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ORIGINAL RESEARCH ARTICLE

The dopamine D4 receptor is essential for hyperactivity and impaired behavioral inhibition in a mouse model of attention deficit/hyperactivity disorder

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The dopamine D4 receptor (D4R) is a candidate gene for attention deficit/hyperactivity disorder (ADHD) based on genetic studies reporting that particular polymorphisms are present at a higher frequency in affected children. However, the direct participation of the D4R in the onset or progression of ADHD has not been tested. Here, we generated a mouse model with high face value to screen candidate genes for the clinical disorder by neonatal disruption of central dopaminergic pathways with 6-hydroxydopamine (6-OHDA). The lesioned mice exhibited hyperactivity that waned after puberty, paradoxical hypolocomotor responses to amphetamine and methylphenidate, poor behavioral inhibition in approach/avoidance conflict tests and deficits in continuously performed motor coordination tasks. To determine whether the D4R plays a role in these behavioral phenotypes, we performed 6-OHDA lesions in neonatal mice lacking D4Rs (*Drd4*$^{-/-}$). Although striatal dopamine contents and tyrosine hydroxylase-positive midbrain neurons were reduced to the same extent in both genotypes, *Drd4*$^{-/-}$ mice lesioned with 6-OHDA did not develop hyperactivity. Similarly, the D4R antagonist PNU-101387G prevented hyperactivity in wild-type 6-OHDA-lesioned mice. Furthermore, wild-type mice lesioned with 6-OHDA showed an absence of behavioral inhibition when tested in the open field or the elevated plus maze, while their *Drd4*$^{-/-}$ siblings exhibited normal avoidance for the unprotected areas of these mazes. Together, our results from a combination of genetic and pharmacological approaches demonstrate that D4R signaling is essential for the expression of juvenile hyperactivity and impaired behavioral inhibition, relevant features present in this ADHD-like mouse model.
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Introduction

The dopamine D4 receptor (D4R) is a G-protein-coupled receptor principally expressed in the prefrontal cortex[1,2] in all mammals studied to date, including the rat, mouse, nonhuman primates and humans.[3,4] This phylogenetic conservation of prefrontal cortical expression strongly suggests a key role for the D4R in the modulation of dopamine (DA)-mediated functions in this brain area, such as the online categorization and filtering of environmental cues and the temporal organization of goal-oriented behaviors.[5] Since D4Rs are localized in both excitatory glutamatergic pyramidal neurons and inhibitory GABAergic interneurons of the prefrontal cortex,[1] it is conceivable that exaggerated or deficient D4R stimulation may alter the exquisite fine tuning of prefrontal cortical circuits. Recently, the D4R has been implicated in attention deficit/hyperactivity disorder (ADHD),[6,7] a neurodevelopmental psychiatric condition characterized by deficits in filtering irrelevant information, poor behavioral inhibition and hyperactivity.[8] Twin, adoption and segregation studies have estimated a heritability of 50–90% for ADHD[9] and several evidences indicate that genes involved in mesocortical DAergic neurotransmission may be candidates for genetic predisposition to this disorder.[10] First, impaired behavioral inhibition, loss of attention and difficulties in concentration are symptoms that indicate malfunction of prefrontal cortical circuits receiving DA innervation.[5] Second, the indirect dopamine agonists methylphenidate and amphetamine exert therapeutical benefits in ADHD patients.[11] Third, neuroanatomical imaging studies demonstrated that dopamine-rich brain areas such

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as the prefrontal cortex and dorsal striatum are smaller in ADHD patients.[12] A distinctive feature that implicates the D4R as a candidate gene for ADHD is that in several case–control and family-based association studies, particular polymorphic D4R alleles were present at higher rates among children with ADHD than in normal children.[6,7] A more recent meta-analysis of all published studies conducted with different population samples confirmed the statistical significance of this association,[13] although it also became apparent that carrying these alleles is neither necessary nor sufficient for the occurrence of ADHD. Moreover, whether the D4R is directly implicated in ADHD still remains to be determined.

Given the high prevalence of ADHD in school-age children (3–6%)[14] and that most of these young patients are medicated chronically with psychostimulants,[11] it is of fundamental interest to investigate the genetic contributions and molecular mechanisms underlying the neurodevelopmental alterations that occur during its onset and progression. Since the etiology of ADHD is completely unknown, a number of animal models have been used during the last several decades to study different aspects of the disease.[15–18] Among them, the rat lesioned neonatally with 6-hydroxydopamine (6-OHDA) has predominated because the early postnatal alteration of central DAergic pathways mimics key hallmarks of the human disease, including hyperactivity and paradoxical response to psychostimulants.[15,16,19–21] However, given the important genetic contribution in the onset of ADHD, the availability of a mouse model to test the participation of targeted gene mutations in the development of ADHD-related phenotypes is of high value. The aim of the present study is to investigate the hypothesis that the D4R is involved in the development of abnormal behaviors that are also present in ADHD. To this end, we adapted into the mouse the neonatal 6-OHDA brain lesion paradigm and demonstrated that recapitulates key features present in ADHD, including hyperactivity, psychostimulant-induced hypoactivity and deficits in behavioral inhibition. Here, we report that these phenotypes are prevented or altered by the genetic ablation of the D4R gene or the pharmacological manipulation of this receptor subtype, demonstrating a direct interaction between D4R stimulation and significant behavioral hallmarks present in this ADHD-like model.

Materials and methods

Animals

All mice tested were male sibling cohorts of CF-1 outbred mice maintained by crossing nonrelated individuals. Male *Drd4*$^{-/-}$ and their wild-type siblings were obtained by mating *Drd4*$^{+/-}$ parents backcrossed for 6–10 generations to CF-1 mice. For details concerning the generation of *Drd4*$^{-/-}$ mice, see Rubinstein *et al*.[22] Mice were housed in groups of five in an animal room at 20–22°C, under a 12 h light/dark cycle (on at 7 : 00 h), with *ad libitum* access to food and water. Animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, United States Public Health Service (USA).

Neonatal lesions with 6-OHDA

Four to six synchronized CF-1 female mice were bred to obtain a large number of cohorts. On postnatal day 2 (P2), male pups received the norepinephrine uptake blocker desipramine hydrochloride (20 mg/kg, s.c.; Sigma-Aldrich, MO, USA). After 30 min, pups were anesthetized by hypothermia (placed on ice for 1 min) and then received 25 μg of 6-OHDA hydrobromide (Sigma-Aldrich, MO, USA) dissolved in 3 μl of ascorbic acid 0.1% into one of the lateral ventricles, at 1.5 μl/min. Control mice received vehicle. Injections were performed manually by penetrating the skin and skull with a 30 G needle (Carpule, Bayer; Osaka, Japan) coupled to a 25 μl Hamilton syringe. The site of injection was determined empirically at 0.6 mm lateral to the medial sagittal suture, 1.5 mm rostral to the lambd and 1.1 mm in depth from the skin. After the injection, the pups were warmed at 37°C on a heating pad until recovery, and then randomly returned in groups of eight to their mothers. After weaning (P24) and within the following 5 days, mice were tested for spontaneous activity and then successful lesions were confirmed by HPLC or tyrosine hydroxylase (TH) immunohistochemistry (see below). After a typical injection day, 10% of the lesioned mice died before weaning, whereas 60–75% developed hyperactivity together with 80–90% DA depletion. Mice that did not meet these last criteria were excluded for the data analyses.

Behavioral tests

All experiments were performed between 13:00 h and 18:30 h under dim illumination, in a separated behavioral room where mice were transferred at least 2 days in advance.

Open field Activity boxes (Med Associates Inc., St Albans, VT, USA) coupled to a computer interface were used to assess horizontal, vertical and stereotyped activity. Animals were placed in one of the four acrylic boxes (40 × 40 × 40 cm) and horizontal and vertical activities were measured by disruption of infrared photobeams separated by 2.5 cm that cross the *x*–*y* plane at two *z*-levels. Stereotyped behavior was measured by repetitive disruptions of single infrared beams. Boxes were carefully cleaned between tests to minimize odor cues in the arena.

Plus maze This test was performed as described elsewhere.[23] Briefly, each mouse was placed in the center of the maze facing one of the closed arms. Entries and the time spent in open or closed arms were recorded for 5 min. An entry was counted only if all four paws were inside the arm. Observations were recorded manually by an investigator blinded to treatment or genotype condition.

Rotarod 8 weeks old mice, were individually placed in a neutral position on the immobile rotarod treadmill (Ugo Basile, Milan, IT, USA). The speed was increased to 16 revolutions/min, and each mouse was given a 10 min training session. After each fall, mice were repositioned on the rod. Mice were tested 2 h later for 3 min.

Ataxia Locomotor incoordination was assessed in a grid-test apparatus, comprising a $20 \times 20 \times 20$ cm clear acrylic box containing a suspended floor built with 5 mm plastic cylindrical rods separated by 1 cm. Each foot slip between two rods was counted as an error. The ratio of errors in proportion to locomotor activity was used as a measure of ataxia. Mice were injected (i.p.) with saline or 2 g/kg ethanol (20% v/v) and placed immediately into the grid-test chamber for a test duration of 30 min, with data collected in 5-min periods.

Pharmacological experiments
Basal locomotor activity of each mouse was determined prior to drug administration for 30 min. Then, mice received an i.p. injection of the test drug or vehicle and were placed again in the open field. At 5 min after the injection, recording of locomotor activity resumed. All treated mice were previously drug naive. DL-amphetamine sulfate (Sigma-Aldrich, MO, USA) and methylphenidate (Ritalin; Novartis, Argentina) were dissolved in saline (NaCl 0.9%), and PNU-101387G (provided by Pharmacia & Upjohn, MI, USA) was dissolved in 2-hydroxypropyl-β-cyclodextrin 15% (Sigma-Aldrich, MO, USA).

Neurochemical assays

DA determination by HPLC Mice were killed by cervical dislocation, the striata dissected and placed at -80°C until use. HPLC determination was performed as described previously.[22] Briefly, samples were homogenized and deproteinized in 0.2 M perchloric acid (1/40 w/v). Supernatants were injected in reversed phase column (Waters) and the electrode potential was set at $+0.7$ V. The peak heights were measured by DATA Jet Integrator (Spectra Physics) and quantified based on standard curves using DATAFIT.

TH immunohistochemistry Tissue preparation and TH detection were performed as described previously.[24] Briefly, mice were transcardiacally perfused with 4% paraformaldehyde. Brains were removed, postfixed and cryoprotected. Coronal brain sections (20 μm) were collected using a freezing sliding microtome (Leica SM2000R, Germany), incubated with a rabbit polyclonal anti-TH antiserum (Chemicon International, CA, USA) and developed with a biotinylated anti-rabbit IgG followed by an avidin—biotin–peroxidase complex (Vector Laboratories, CA, USA) and diaminobenzidine. TH-positive neurons were counted at the level of the midbrain in at least four sections per mouse.

Statistical analysis
Data were analyzed using STATISTICA-Kernel 5.5 (Statsoft, Inc.; OK, USA). Unpaired two-tailed Student's *t* test was used when only two groups were compared. One-way ANOVA followed by Tukey HSD test was used when more than two groups were compared and data were collected in a single trial. Repeated measures ANOVAs were used when data were collected in multiple trials of a single session, followed by the Fisher LSD test.

Results

Neonatal disruption of the nigrostriatal dopaminergic pathway induces hyperactivity and paradoxical response to psychostimulants
At weaning age (P24), 6-OHDA-treated mice exhibited a two-fold spontaneous hyperlocomotion when tested in an open field (Figure 1a). This increase in horizontal activity was due to a higher number of movement initiations but not to an increase in velocity. Vertical exploratory activity was also increased in DA-depleted mice although to a lower extent (58%, Figure 1b), whereas qualitative and quantitative stereotyped behavior was similar between mice treated neonatally with vehicle (control mice) or 6-OHDA (data not shown). At 5 weeks, hyperactive mice showed an irreversible nigrostriatal DA cell loss evidenced by an $83.0 \pm 2.7\%$ reduction in TH immunoreactivity in the substantia nigra pars compacta (A9; Figure 1c). TH immunoreactivity was also drastically reduced in the striatum (Figure 1d) in agreement with an 88% depletion of striatal DA contents determined by HPLC (Figure 1e, left). DAergic neurons in the ventral tegmental area (A10) were less vulnerable to the toxin showing a reduction of $34.7 \pm 3.3\%$ (Figure 1c). Hyperlocomotor scores in 6-OHDA-treated mice persisted for several weeks, but waned during puberty until reaching normal levels that were maintained throughout adulthood (Figure 1f), despite the fact that striatal DA contents remained below 15% (Figure 1e, right).

The two-fold difference in horizontal activity scores between control and 6-OHDA-lesioned mice persisted for 60 min and even after an i.p. injection of saline (Figure 2a). We then tested a different group of mice with amphetamine or methylphenidate, two psychostimulants that increase extracellular DA levels by inducing DA release from neuronal terminals or by blocking the DA reuptake transporter, respectively. When given to control mice, these two drugs induced a considerable increase in locomotor activity (Figure 2b and c). Conversely, the same doses of amphetamine (4 mg/kg, i.p.) or methylphenidate (10 mg/kg, i.p.) induced a paradoxical hypolocomotor effect when given to DA-depleted mice (Figure 2b and c, respectively). No stereotypic behaviors were observed in either control or 6-OHDA-lesioned mice at these doses of amphetamine and methylphenidate.

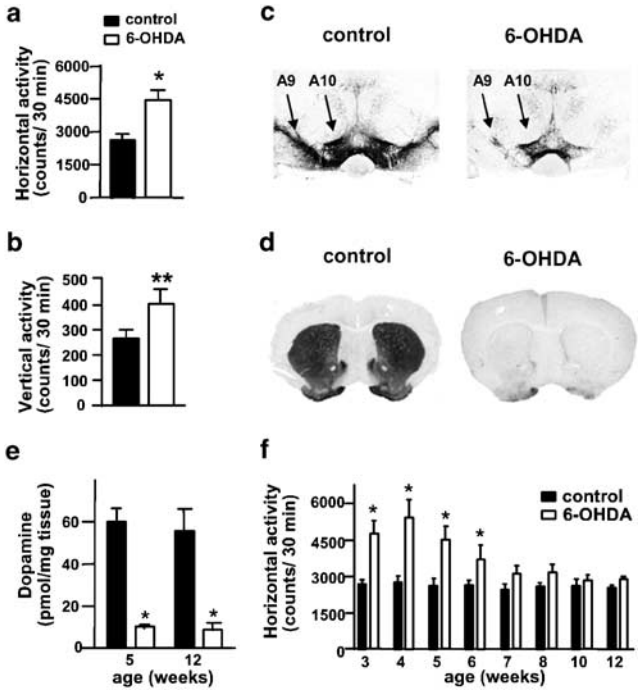

Figure 1 Neonatal disruption of the nigrostriatal dopaminergic pathway. Horizontal (a) and vertical (b) spontaneous activity were enhanced in 6-OHDA-neonatal-lesioned mice ($n=11$) compared to their control siblings ($n=12$) when tested in the open field at weaning age (P24). (c) TH immunohistochemistry in coronal brain slices of 5-week-old mice at the level of the substantia nigra pars compacta (A9) and ventral tegmental area (A10) or the striatum (d). (e) Striatal DA contents in control and 6-OHDA-lesioned mice at 5 weeks (left; control, ($n=6$); 6-OHDA, ($n=8$)) or 12 weeks old (right; ($n=5$) in both groups). (f) Horizontal activity of control and 6-OHDA-lesioned mice evaluated at weekly intervals in a longitudinal design and comparisons were performed between subjects (($n=6$) in all groups, $^{*}P<0.001$, $^{**}P<0.01$ *vs* control; Student's *t* test). Bars represent the mean + SEM. Black bars represent control mice and white bars represent 6-OHDA-treated mice.

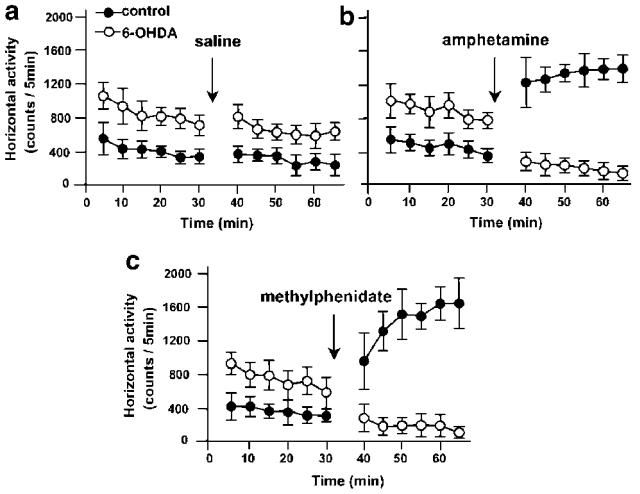

Figure 2 Paradoxical hypolocomotor response to psychostimulants in DA-depleted mice. (a) DA-depleted mice ($n=6$) showed a two-fold hyperactivity compared to control mice ($n=6$) at 4 weeks (group difference, $F(1,21)=27.71$, $P<0.001$, repeated measures ANOVA), which persisted after a saline injection. (b) Amphetamine (4 mg/kg, i.p.) induced hyperlocomotion in control mice ($n=6$) but hypolocomotion in 6-OHDA-lesioned mice ($n=6$) (amphetamine × group interaction, $F(1,10)=184.58$, $P<0.0001$, repeated measures ANOVA; followed by the Fisher LSD test, effect of amphetamine in control mice $P=0.001$; in 6-OHDA-lesioned mice $P<0.001$). (c) A similar effect was observed with the administration of methylphenidate (10 mg/kg, i.p.) (methylphenidate × group interaction, $F(1,13)=358.45$, $P<0.0001$, repeated measures ANOVA; Fisher LSD test: control $P<0.001$ ($n=8$); 6-OHDA $P=0.003$, $n=7$)). Circles represent the mean ± SEM.

Stimulation of D4Rs plays a critical role in hyperactivity

To investigate whether the D4R was implicated in the behavioral and pharmacological phenotypes observed in this mouse model, we treated P2 *Drd4*$^{-/-}$ male mice and their wild-type siblings with 6-OHDA or vehicle. Neonatal 6-OHDA treatment was equally effective in *Drd4*$^{-/-}$ mice as in wild-type mice to disrupt nigrostriatal DA neurons as evidenced by the decrease in striatal DA contents determined at 5 weeks (control *Drd4*$^{-/-}$: 55.37 ± 1.50, $n=6$ *vs* 6-OHDA *Drd4*$^{-/-}$: 8.12 ± 1.02 pmol/mg tissue, $n=6$; 85% depletion). Interestingly, neonatally DA-depleted *Drd4*$^{-/-}$mice did not develop hyperlocomotion, but displayed identical horizontal activity scores to those observed in control mice of both genotypes (Figure 3). When treated with amphetamine (4 mg/kg, i.p.), control *Drd4*$^{-/-}$ mice showed a classical hyperlocomotor effect, whereas 6-OHDA-lesioned *Drd4*$^{-/-}$ mice decreased their locomotor activity scores by 50% (Figure 3b) as was observed in their wild-type siblings (Figure 3a). A control group of *Drd4*$^{-/-}$ mice showed no difference in locomotor activity after receiving saline i.p. (data not shown), similar to what we observed with wild-type mice (Figure 2a).

To further study the importance of D4R stimulation in hyperactivity and paradoxical response to psychostimulants observed in this mouse model, we treated wild-type 6-OHDA-lesioned mice with the D4R antagonist PNU-101387G.[25] A working dose of 10 mg/kg, i.p. was selected after pilot studies showed no effects between 1 and 5 mg/kg and lack of selectivity above 10 mg/kg because of noticeable hypolocomotor effects in *Drd4*$^{-/-}$ mutant mice. Control mice reduced their spontaneous locomotor activity scores by 50% after receiving PNU-101387G (10 mg/kg, i.p.), but still displayed amphetamine-induced hyperlocomotion (Fig 3c). This is different from what we have observed using the nonselective D2-like antagonist haloperidol that induced bradykinesia, and also prevented amphetamine-induced hyperactivity (data not shown). In DA-depleted mice, hyperactivity was reverted by PNU-101387G to locomotor scores similar to those observed in control

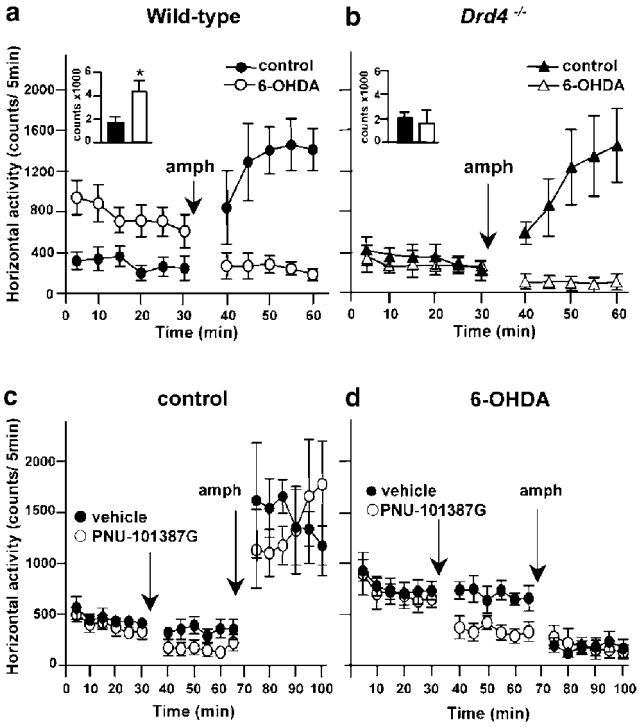

Figure 3 Stimulation of D4Rs plays a critical role in hyperactivity. (a,b) Wild-type and *Drd4*$^{-/-}$ mice, 4 weeks old, treated with vehicle (control) or 6-OHDA were tested for spontaneous locomotor activity during 30 min and then injected with amphetamine (4 mg/kg, i.p.). 6-OHDA-lesioned *Drd4*$^{-/-}$ mice did not display the characteristic hyperlocomotion observed in wild-type-lesioned siblings (genotype × lesion interaction, $F(1,35)=32.02$, $P<0.0001$, repeated measures ANOVA). Amphetamine enhanced locomotion in control mice of both genotypes (Fisher LSD test, for wild type: $P=0.008$ ($n=10$); *Drd4*$^{-/-}$: $P=0.0018$ ($n=10$)) and reduced activity in 6-OHDA-lesioned mice of both genotypes (Fisher LSD test for wild type: $P=0.024$ ($n=8$); *Drd4*$^{-/-}$: $P=0.014$ ($n=11$)). Insets show the total horizontal activity during the first 30 min. (c,d) The D4R antagonist PNU-101387G (10 mg/kg, i.p.) or vehicle were administered to wild-type control (c) and 6-OHDA -lesioned (d) mice after 30 min of spontaneous locomotor activity. PNU-101387G reduced locomotor activity in both groups (drug effect in control mice: $F(1,10)=5.53$, $P<0.04$ ($n=7$); in 6-OHDA: $F(1,9)=10.04$, $P<0.001$ ($n=6$), repeated measures ANOVA)]. PNU-101387G pretreatment did not affect the response to amphetamine in either group. Symbols represent the mean ± SEM.

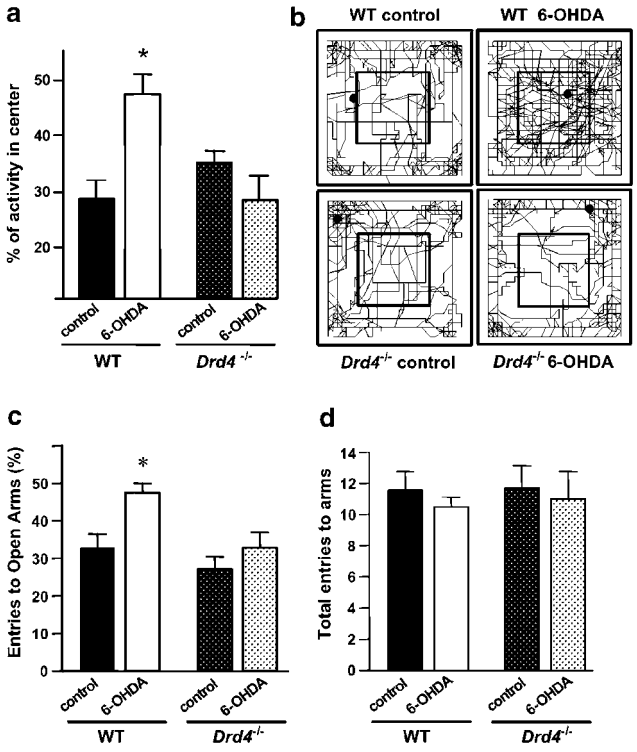

Figure 4 DA-depleted wild-type (WT) but not *Drd4*$^{-/-}$ mice exhibit attenuated behavioral inhibition. (a,b) Horizontal activity in the open field was quantified in the central area and the periphery during the first 10 min in 4-week-old mice. (a) Percentage of distance traveled in the central area by wild-type and *Drd4*$^{-/-}$ mice lesioned with vehicle (control; WT $n=6$, *Drd4*$^{-/-}$ $n=4$) or 6-OHDA (WT $n=6$, *Drd4*$^{-/-}$ $n=5$) ($F(3,31)=7.154$, one-way ANOVA followed by Tukey's HSD test, $^{*}P<0.01$). (b) Representative trajectories of mice from each group. (c,d) Mice, 8 weeks old, from the four groups were challenged in the elevated plus maze. (c) Percentage of entries into open arms ($F(3,39)=7{,}58$; one-way ANOVA followed by Tukey's HSD test, $^{*}P<0.01$); (d) Total entries into all arms showed no difference among all groups (WT: control $n=10$, 6-OHDA $n=10$; *Drd4*$^{-/-}$: control $n=6$, 6-OHDA $n=6$). Bars represent the mean + SEM.

mice receiving vehicle (Figure 3d). Amphetamine did not produce a further decrease in locomotor activity scores in the 6-OHDA- and PNU-101387G-treated mice probably due to a floor effect (Figure 3d).

Impaired behavioral inhibition in DA-depleted mice is dependent on D4R stimulation

A topographical analysis of mouse activity across the *x*–*y* axes of the open field revealed that whereas control mice avoided crossing the central area (28% of the total distance traveled) and instead preferred the periphery and, in particular, the corners; 6-OHDA-lesioned mice spent 47% of the traveled distance in the central zone (Figure 4a and b). In contrast, analysis of the activity pattern in *Drd4*$^{-/-}$ mice revealed that both control and 6-OHDA-lesioned mutant mice avoided visiting the central area of the open field (Figure 4a and b). Together, these results suggested poor behavioral inhibition of wild-type 6-OHDA-lesioned mice to enter into a less protected part of a novel environment. The normal behavior observed in DA-depleted mice lacking D4Rs indicated to us that the behavioral impairment shown by 6-OHDA-lesioned wild-type mice is dependent on D4R stimulation.

To further study the effects of neonatal DA denervation and the participation of the D4R in behavioral inhibition, we challenged mice of both genotypes on the elevated plus maze, another

approach/avoidance conflict test that is solved on the basis of a proper evaluation of environmental cues. All four experimental groups corresponded to 8-week-old mice that showed normal locomotor scores in the open field. Whereas control wild-type mice showed higher avoidance for the open unprotected arms of the maze, 6-OHDA-lesioned mice displayed no preference for either type of arm, indicating an abnormal evaluation of the potential risk existing on the open arms of the maze (Figure 4c). Conversely, *Drd4*$^{-/-}$ mice treated neonatally with 6-OHDA avoided entering into the open arms, indicating a normal appraisal of the riskier environment as exhibited by control mice from both genotypes. The total number of entries to all arms was not different among the four groups, indicating that locomotor activity and motivation to explore the arms of the maze were similar across treatments and genotypes (Figure 4d). The results observed in the open field and the elevated plus maze suggest that the deficits in behavioral inhibition observed in 6-OHDA-lesioned mice depend on D4R signaling.

Continuous motor performance is impaired in DA depleted mice in a D4R-independent manner

To assess whether DA depletion impaired continuously performed motor coordination, we challenged 8-week-old control and 6-OHDA-treated mice on the Rotarod. 6-OHDA-treated mice performed poorly compared to their control siblings as evidenced by a higher number of falls and reduced latency to fall from the rotating rod (Figure 5a and b, left). Similarly, *Drd4*$^{-/-}$ control mice displayed normal continuous motor coordination, whereas *Drd4*$^{-/-}$ 6-OHDA-lesioned mice showed deficits in the Rotarod as severe as their wild-type counterparts (Figure 5a and b, right). When tested in a foot-slip error paradigm used to score for ataxia, control and 6-OHDA-lesioned mice showed a low number of unforced errors (Figure 5c) that increased identically in both groups after ethanol treatment. Together, these results indicate that 6-OHDA-lesioned mice exhibited a motor coordination deficit only in continuous executive tasks and that this deficit is independent of the presence of D4Rs.

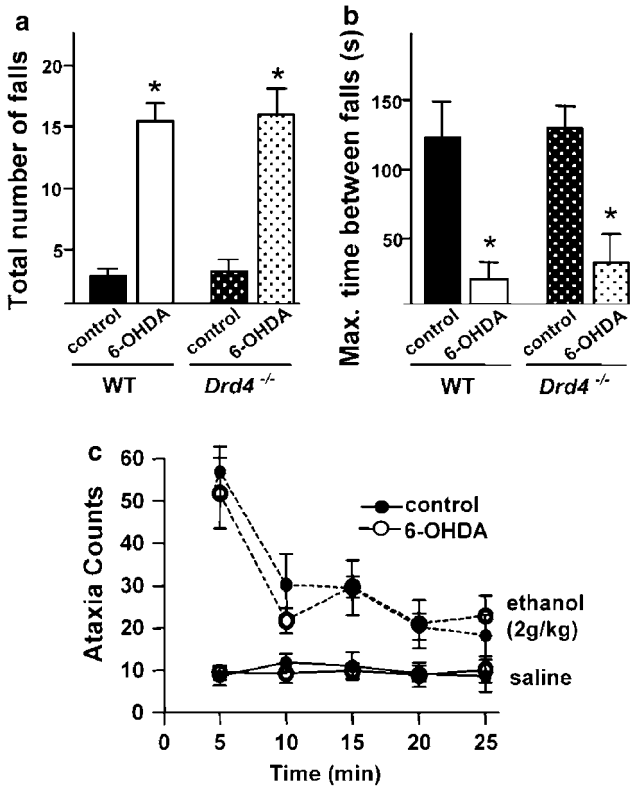

Figure 5 Continuous performance in a motor coordination task. The number of falls (a) and maximum time between falls (b) were counted during a 3 min test in 8-week-old control and 6-OHDA-lesioned mice of both genotypes (one-way ANOVA followed by Tukey's HSD test, $^*P<0.001$; WT: control $n=5$, 6-OHDA $n=6$; *Drd4*$^{-/-}$: control $n=6$, 6-OHDA $n=6$). (c) Ataxia ratios (errors $\times 10$/activity counts) were assessed during 5 min in wild-type control ($n=5$) and 6-OHDA-lesioned mice ($n=7$) receiving saline (filled line) or ethanol (2 g/kg, i.p., dotted line). Bars and circles represent the mean $\pm$ SEM.

Discussion

Challenging the D4R candidate gene hypothesis in a mouse model for ADHD

With the aim of studying the participation of the D4R in the development of behavioral symptoms related to ADHD, we used a combination of genetic and pharmacological approaches in a mouse model produced by neonatal lesion of midbrain DAergic neurons. Several behavioral hallmarks observed in children with ADHD were reproduced in this model: (1) juvenile mice (3–6 weeks old) displayed spontaneous hyperactivity, (2) mice responded with a paradoxical hypolocomotor effect to psychostimulant drugs such as amphetamine or methylphenidate, (3) hyperactivity started to wear off at puberty, although other signs of the syndrome persisted throughout adulthood, (4) DA proved to be implicated in this mouse syndrome since its contents were severely diminished in the striatum at all ages, (5) mice displayed poor behavioral inhibition evidenced by an impaired reaction to aversive contextual cues in approach/avoidance conflict paradigms, (6) mice showed difficulties in executing a continuous performance motor coordination task without signs of ataxia. Manifestation of this syndrome was only observed when striatal DA levels fell between 80 to 90% from control values; milder DA depletions were overcome by compensatory mechanisms, whereas more severe DA denervation led to a state of akinesia and aphagia that was observed at higher doses (50–60 μg of 6-OHDA) in the initial pilot studies aimed at finding the appropriate working dose (data not shown).

By using mutant mice lacking D4Rs, we observed that stimulation of D4Rs was essential for the developmental onset of juvenile hyperactivity follow-

ing a neonatal 6-OHDA lesion. In contrast to the hyperactivity observed in wild-type lesioned mice, locomotor activity scores of 6-OHDA-lesioned *Drd4*$^{-/-}$ mice were normal and indistinguishable from those of wild-type or *Drd4*$^{-/-}$ control mice at all ages tested. However, mice of both genotypes experienced an equivalent degree of DA depletion and denervation induced by the neurotoxin. In addition, administration of the selective D4R antagonist PNU-101387G to wild-type lesioned mice decreased hyperlocomotion to normal activity levels, confirming the importance of D4R signaling for the expression of hyperactivity. At the dose used, PNU-101387G appeared to block D4Rs selectively not only because it produced no locomotor effects in *Drd4*$^{-/-}$ mice (data not shown) but also because it allowed amphetamine to induce hyperlocomotion in control mice in contrast to the classical nonselective 'D2-like' blockers that prevented the reversal of bradikinesia even at high doses of amphetamine (data not shown). Therefore, either the chronic absence or acute blockade of D4Rs revealed a net deficit in DA-mediated motor control that may be beneficial to attenuate neurodevelopmental-induced hyperactivity. In addition, PNU-101387G reduced locomotor scores in nonlesioned mice, indicating that D4Rs also participate in the overall locomotor activity of normal mice. These interpretations are in agreement with our initial studies performed on *Drd4*$^{-/-}$ mice (C57Bl/6J $\times$ 129SvEv F2s) that showed a small but significant reduction in spontaneous horizontal activity compared to their wild-type siblings.[22] Attenuation of hyperactivity in 6-OHDA-lesioned rats has also been observed with other D4R antagonists,[21] and whether the use of D4R blockers will provide therapeutical benefits to ADHD-affected children remains to be investigated.

Other reported mouse models of hyperactivity such as the DA transporter (DAT) knockout[17] and DAT hypomorph mice[18] are associated with elevated levels of synaptic DA in which DAT deficiency leads to hyperactivity that persists throughout adulthood. In DAT knockout mice, the calming effect of psychostimulants has been interpreted as an increase in 5-HT transmission, whereas in the DAT knockdown mice the hypothesis of autoreceptor stimulation that decreases DA release has been favored. The mechanism of psychostimulants' therapeutic 'calming' effects in ADHD children is an important area of debate.[26] It is unlikely that DA autoreceptors play a role because it has been demonstrated that indirect dopamine agonists do not exert a preferential action on presynaptic DA receptors[27] and, particularly in our hypodopaminergic model the density of DA terminals is too drastically diminished to have such a robust effect. However, we cannot rule out a 5-HT-related mechanism because we have observed a significant increase in 5-HT terminal density and 5-HT levels in the striatum in the lesioned mice (data not shown) as has been observed in rats.[28] Although there is sufficient evidence to support the idea that an animal model that resembles at least part of the developmental features of ADHD must have impaired DAergic transmission, it is not clear whether a hyper- or a hypodopaminergic state is present in ADHD.[10] Recent studies using functional magnetic resonance imaging in brains of ADHD children sitting still[12] or performing a continuous task[29] showed smaller sizes of DA target areas, including the prefrontal cortex and striatum and deficits in the basal ganglia.

D4R deficiency was also demonstrated to be critical in a phenotype observed in this mouse model that may be interpreted as lack of proper behavioral inhibition. The open field and the elevated plus maze are two approach/avoidance conflict tests in which mice evaluate the potential rewards or risks associated with safer or less safe areas of the novel arenas. Control mice of both genotypes showed aversion to explore the unprotected areas of these mazes. Remarkably, wild-type mice lesioned with 6-OHDA did not manifest any behavioral sign of such a conflict entering indiscriminately into either protected or unprotected areas. Together, these results may be interpreted as if the lesioned mice underestimated the potential risks existing in the unprotected zones of the maze or, alternatively, the lesioned mice developed a deficit to inhibit their approaching behavior when facing a 'no-go' signal. Poor behavioral inhibition is probably the most consistent hallmark of ADHD-affected children[11] and is often diagnosed after performance deficits are revealed in go/go-no tests,[30] a sign attributed to prefrontal malfunction. Strikingly, D4R-deficient mice lesioned with 6-OHDA exhibited a normal reaction to the relatively riskier environment present in the unprotected zones of both mazes. Therefore, both relevant motor and emotional features of this ADHD-like mouse model depend on *Drd4* expression. The observation of impaired behavioral inhibition in mice lesioned with 6-OHDA was not appreciated previously in the rat[16] and it strengthens its usefulness as an ADHD-like model. It still remains to be determined whether the 6-OHDA-lesioned mouse will be a valuable tool to study the role of candidate genes in attention deficit and impulsivity.

Possible neurodevelopmental mechanism involving the D4R

In rodents, DAergic activity of nigrostriatal neurons during the first 2 weeks of postnatal development is critical for the final maturation of corticostriatal excitatory synapses by decreasing the probability of glutamate release.[31] Selective disruption of this DAergic pathway during this time frame maintains an elevated efficacy of glutamatergic transmission.[32] Thus, the behavioral abnormalities observed in mice exposed neonatally to 6-OHDA are probably due to insufficient DA-mediated maturation of corticofugal glutamatergic synapses projecting to the striatum and/or nucleus accumbens.[32,33] The absence of hyperlocomotion observed in 6-OHDA-lesioned *Drd4*$^{-/-}$ mice together with the lack of impaired behavior in approach/avoidance conflict tests are the strongest

behavioral phenotypes identified to date in these knockout mice. These salient phenotypes have to be interpreted in light of the role that the D4R plays within the prefrontal cortex and subcortical circuits of the basal ganglia. D4Rs are expressed in both glutamatergic corticofugal neurons as well as in GABAergic cortical interneurons[1] which may exert a negative regulation over the excitatory pyramidal neurons. Since D4Rs induce neuronal hyperpolarization by activating G-protein coupled inwardly rectifying K^+ channels,[34] we postulate that D4R signaling buffers cortical excitability. 6-OHDA toxic effects are not as severe in DA neurons of the VTA compared to the substantia nigra, suggesting that cortical and limbic D4Rs are still being stimulated by DA in lesioned mice. In addition to the residual DA present in 6-OHDA-lesioned mice, norepinephrine neurotransmission may contribute to D4R-mediated effects because norepinephrine terminals are present in the prefrontal cortex and striatum and this transmitter showed to be equipotent with DA to bind to D4Rs and to stimulate D4R-mediated adenylyl cyclase inhibition.[35] Therefore, a lack of D4R-mediated inhibition of GABAergic cortical interneurons would indirectly diminish the exaggerated glutamatergic input into the basal ganglia and limbic system allowing the expression of normal behavioral inhibition and locomotion. Alternatively, the absence of D4R stimulation in ventropallido-thalamic GABAergic neurons[2] may increase the inhibition of glutamatergic thalamic input into the cortex[36] providing a similar effect. Other phenotypes present in this ADHD-like mouse model that are hallmarks of the human disease such as paradoxical response to psychostimulants and deficits in continuously performed tasks of motor coordination are independent of D4R stimulation, probably because they are more directly related to intrinsic striatal mechanisms involving other DA receptors or neurotransmitters.

Together, the results reported here demonstrate that neonatal 6-OHDA-lesioned mice constitute a valuable platform to study the importance of individual candidate genes for the occurrence of ADHD and, in particular, that the D4R plays a direct role in the establishment of critical aspects of this model. Therefore, it is tempting to speculate that the various human polymorphic variants of the D4R participate differentially during the onset and maturation of brain circuits that may be altered in the human disease.

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