor deficient mice. Mice were injected with cocaine (30 mg/kg, i.p.) or saline and locomotor activity was recorded (n = 6-8 mice per group). Cocaine induced a significant increase in locomotion in the three groups of mice (**p<0.01 as compared to saline-treated mice of the same genotype). C. Haloperidol-induced catalepsy in D₃^{+/+}, D₃^{+/-} and D₃^{-/-} mice. Catalepsy was assessed 1 h after administration of haloperidol (1 mg/kg, i.p.), using the bar test. Values represent mean + SEM descent latency (time in seconds to remove both forepaws from the bar) during a 5 min test (n = 4-5 mice per group).

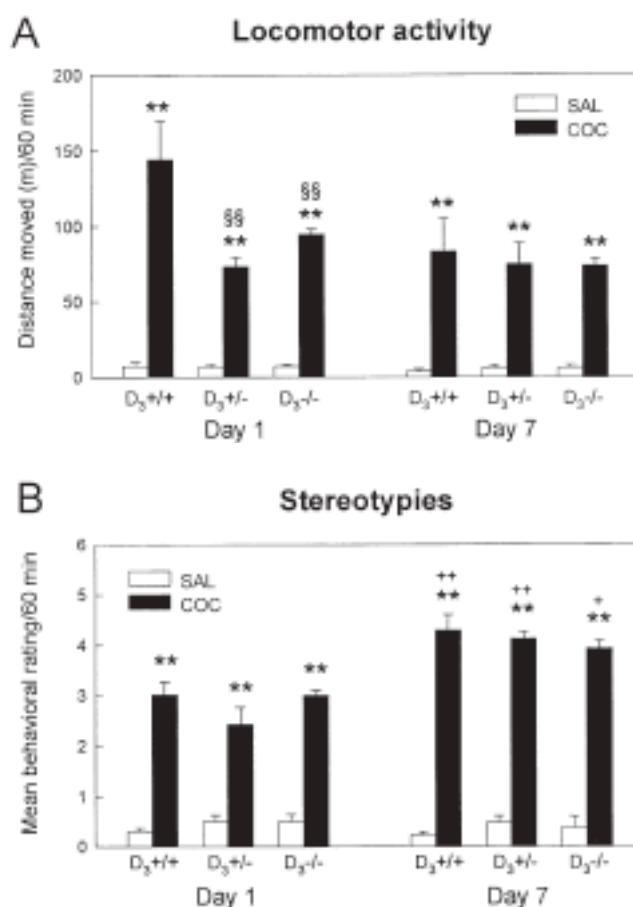


Figure 7. Effect of acute and repeated cocaine on the locomotor activity (A) and stereotyped behavior (B) of D3+/+, D3+/- and D3-/- mice. Animals received seven daily injections of cocaine (20 mg/kg, i.p.) or saline, and their behavioral response was recorded on the first (day 1) and the last (day 7) injections. A. Locomotor activity is expressed as mean \pm SEM total distance moved (m) during the first hour after the injection ($n = 6-8$ mice per group). Cocaine increased locomotion in the three groups of mice on days 1 and 7 (** $p < 0.01$ as compared to saline-treated mice of the same genotype, ANOVA followed by Tukey test), although D3+/- and D3-/- mice showed lower activity compared to wild-type animals (§§ $p < 0.01$, Tukey test). No increase in horizontal locomotion (behavioral sensitization) was observed in wild-type or mutant mice on day 7 when compared to day 1. B. Values represent mean \pm SEM behavioral rating during the first hour after the injection. Cocaine-treated mice had a higher behavioral rating than saline-treated animals on days 1 and 7 (** $p < 0.01$ as compared to saline-treated mice of the same genotype). The increased behavioral rating on day 7 indicates the development of behavioral sensitization, characterized by the appearance of stereotypes (repetitive sniffing and head bobbing) in animals exposed repeatedly to the psychostimulant (+ $p < 0.05$, ++ $p < 0.01$ as compared to mice of the same genotype on day 1).