European Journal of Neuroscience, Vol. 15, pp. 158–164, 2002

Absence of dopamine D4 receptors results in enhanced reactivity to unconditioned, but not conditioned, fear

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Keywords: anxiety, dopamine receptors, fear, frontal cortex, knockout mice, mouse

Abstract

The prefrontal cortex receives a major dopaminergic input from the ventral tegmental area, which plays an important role in the integration of neuronal signals influencing behavioural responses to stressful environmental stimuli. The dopamine D4 receptor (D4R) is expressed at highest levels in the prefrontal cortex and is the predominant D2-like receptor localized in this brain area. To investigate the functional significance of D4Rs in dopamine-mediated responses we have analysed a strain of mice lacking this receptor subtype (*Drd4*$^{-/-}$). Wild-type and *Drd4*$^{-/-}$ mice were challenged in two different approach/avoidance conflict paradigms: the elevated plus maze and the light/dark preference exploration test. By these behavioural measures *Drd4*$^{-/-}$ mice showed heightened avoidance to the more fear-provoking areas of each maze as demonstrated by reduced exploration of the open arms of the plus maze and longer latencies to explore the illuminated compartment of the light/dark shuttle box. These exaggerated avoidance behaviours were further enhanced by an additional handling stress but completely prevented by anxiolytic agents such as the benzodiazepine midazolam and ethanol. Although *Drd4*$^{-/-}$ mice displayed heightened anxiety, they exhibited normal ethanol preference and consumption in a two-bottle choice test. Learned fear responses evaluated by contextual, cued and instrumental fear-conditioning tests showed no difference between wild-type and *Drd4*$^{-/-}$ mice. Taken together these results indicate that the absence of D4Rs increases avoidance behaviour to unconditioned stimuli and does not impair behavioural reactions to Pavlovian fear-conditioning, suggesting that the D4R could play a key role in the dopaminergic modulation of cortical signals triggered by environmental stimuli.

Introduction

The prefrontal cortex (PFC) participates in the temporal organization and execution of motor behaviours through the integration of diverse neuronal signals that are capable of detecting, with a unique sensitivity, subtle changes in environmental stimuli and contextual cues (Fuster, 1997). Mesoprefrontal dopamine (DA) transmission plays a key role in the modulation of these signals influencing complex processes such as arousal, attention, vigilance, novelty-induced stress and learning (LeMoal & Simon, 1991; Williams & Goldman-Rakic, 1995; Bardo *et al*., 1996). This DA system exerts an exquisite susceptibility to react to stress by increasing DA release and turnover rate (Roth *et al*., 1988). Selective lesions and pharmacological manipulations that modify DA transmission in the PFC impair alertness, behavioural reactions to stress and working memory performance (Simon *et al*., 1980; Roth *et al*., 1988; Williams & Goldman-Rakic, 1995; Espejo, 1997).

D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors have been identified in the PFC (Goldman-Rakic, 1996; Khan *et al*., 1998) and electrophysiological studies have provided evidence for both inhibitory and excitatory actions following the stimulation of DA receptors on PFC neurons (Cepeda *et al*., 1992; Goldman-Rakic, 1996). Whereas D1-like agonists promote seizure activity, stimulation of D2-like DA receptors is anticonvulsant, diminishing the electrical excitability of the PFC (Starr, 1996). Conversely, typical and atypical neuroleptics that block D2-like receptors can induce epilepsy in a dose-dependent manner presumably by lowering seizure threshold (Starr, 1996). Of the DA receptor subtypes present in the PFC the dopamine D4 receptor (D4R) is of fundamental interest because it is expressed at its highest density in this brain area (Mrzljak *et al*., 1996; Ariano *et al*., 1997) and is the most abundant cortical D2-like receptor (Khan *et al*., 1998). Although the relative abundance of D4Rs in total brain is low, this unique prefrontal cortical localization of D4Rs in rat, mouse, nonhuman primate and man has been substantiated by numerous techniques (reviewed in Tarazi & Baldessarini, 1999 and Oak *et al*., 2000). The phylogenetic conservation of area-specific D4R expression strongly suggests a priveleged function for this receptor subtype in cortical function. In addition, the D4R has been implicated in the pathophysiology of psychiatric diseases that involve PFC malfunction such as schizophrenia (Seeman *et al*., 1993) and attention deficit with hyperactivity disorder (LaHoste *et al*., 1996; Swanson *et al*., 1998; Faraone *et al*.,

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Received 19 June 2001, revised 14 November 2001, accepted 16 November 2001

2001). Lack of proper inhibitory control is one of the hallmarks of these psychiatric conditions characterized by distractibility and loss of attention in the former and hallucinations and perseverant behaviour in the latter.

Unraveling the mechanisms by which DA modulates cortical excitability will allow an improved understanding of behavioural reactions to environmental stimuli in normal conditions and in disease. *In vitro* studies have demonstrated that activated D4Rs couple to inwardly rectifying potassium channels that hyperpolarize neurons (Werner *et al*., 1996). More recently, we have shown that mice lacking D4Rs display increased cortical excitability in pyramidal neurons recorded from PFC slices and are supersensitive to the convulsant effect of the $GABA_A$ blocker bicuculline (Rubinstein *et al*., 2001). These results have been strengthened by the fact that the D4R antagonist PNU-101387G increased pyramidal cell activity in PFC slices taken from normal mice but not from D4R deficient mice (Rubinstein *et al*., 2001). Therefore, given that the D4Rs are relatively abundant in the PFC and that they participate in setting the fine tuning of the threshold levels of cortical excitability it was of interest to evaluate the behavioural performance of mice deficient in D4Rs when exposed to different approach/avoidance conflicts and to increasing levels of stressful stimuli.

Materials and methods

Behavioural studies

All mice tested were sibling cohorts of 8–12-week-old F2s that weighed 20–35 g and derived from the crossing of $Drd4^{+/-}$ mice (129/Ola × C57Bl/6J) for more than 10 generations. Molecular, immunocytochemical, neurochemical and pharmacological data indicate the total absence of functional D4Rs in dopamine D4 receptor-deficient mice ($Drd4^{-/-}$) mutant mice (Rubinstein *et al*., 1997; Defagot *et al*., 2000; Rubinstein *et al*., 2001). For details concerning the generation of $Drd4^{-/-}$ mice see Rubinstein *et al*. (1997). Animals were housed in same-sex groups of five or six with free access to food and water with a 12-h light : 12-h dark cycle (lights on at 07.00 h). Experiments were performed between 1400 and 1900 by individuals who were blind to the animal's genotype. All animal procedures were performed in accordance with the ethical standards set by both the Animal Care Committee of the School of Sciences of the University of Buenos Aires, Argentina and the guidelines for the care and use of laboratory animals of the National Institutes of Health (US).

Elevated plus maze

This test was conducted in a dimly illuminated room where the animals were habituated for 2 h in their home cages. For the test, mice were individually removed and placed on the centre of the elevated plus maze custom-made apparatus 50 cm above the floor. Each arm of the maze is 5 cm wide and 30 cm long with black acrylic parts. The closed arms have transparent acrylic walls, 12 cm high; the open arms have 2-mm rims. Entries into all arms and the time spent on the open or closed arms were recorded for 5 min. An entry was counted only if all four paws were inside the arm. Solutions of ethanol and midazolam (Hoffmann-La Roche, Switzerland) were prepared in physiological saline (0.9% NaCl). Because no gender differences were observed within genotypes, experimental groups contained 50% of mice of each gender.

Light/dark exploration test

A custom-made two-chamber shuttle box containing a dividing wall with a 4 × 5-cm hole in the centre that allows mice free access to both sides was used. Each chamber is 20 (w) × 26 (l) × 14 cm (h) with opaque walls and normal cage bedding on the floor. Mice were placed on one side of the shuttle box that was then immediately covered by a black acrylic ceiling. The other chamber received ambient illumination. Entries to both compartments were recorded during a 5-min test period to determine the length of time prior to entering the lit compartment (latency) and the percentage of time spent on the illuminated side. This test was performed with male and female mice as above.

Two-bottle choice test

Male mice were individually caged 48 h before beginning the study where they had free access to regular water bottles. During the nine following days mice had free access to two inverted graduated cylinders containing either water or 10% ethanol. Each day the consumed volumes were evaluated between 10.00 and 11.00 h, fluids were discarded and they were filled with fresh solutions. The position of the bottles was reversed daily to control for side preference and mice were weighed to calculate ethanol consumption (in g/kg).

Fear conditioning

Context- and tone-dependent fear conditioning experiments were conducted in a small rodent custom-made chamber made of black acrylic (30 × 30 × 20 cm) containing 5-mm stainless steel rod floor, spaced 1 cm, through which scrambled foot shocks could be administered. Animals were placed in the conditioning context for 3 min and then presented with three tone–shock pairings 3 min apart. Tone duration was 10 s at 80 dB immediately followed by a 0.5-mA 1-s shock. Place conditioning testing was conducted 24 h after the training in the same chamber for 3 min. Testing of tone conditioning was performed 2 h later in a different acrylic transparent chamber (30 × 22 × 16 cm) and room where mice were exposed to the same tone as the previous day. Contextual and cued freezing were assessed when the animal persisted for > 2 s without moving by an observer blind to the mouse genotype.

Passive avoidance

Male mice were habituated to a dimly illuminated room for 2 h. They were then placed on the elevated and brightly illuminated platform (9 × 6 cm) of a custom-made step-through two-chamber shuttle box. A 4 (w) × 3 cm (h) hole communicates to a dark chamber made of black acrylic containing a 5-mm stainless steel rod floor, spaced 1 cm apart. On the first day mice received one footshock (0.5 mA, 1 s) immediately after entering with their four paws into the dark chamber. Latency to enter the dark compartment was recorded. After the shock, mice were removed and then returned to their homecage. Twenty-four hours later mice were placed again on the bright platform and latency to reenter into the dark chamber was recorded. No shock was delivered on the second day.

Results

Drd4$^{-/-}$ *mice exhibit increased anxiety in approach/avoidance conflicts*

Novel environmental stimuli can elicit approach/avoidance conflicts that activate DA transmission in the rodent's PFC (Tassin *et al*., 1980; Roth *et al*., 1988). With the demonstration that most D4R immunoreactivity in the rat and mouse brain is found in cortical

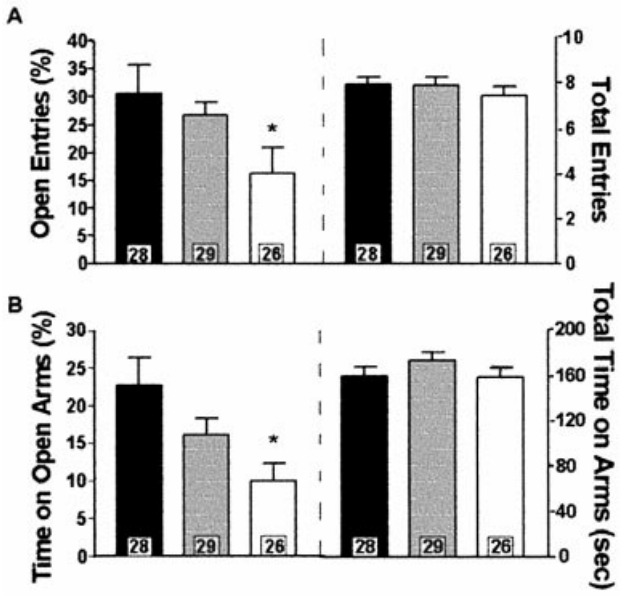

FIG. 1. *Drd4*$^{-/-}$ mice displayed increased avoidance to the unprotected arms of the elevated plus maze. (A, left) The percentage of entries into the open arms, (A, right) the total number of entries into all four arms, (B, left) the percentage of time spent on the open arms and (B, right) the total time spent on all four arms of an elevated plus maze were calculated for wild-type (black), heterozygote (grey) and homozygote mutant (white) mice. Group sizes are indicated within each bar. Bars represent the mean + SEM. ANOVAs followed by Dunnett's *t*-test revealed significant differences between wild-type and mutant mice (*$P < 0.05$ vs. wild-type group under the same conditions).

FIG. 2. Intense handling further increased avoidance to the open arms of the elevated plus maze in wild-type and *Drd4*$^{-/-}$ mice. Percentage of entries into the open arms of the maze of wild-type and D4R-deficient mice that received or did not receive an i.p. saline injection 5 min prior to the test. Analysis of the data was performed by ANOVA followed by a Dunnett's *t*-test. (*$P < 0.05$ vs. wild-type group under the same conditions; $^{\S}P < 0.05$ vs. control mice from same genotype.)

neurons (Ariano *et al*., 1997; Rubinstein *et al*., 2001) it was of interest to compare the behavioural responses of wild-type and D4R-deficient mice in different stressful conflict paradigms that have been ethologically and pharmacologically validated for quantitating anxiety in rodents.

When challenged in the elevated plus maze (Dawson & Tricklebank, 1995) all mice showed a preference for the closed arms; however, *Drd4*$^{-/-}$ mice made significantly fewer open arm entries and spent less time in the open arms (Fig. 1A and B, left). Interestingly, prior to entering an open arm both wild-type and mice heterozygous for the mutant *Drd4* allele carefully inspected the zone of transition adopting a crouch-down position for several seconds before deciding whether to enter or retreat from the open arms. Homozygous mutant mice engaged in a more intense exploratory behaviour as evidenced by repetitive forward and backward movements of the head and shoulders accompanied by pronounced sniffing. The total number of entries into either the open or closed arms was similar between the three genotypes, indicating that the reduced number of open-arm entries exhibited by the mutant mice cannot be interpreted as a locomotor or motivational deficit (Fig. 1A, right). Similarly, there were no differences among genotypes in total time spent in the arms (Fig. 1B, right) despite the significant reduction in the percent time spent in the open arms by *Drd4*$^{-/-}$ mice (Fig. 1B, left). These results were consistently observed over the course of the analysis of four different cohorts of mice ($n = 6$–8 per genotype) performed over 8 months. In each individual experiment mouse groups were equally sex- and age-matched (see Materials and methods) and the reduction in percentage of entries into the open arms reached statistical significance in each trial ($P < 0.05$). Therefore, we decided to collapse all data as presented in Fig. 1. As a consequence of the apparent increased appraisal of potential risk only nine out of 26 (34.6%) *Drd4*$^{-/-}$ mice stepped out onto the open arms more than once whereas 20 out of 28 (71.4%) wild-type mice (Fig. 1A, left) entered onto the open arms more than once during the test (χ^2 $P = 0.04$).

Several procedures like intense handling are known to elevate anxiety in mice. To evaluate how D4R-deficient mice would react to an additional stressful stimulus we tested a new cohort of wild-type mice and their *Drd4*$^{-/-}$ littermates that received an intraperitoneal saline injection 5 min before being placed on the centre of the elevated plus maze. Both wild-type and *Drd4*$^{-/-}$ mice receiving the saline injection displayed a significant reduction in the percentage of both entries onto the open arms and time spent on open arms than did mice that had been placed directly on the maze, indicating a significant increase in the levels of anxiety after an inescapable stressor. Interestingly, under these conditions the *Drd4*$^{-/-}$ mice continued to display exaggerated avoidance behaviour compared to their wild-type siblings (Fig. 2).

The reduced exploratory activity displayed by *Drd4*$^{-/-}$ mice in the more conflict-producing environments could be the result of enhanced avoidance behaviour associated with heightened anxiety. To examine this possibility we evaluated the behavioural responses to novel environmental stimuli in mice receiving anxiolytic drugs. The benzodiazepines and ethanol are known to diminish novelty-induced anxiety, in part through the modulation of cortical GABAergic neurotransmission, without increasing motivation or novelty-seeking behaviour (Widgiz & Beck, 1990; Simon *et al*., 1994). In the elevated plus maze ethanol produced anxiolytic effects in both genotypes (Fig. 3A). At 1 g/kg, i.p. ethanol produced anxiolysis only in *Drd4*$^{-/-}$ mice, making their behaviour indistinguishable from that of wild-type mice. At 2 g/kg, i.p. ethanol produced marked anxiolysis in both wild-type and mutant mice as measured by the number of indiscriminate entries into either open or closed arms of the maze (Fig. 3A). The benzodiazepine midazolam (1 mg/kg, i.p.) also produced a significant anxiolytic effect in both wild-type and mutant mice, which

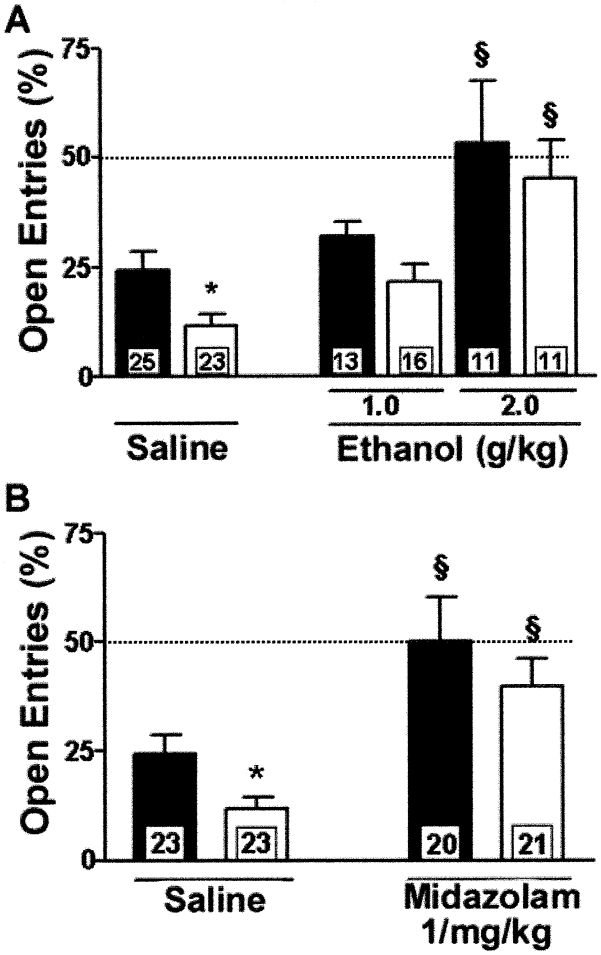

FIG. 3. Anxiolytic drugs increase approach behaviour to the unprotected open arms of the elevated plus maze in wild-type and *Drd4*$^{-/-}$ mice. Both (A) ethanol (1 and 2 g/kg, i.p) and (B) midazolam (1 mg/kg, i.p) injected 5 min prior to the test increased the number of open entries in wild-type and mutant mice. The dotted line indicates 50% of preference for open or close arms. Analysis of the data was performed by ANOVA followed by a Dunnett's *t*-test. (*$P < 0.05$ vs. wild-type group under the same conditions; $^{§}P < 0.05$ vs. control mice from same genotype.)

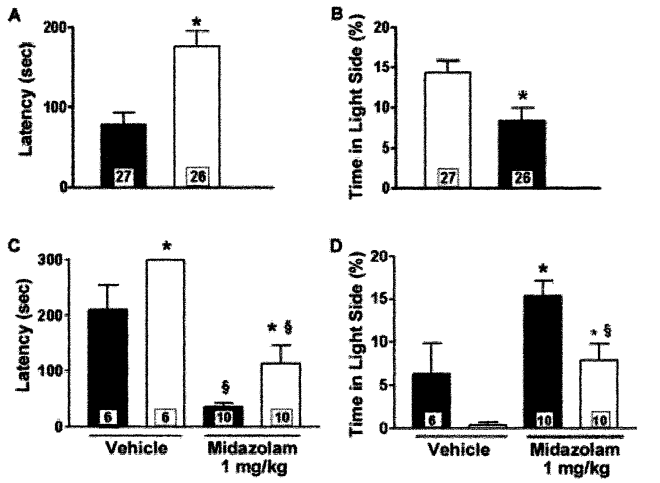

FIG. 4. Mice lacking D4 receptors display increased anxiety-like behaviour in a light/dark preference paradigm. (A) The latency to enter into the illuminated compartment of a shuttle box and (B) the percentage of time spent in the lit side were recorded for both wild-type (black) and *Drd4*$^{-/-}$ mice (white). (C and D) Midazolam (1 mg/kg, i.p.) exerted an anxiolytic effect in both wild-type and mutant mice as demonstrated by (C) the reduced latency to enter the lit side of the test apparatus and (D) the increased time spent in the illuminated side. Sample sizes are indicated by the numbers within the bars. Bars represent means + SEM. Analysis of the data was performed by Student's *t*-test (*$P < 0.01$ vs. wild-type in A and B). In C and D, ANOVA followed by a Newman–Keuls multiple comparison test revealed a significant effect (*$P < 0.01$ vs. the wild-type group under the same conditions; $^{§}P < 0.01$ vs. control mice of the same genotype).

entered with equal preference onto the open or close arms of the maze (Fig. 3B).

The light/dark preference test is another paradigm for quantifying approach/avoidance conflicts and is widely employed to evaluate anxiety in rodents (Costall *et al*., 1989). The initial latency to enter the more aversive illuminated compartment and the percentage of time spent on the light side are accepted measures of anxiety. In this paradigm both genotypes preferred the dark side of the box. However, mice lacking D4Rs displayed a 120% increase ($P < 0.01$; Fig. 4A) in their initial latency to enter the lit compartment and spent 42% less time on the illuminated side compared to wild-type mice ($P < 0.01$; Fig. 4B). Treatment of wild-type and *Drd4*$^{-/-}$ mice with 1 mg/kg midazolam yielded similar results in the light/dark preference test as demonstrated by a reduction in the latency to enter the lit side (Fig. 4C) and an increase in the time spent on the lit side (Fig. 4D).

It has been proposed that the anxiolytic effects of ethanol contribute to the initiation of ethanol consumption and lead to the self-medication hypothesis in individuals with heightened anxiety (Kushner *et al*., 1990). Because it has been shown that the basal level of anxiety may play an important role in the vulnerability to ethanol consumption in rats (Spanagel *et al*., 1995) and that DA neurotransmission plays a critical role in ethanol preference and consumption (El-Ghundi *et al*., 1998; Phillips *et al*., 1998) we decided to test wild-type and *Drd4*$^{-/-}$ mice in a two-bottle choice preference test. Individually-caged male mice had free access to both water and ethanol 10% (v/v). During the nine consecutive days of the test wild-type mice showed a slight preference for the ethanol solution that was indistinguishable from that displayed by *Drd4*$^{-/-}$ mice (Fig. 5A). In contrast to what has been reported for the D1R- (El-Ghundi *et al*., 1998) and the D2R-deficient mice (Phillips *et al*., 1998), no difference was observed in ethanol preference and total ethanol consumption between wild-type and D4R mutant mice (Fig. 5A and B).

Normal emotional reactions to conditioned fear in Drd4$^{-/-}$ *mice*

Exposure to a novel environment or to an aversive stimulus increase DA turnover and DA release in the PFC of the rat and are both prevented by the preadministration of anxiolytic doses of benzodiazepines (Reinhard *et al*., 1982; Claustre *et al*., 1986; Yoshioka *et al*., 1996). DA metabolism also increases in the amygdala in response to conditioned fear stimuli (Coco *et al*., 1992; Inoue *et al*., 1994) and D2-like receptors have been identified in this brain region as well as in the hippocampus (Khan *et al*., 1998). After observing that *Drd4*$^{-/-}$ mice display increased reactivity to environmental aversive cues we decided to investigate the behavioural reactions triggered by learned fearful stimuli to evaluate the potential contribution of the D4R in DA-mediated expressions of conditioned fear responses. To this end we evaluated the response of *Drd4*$^{-/-}$ mice in two different classical conditioning paradigms. First, a group of mice was evaluated in a contextual and cued fear-conditioning test in which three footshocks were paired with a tone in a novel grid-bottom cage. Twenty-four

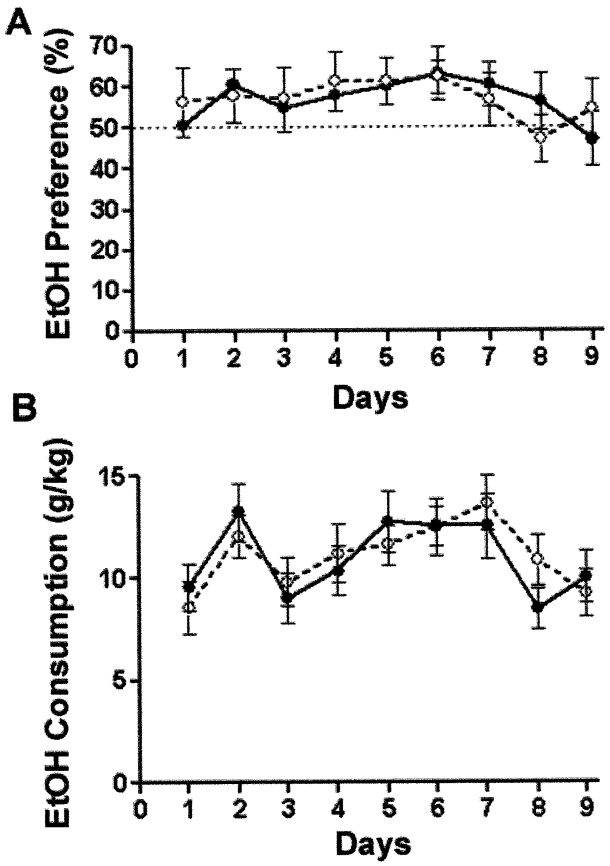

FIG. 5. Normal ethanol (A) preference and (B) consumption in *Drd4*$^{-/-}$ mice. Twelve male mice from each genotype were individually housed 2 days before beginning the drinking test. During the following 9 days mice had free access to a bottle containing water and a similar bottle containing ethanol 10% (v/v).

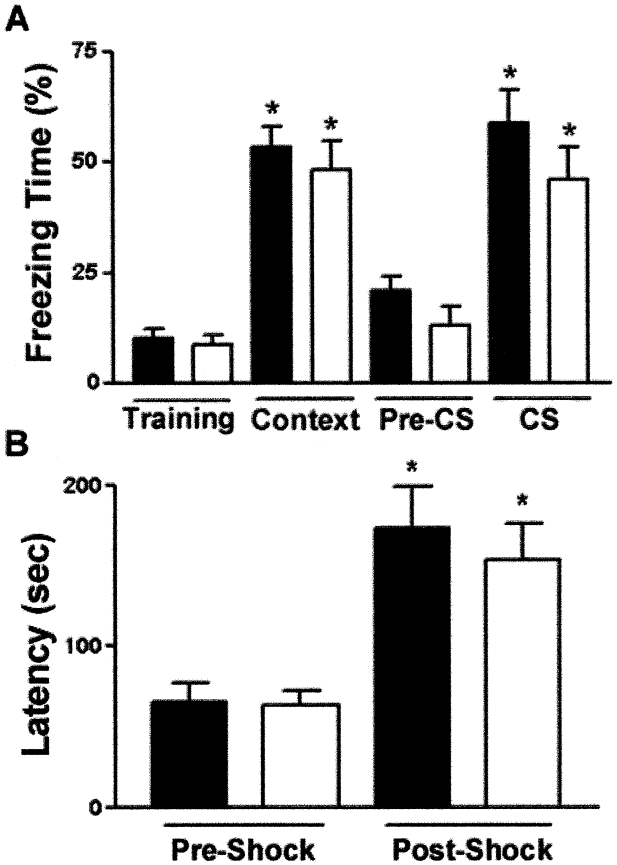

FIG. 6. Mice lacking D4Rs showed normal contextual and tone-cued fear-conditioning responses as well as normal conditioned suppression of instrumental responses. (A) Freezing behaviour was monitored in mice 24 h after receiving an electric footshock paired with a tone. Contextual conditioning was determined in the same chamber and room whereas tone-cued conditioned stimulus (CS) memory was tested in a novel chamber and room. Basal freezing time was determined during training. (B) Passive avoidance. Latency to enter into the dark chamber was determined immediately before (Pre-shock) and 24 h after receiving an electric footshock (Post-shock). Analysis of the data was performed by ANOVA followed by a Newman–Keuls multiple comparison test. (*$P < 0.01$ vs. control mice from same genotype).

hours later mice were evaluated for their context fear conditioning response after being returned to the same grid-bottom cage where they had received the footshocks. Wild-type and D4R-deficient mice displayed an intense freezing response that did not differ between the two genotypes. After 3 min mice were returned to their home cage for 2 h and then transferred to a different novel flat-bottomed cage located in another room for the cued fear-conditioning test. Freezing behaviour in this altered context was no different from the basal levels observed during the training phase for wild-type and D4R-deficient mice. However, when exposed to the tone previously paired to the shock, mice from both genotypes exhibited similar levels of freezing response suggesting that acquisition and retention of a classical fear conditioning response was intact in the absence of D4Rs.

In a separate set of experiments, another group of mice was evaluated in a step-through passive avoidance test that measures conditioned suppression of instrumental behavioural responses. In this test mice face a conflict between responding to obtain a positive reinforcement (entering into a dark chamber) and not responding to avoid an electric footshock. The latency to enter into the dark chamber in which a three-footshock train was given 24 h before was much larger than when mice were initially placed into the brightly lit compartment. Pre- and postshock latencies were indistinguishable between wild-type and *Drd4*$^{-/-}$ mice (Fig. 6).

Discussion

Increased anxiety and vigilance in mice lacking D4 receptors

D4Rs are localized in the frontal cortex following a decreasing rostrocaudal gradient of expression (Ariano *et al.*, 1997) that parallels those observed for DA levels and terminals (MacBrown & Goldman, 1977; Gaspar *et al.*, 1989) being all three highest in the PFC. This brain region is specialized in integrating informative environmental cues while filtering out irrelevant distracting stimuli by using a complex fine network of stimulatory and inhibitory synapses. Therefore, successful planning of goal-orientated behaviours as well as harm avoidance reactions will strongly depend on a proper cortical activation elicited during the initial evaluation of contextual stimuli. Given that DA plays a key role in the modulation of cortical activity together with our recent demonstration that the genetic disruption of D4Rs increases cortical excitability of pyramidal neurons (Rubinstein *et al.*, 2001) we decided to investigate whether the lack of a normal inhibitory cortical component would impair behavioural reactions to stress in mice. To this end, wild-type and

mutant mice lacking this receptor subtype were challenged in two different approach/avoidance conflict paradigms: the elevated plus maze and the light/dark preference exploration test. By these behavioural measures *Drd4*$^{-/-}$ mice showed an increased avoidance to the more aversive places of each maze as demonstrated by significantly reduced exploration of the unprotected open arms of the plus maze and longer latencies to explore the illuminated compartment of the light/dark shuttle box. Although is has been shown that *Drd4*$^{-/-}$ mice exhibit reduced exploration of novel stimuli (Dulawa *et al*., 1999), the behaviours reported here reflect an increase in anxiety because they were completely reversed by anxiolytic drugs such as midazolam and ethanol, two compounds that do not increase motivation or novelty-seeking behaviour (Widgiz & Beck, 1990; Simon *et al*., 1994). These results are in agreement with a considerable body of evidence in favour of a direct involvement of PFC dopamine in anxiety-related behavioural responses (Roth *et al*., 1988; Broersen *et al*., 1995). Because DA synthesis and turnover are normal in the PFC of *Drd4*$^{-/-}$ mice (Rubinstein *et al*., 2001), the possibility that the heightened anxiety observed in the mutant animals was due to increased stimulation of D1-like receptors can be ruled out. In light of our current findings it is tempting to speculate that one of the functions of stress-induced DA release in the PFC is to regulate cortical excitability via D4R stimulation. In the absence of inhibitory modulation by this receptor subtype, environmental stimuli that produce normal arousal in wild-type mice may trigger a hypervigilant state in the D4R-deficient mice that results in exaggerated avoidance behaviour. In humans, anxiety disorders such as agoraphobia and panic attacks are clinically recognized conditions that are normally treated with drugs capable of diminishing cortical excitability, such as benzodiazepines. Functional brain imaging studies in humans subjected to anticipatory anxiety have shown a positive correlation between regional blood flow in the PFC and anxiety scores (Simpson *et al*., 2001). In addition, it is interesting to note that anxiety is highly comorbid with Parkinson's disease (Menza *et al*., 1993; Maricle *et al*., 1995) and ADHD (Milberger *et al*., 1995; MTA Coopertaive Group, 1999), two conditions, characterized by compromised midbrain DAergic neurotransmission, that are treated with drugs that enhance DAR stimulation such as the DA precursor L-DOPA and the DA transporter blocker methylphenidate, respectively.

The fact that D4R-deficient mice show normal emotional reactions to contextual and cued associative fear conditioning is consistent with the idea that circuits involved in implicit forms of learning such as the amygdala and the hippocampus are unaffected by the lack of this DA receptor subtype. D-1-like and D-2-like receptors have been identified in the amygdalar complex (Guarraci *et al*., 1999; Guarraci *et al*., 2000) and there is accumulative evidence that DA activation in the amygdala is implicated in conditioned fear (Coco *et al*., 1992; Inoue *et al*., 1994). Our results showing enhanced behavioural responses to unconditioned fear or anxiety responses together with normal reactions to conditioned fear suggest that the participation of the D4R in the PFC is more critical that in the amygdala or hippocampus in agreement with its relative expression levels in these brain regions.

Twin, adoption and family studies have shown the existence of genetic predisposition for anxiety disorders (Gershenfeld & Paul, 1998) and quantitative trait loci analyses in mice have revealed multiple loci linked to anxiety-like behaviours (Turri *et al*., 1999). Although a number of genetically engineered mouse mutants, including the γ2-subunit of the $GABA_A$ receptor (Crestani *et al*., 1999) and the α4-subunit of the nicotinic receptor (Labarca *et al*., 2001), displayed anxiety-like behaviours; this is the first evidence of a heightened anxiety phenotype after deletion of a gene that is expressed predominantly in the PFC and participates in mesoprefrontal DA neurotransmission. The human DRD4 gene is highly polymorphic at two levels. In its coding region, up to 27 different haplotypes have been identified carrying different number and sequence of a 48-bp repeat (Van Tol *et al*., 1992). Upstream of the 5′ transcription initiation site there is, in addition, a 120-bp repetitive element (McCracken *et al*., 2000). Given that numerous reports have described genetic associations of some of the human DRD4 alleles with the occurrence of psychiatric diseases (LaHoste *et al*., 1996; Swanson *et al*., 1998; McCracken *et al*., 2000; Faraone *et al*., 2001) together with the heightened anxiety presented here in mice lacking the D4R, it is tempting to speculate that DRD4 might be a candidate gene for traits related to emotional reactions to stress or anxiety-like behaviours.

Acknowledgements

Our thanks to M. Garibaldi, N. Malarini and A. Paladini for their excellent technical assistance. This work has been supported in part by an International Research Scholar Grant of the Howard Hughes Medical Institute (M.R.), Agencia Nacional de Promoción Científica y Tecnológica (M.R.), CONICET (M.R.), National Science Foundation (M.J.L.), National Institute of Drug Abuse (D.K.G., M.J.L.), and National Institute of Mental Heath (M.J.L.). T.L.F., D.M.G. and J.I.Y. are recipients of doctoral fellowships of the Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina.

Abbreviations

DA, dopamine; D4R, dopamine D4 receptor; *Drd4*$^{-/-}$, dopamine D4 receptor-deficient mice; PFC, prefrontal cortex.

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