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A targeted mutation of the D_3 dopamine receptor gene is associated with hyperactivity in mice

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ABSTRACT **While most effects of dopamine in the brain are mediated by the D_1 and D_2 receptor subtypes, other members of this G protein-coupled receptor family have potentially important functions. D_3 receptors belong to the D_2-like subclass of dopamine receptors, activation of which inhibits adenylyl cyclase. Using targeted mutagenesis in mouse embryonic stem cells, we have generated mice lacking functional D_3 receptors. A premature chain-termination mutation was introduced in the D_3 receptor gene after residue Arg-148 in the second intracellular loop of the predicted protein sequence. Binding of the dopamine antagonist [^{125}I]iodosulpride to D_3 receptors was absent in mice homozygous for the mutation and greatly reduced in heterozygous mice. Behavioral analysis of mutant mice showed that this mutation is associated with hyperactivity in an exploratory test. Homozygous mice lacking D_3 receptors display increased locomotor activity and rearing behavior. Mice heterozygous for the D_3 receptor mutation show similar, albeit less pronounced, behavioral alterations. Our findings indicate that D_3 receptors play an inhibitory role in the control of certain behaviors.**

The neurotransmitter dopamine exerts a broad array of effects on the central nervous system, affecting cognitive, motor, and reward processes among others. The effects of dopamine are mediated by a family of guanine nucleotide-bindindg regulatory protein (G protein)-coupled receptors, which include D_1–D_5 receptor subtypes. Based on genomic organization and pharmacological and signaling properties, dopamine receptors have been assigned to one of two subclasses, termed D_1-like and D_2-like (1–4). D_1-like receptors include the products of the D_1 receptor and D_5 receptor genes; agonist binding to these receptors stimulates adenylyl cyclase through receptor coupling to G_s. On the other hand, activation of D_2-like receptors produces multiple effects, including inhibition of adenylyl cyclase by G_i. The D_2-like family is composed of the D_2, D_3, and D_4 gene products.

The D_3 receptor has a distinct distribution in limbic areas of the brain, including the nucleus accumbens, olfactory tubercle, islands of Calleja, and hippocampus (5–12). Functional studies suggest that the D_3 receptor may be involved in motivational aspects of behavior (13). Moreover, this receptor binds most antipsychotic drugs with high affinity (7, 12) and may mediate some effects of "atypical" neuroleptics, such as clozapine, on gene regulation (14). These findings have led to the suggestion that D_3 receptors may play a role in schizophrenia. This conclusion, however, remains controversial (3, 15).

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The role of the D_3 receptor has been difficult to study because of its low abundance (≈1% of D_2 receptors) (3) and the absence of selective ligands. Using a strategy of gene targeting in mouse embryonic stem cells (ES cells), we have begun to elucidate the function of dopamine receptor subtypes by generating mouse strains that lack individual receptor subtypes. We have previously reported the generation of a D_1-deficient mouse (16). Here, we report the generation of a D_3 receptor mutant mouse, which is characterized by a hyperactive behavioral phenotype. Preliminary results of this study have been presented (30).

MATERIALS AND METHODS

Construction of Targeting Vector. A mouse genomic library derived from the 129/sv strain (Clontech) was screened using a mouse D_3 cDNA probe (17). A positive clone encompassing exon 2 of the murine D_3 gene was isolated and further characterized. A 7-kb *Xho* I–*Asp*718 fragment was engineered for targeted mutagenesis by introducing the GKNeo cassette (16) in antisense orientation at the *Sal* I site in exon 2 (17). Integration of sequences derived from the pGKNeo cassette generates a novel open reading frame, resulting in the following peptide sequence appended after Arg-148: PASDGIRTWQNNTENEVYVEQRLLISFFRL Opal (Stop). The sequence of the mutant allele was confirmed by direct sequencing of reverse transcription–PCR (rPCR) products derived from brain mRNAs of −/− and +/− mice (data not shown).

Transfection of ES Cells and Embryo Manipulations. J-1 ES cells (a kind gift of R. Jaenisch, Massachussetts Institute of Technology) at passage 13 were grown on mitomycin C-treated embryonic fibroblasts derived from a homozygous neomycin (Neo)-resistant transgenic mouse (16). Cells (2×10^7) were electroporated in a 1-ml cuvette (path length-0.2 cm) at 0.4 kV and 25 μF. Cells were plated onto 40 gelatin-coated Petri dishes (6 cm) on embryonic feeder cells. Selection with G418 (0.3 mg/ml; active concentration of 0.66 μg/mg of dry powder; GIBCO) was applied 24 hr after plating and was continued for 7–9 days. Individual Neo-resistant colonies were picked using a dissection microscope and expanded as described (16). Genomic DNA was prepared from an aliquot of cells for each clone using previously described techniques and analyzed by Southern blotting (18). Recovery, microinjection, and transfer of 3.5 day postcoitus embryos was performed as described (16). Chimeric males (80–100% chimerism based on coat color)

Abbreviations: G-protein, guanine nucleotide-binding regulatory protein; rPCR, reverse transcription–PCR; ES cell, embryonic stem cell; Neo, neomycin.

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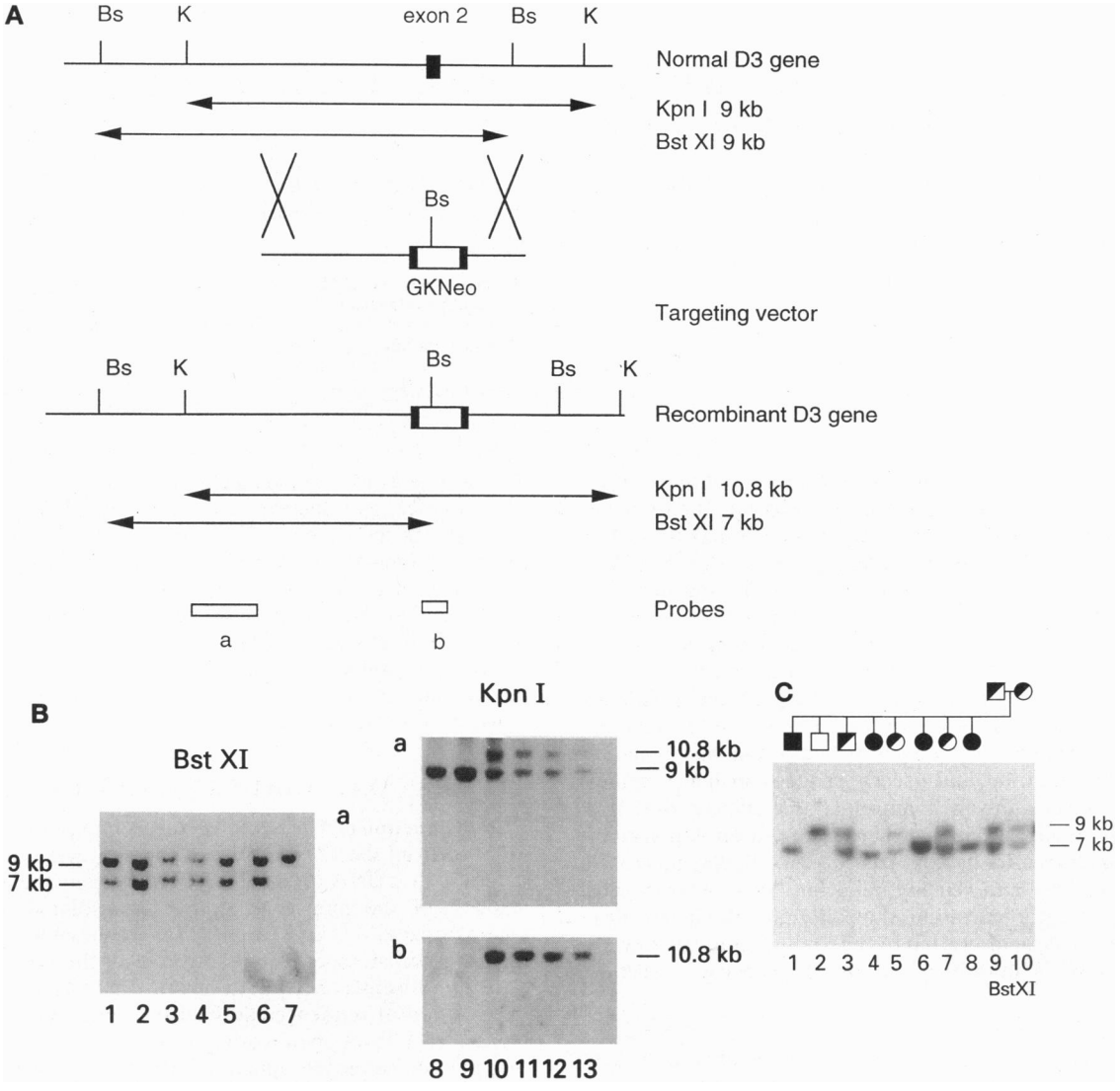

FIG. 1. (*A*) Genomic map of the murine D_3 receptor locus surrounding exon 2. The location of crossover sites is indicated at either end of the targeting vector. Probes used for Southern analysis are indicated by the open bars. Relevant restriction enzyme sites are indicated as follows: K, *Kpn* I; Bs, *Bst*XI. (*B*) Southern blotting analysis of targeted ES cells. DNA derived from control ES cells (lanes 7–9) and from individual clones of transfected ES cells (lanes 1–6 and 10–13) was digested with *Bst*XI (lanes 1–7) or with *Kpn* I (lanes 8–13) and fractionated on an agarose gel. Filters were hybridized with a DNA probe located 5′ to sequences included in the targeting vector (a), as well as a Neo probe (b). (C) Inheritance of the mutant allele of the D_3 receptor gene in mice. Heterozygous F_1 animals were intercrossed to obtain $-/-$ mice. A representative pedigree is shown. Genomic DNA was isolated from tail biopsies and analyzed by restriction digestion with *Bst*XI. Southern blotting was performed as described (20).

were mated with C57BL/6 females. The genotype at the D_3 receptor locus was determined by Southern blotting of tail DNA.

rPCR Analysis of D_3 Receptor Gene Expression in Mouse Brain. Total cellular RNA was isolated from the brains of $+/+$, $+/-$, and $-/-$ mice as described (18). Two sets of primers were employed to analyze D_3 receptor gene expression. Wild-type gene: upstream primer, 5′-GCAGTGGTCATGCCAGTTCACTATCAG-3′ (nt 391–417; ref. 19); downstream primer, 5′-CCTGTTGTGTTGAAACCAAAGAGGAGAGG-3′ (nt 526–498; ref. 19); mutant allele: upstream primer, 5′-GCAGTGGTCATGCCAGTTCACTATCAG-3′ (nt 391–417; ref. 19); downstream primer, 5′-ATATTGCTGAAGAGCTTGGCG-3′ (GKNeo) (18). The sizes of the expected products are 135 nt for the wild-type gene and ≈600 nt for the mutant gene. Reverse transcription and PCR conditions have been described in a previous publication (18). As a negative control, RNA was omitted from the reverse transcription reaction.

Autoradiographic Analysis of D_3 Binding to the Islands of Calleja. Receptor autoradiography was generally performed as described (20) using 14-μm-thick coronal brain sections. Serial sections were collected from each brain throughout the region of the olfactory tubercles to ensure inclusion of the islands of Calleja. Frozen sections were preincubated in binding buffer (50 mM Tris·HCl, pH 7.4/120 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/5 mM ascorbic acid) for 15 min prior to a 30-min 25°C incubation with 0.23 nM [^{125}I]iodosulpride (Amersham) and the appropriate competing ligands (100 nM). Nonspecific binding was defined using 1 μM (−)-sulpiride. Preliminary experiments were carried out to determine the optimal concentration of domperidone and quinelorane to visualize D_3- and D_2-specific binding, respectively. After washing and drying, sections were apposed to autoradiography film for up to 2 weeks.

Behavioral Analysis in an Open Field Test. Animals were kept on a 12-hr light/12-hr dark cycle (lights on at 7:00 a.m.) and housed individually in standard cages for at least 7 days

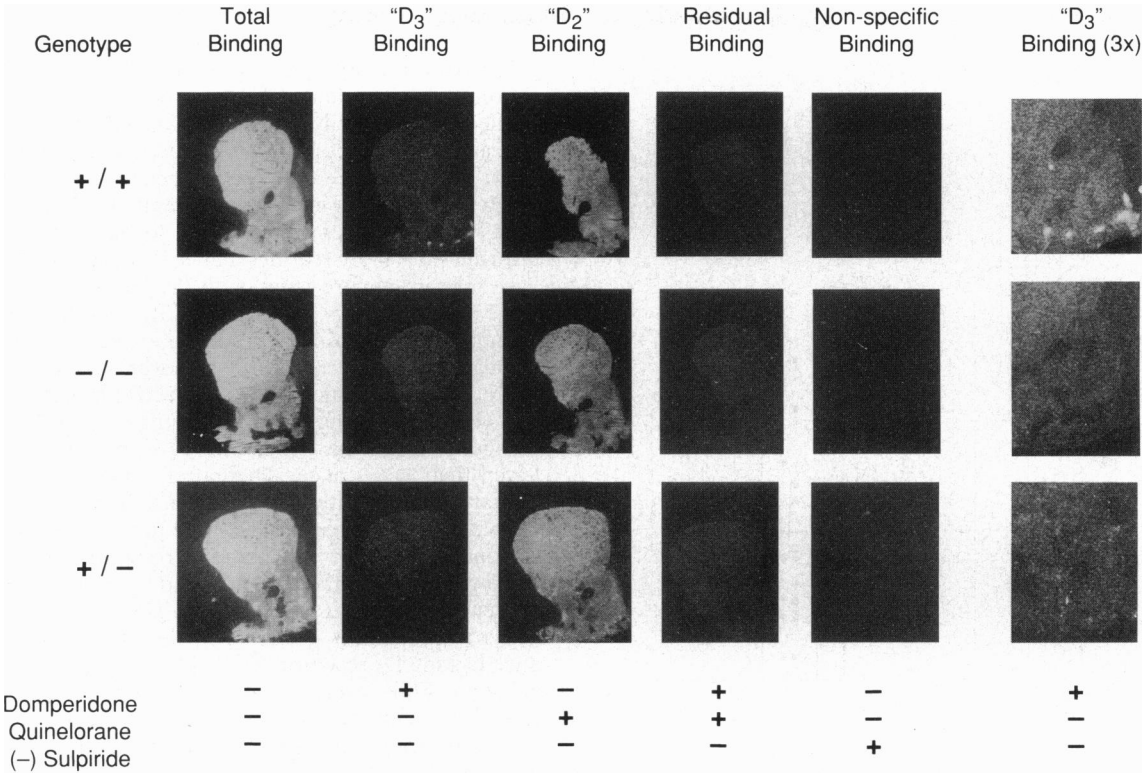

FIG. 2. Visualization of D_3 and D_2 receptors using [^{125}I]iodosulpride autoradiography. Coronal brain sections through the striatum, nucleus accumbens, olfactory tubercles, and islands of Calleja were incubated with [^{125}I]iodosulpride in the absence of competing unlabeled ligands (total binding) or in the presence of 100 nM domperidone to inhibit binding to D_2 receptors ("D_3" binding) or 100 nM quinelorane to inhibit binding to D_3 receptors ("D_2" binding). Residual binding was defined as the amount of radioactivity in the presence of both unlabeled competitors. Likewise, nonspecific binding was defined as radioactivity that could not be blocked by competition with an excess of unlabeled sulpiride. The far right column shows a ×3 magnification of "D_3-specific" binding in the area of the islands of Calleja. The genotypes of the mice are indicated on the left.

before testing. Behavioral tests were performed between 1 p.m. and 6 p.m. Eight- to 12-week-old litter mates (+/+, n = 17; +/−, n = 7; −/−, n = 11) were examined in a square open field (60 × 60 cm, divided into nine quadrants by lines on the floor) over a 15-min period (16). Locomotor activity was assessed by counting the number of lines crossed with all four feet (crossings). Rearing and grooming events were also recorded. Behavior was rated by two observers who were unaware of the genotype. The test sessions were also videotaped. Behavioral scores were subjected to a nonparametric analysis of variance (Kruskal–Wallis test) followed by post hoc Mann–Whitney U tests to compare the individual groups.

RESULTS

Targeting of the Murine D_3 Gene. The mouse D_3 receptor gene has been mapped and shown to be expressed as two variably spliced isoforms, which differ by the presence of a 21-amino-acid sequence in the third intracellular loop (17, 19). To generate mice lacking D_3 receptors, a gene replacement-type targeting vector was constructed. The pGKNeo cassette (16) was introduced in an antisense orientation at the *Sal* I site in exon 2 of the murine D_3 gene (Fig. 1*A*) (17). Homologous integration of the targeting construct was predicted to result in the generation of a mutant allele in which sequences downstream of amino acid residue Arg-148 in the second intracellular loop of the D_3 receptor are replaced by sequences derived from the Neo gene. We refer to the mutant allele as Δ148(+30). Three targeted clones (Fig. 1*B*) were used to generate chimeric mice by blastocyst injection. Chimeric mice were scored for germ-line transmission of the mutant allele by crossing with C57BL/6 mice. Four chimeras gave rise to germ-line transmission of the mutant D_3 receptor allele. Southern analysis of tail DNA from progeny of heterozygous matings revealed the predicted ratios of homozygous mutant (−/−), heterozygous (+/−), and normal mice (+/+) (Fig. 1*C*).

Detection of Wild-Type and Mutant D_3 mRNA by rPCR Analysis. To determine whether the mutation introduced in the D_3 receptor gene resulted in a loss of expression of normal D_3 mRNA, we isolated total RNA from brains of +/+, +/−, and −/− mice and performed rPCR using D_3 receptor-specific primers (18, 19). Two sets of primers were employed, one that would detect mRNA transcribed from the wild-type allele and one that would only detect mRNA transcribed from the Δ148(+30) allele. Wild-type mRNA could readily be identified in brains of +/+ and +/− mice but not in −/− mice. In contrast, mutant mRNA derived from the Δ148(+30) allele was detected in +/− and −/− but not in +/+ mice (data not shown). These findings suggest that the Δ148(+30) mutation effectively prevents normal transcription of the murine D_3 receptor gene.

Autoradiographic Studies of "D3-Specific" Binding. To demonstrate that the mutant mice are lacking functional D_3 receptors, we performed autoradiographic binding studies on brain sections of +/+, +/−, and −/− mice using the D_2-like receptor antagonist [^{125}I]iodosulpride (Fig. 2). Our goal was to examine the D_3 receptor binding in the islands of Calleja as these areas are enriched for D_3 receptors (5–8). Since [^{125}I]iodosulpride labels both D_2 and D_3 receptors, we used the method described in ref. 8 to selectively visualize these receptor subtypes. Preferential labeling of D_3 receptors was achieved by attenuating the majority of D_2 labeling with the D_2-selective ligand domperidone (8). Conversely, D_2 receptors were selectively visualized by the addition of quinelorane, which is D_3-preferring (6). In +/+ mice, [^{125}I]iodosulpride

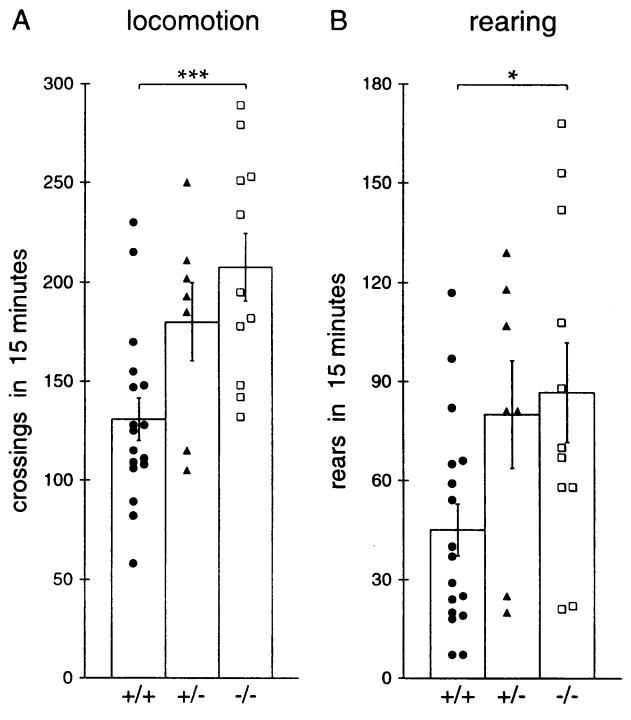

FIG. 3. D_3 mutants are hyperactive in an exploratory test. D_3 receptor +/+, +/−, and −/− mice were examined for 15 min in an open field test. Locomotor activity (crossings) (*A*) and rearing behavior (*B*) are depicted for individual animals and as the mean ± SEM. Nonparametric analysis of variance (Kruskal–Wallis test) revealed significant differences for crossings ($P < 0.005$) and rearing ($P < 0.05$), which were mainly due to greater values of −/− mice than those of +/+ mice ($P < 0.001$ and $P < 0.05$, respectively, Mann–Whitney *U* test). ***, $P < 0.001$; *, $P < 0.05$.

binding in the islands of Calleja is distinctly visible in the presence of domperidone (see bottom of +/+ D_3 binding panel in Fig. 2), consistent with the fact that D_3 receptors are expressed in this region. In contrast, D_3 receptor binding in the islands of Calleja is absent in −/− mice and minimal in +/− mice. Interestingly, +/− mice showed a nearly complete absence of D_3-specific binding, to a greater extent than expected for a mutation affecting expression of one allele of the D_3 receptor gene. This observation raises the possibility that this mutation acts in a dominant-negative fashion to inhibit binding to D_3 receptors expressed from the wild-type allele. D_2 receptor binding is comparable in control, heterozygous, and homozygous mutant mice, suggesting that disruption of the D_3 receptor gene does not impair D_2 receptor expression (Fig. 2).

Behavioral Analysis of Mutant Mice in an Open Field Test. D_3 receptor +/− and −/− mice develop normally and are fertile. Neurologically, they display normal gait and coordination and have intact primitive reflexes. Exploratory behavior of D_3 +/− and −/− mice was studied in an open field test. In this test, D_3 mutant mice were hyperactive. D_3 receptor −/− mice showed on average 57% more locomotor activity (crossings) and 93% more rearings than +/+ mice (Fig. 3). Likewise, +/− mice were more active, with 38% more crossings and 77% more rearings than +/+ mice (Fig. 3). Time course analysis of locomotor activity revealed that −/− mice were more active throughout the test (Fig. 4). Moreover, this analysis demonstrated that +/− mice were hyperactive in later parts of the test (Fig. 4). During the first 4 min, +/− mice showed a number of crossings similar to +/+ mice and significantly fewer than −/− mice. Thereafter (min 5–15), +/− mice displayed significantly more crossings than +/+ mice, with a degree of hyperactivity comparable to the −/− mice (Fig. 4). Grooming behavior did not differ significantly among the three genotypes, although +/− and −/− mutants showed a tendency for less grooming and a delayed onset (data not shown). The results of the open field test demonstrate that mice lacking D_3 receptors are hyperactive during exploratory behavior. Thus, even though the D_3 receptor subtype represents only a minor fraction of all dopamine receptors, ablation of D_3 receptors is associated with significant behavioral changes.

DISCUSSION

We have generated mice lacking D_3 dopamine receptors. Autoradiographic studies show that D_3-specific binding is absent in the islands of Calleja, a brain structure in which D_3 receptors are normally very highly expressed. Lack of functional D_3 receptors is also supported by studies of rPCR amplification, which indicate the absence of mRNA transcribed from the wild-type allele in −/− mice. Thus, two independent lines of evidence support the notion that the Δ148(+30) mutation results in the generation of D_3-deficient mice.

Greatly reduced D_3 binding in +/− mice suggests that the Δ148(+30) mutation may act in a dominant-negative fashion to inhibit binding to D_3 receptors expressed from the wild-type allele. The mutant allele encodes a truncated receptor lacking sequences downstream of the second intracellular loop. Interestingly, a truncated D_3 receptor has also been detected in

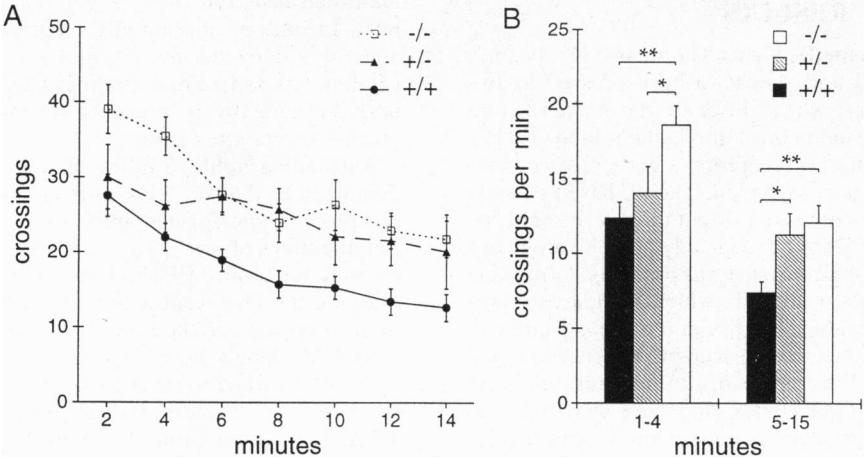

FIG. 4. Time course analysis of locomotor activity (crossings) in D_3 mutant mice. Data are presented as the mean ± SEM values for each of seven 2-min blocks (*A*) or as averages per minute for minutes 1–4 vs. 5–15 during the open field test. **, $P < 0.01$; *, $P < 0.05$.

brains of normal human subjects as well as schizophrenic patients (15).

Dominant-negative mutations have been identified in several G protein-coupled receptors. For instance, mutations of the rhodopsin gene have been shown to cause dominantly inherited forms of retinitis pigmentosa in humans (21). A more detailed characterization of the biochemical mechanism underlying the dominant-negative effects of the Δ148(+30) mutation will require further studies in transfection systems.

The development of a mouse strain with a targeted mutation of the D_3 receptor gene is a first step toward understanding the function of this subpopulation of dopamine receptors. We have demonstrated that mice lacking D_3 dopamine receptors are hyperactive in a test for exploratory behavior. These mice show increased locomotor activity and rearing. Therefore, data derived from genetic ablation of the D_3 gene in mice are consistent with recent results of pharmacological studies. Administration of 7-OH-DPAT, a dopaminergic agonist that binds preferentially to D_3 receptors, exerts an inhibitory effect on locomotor activity (22–24). Furthermore, a D_3-preferring antagonist caused hyperactivity (25). Although the selectivity of these drugs remains controversial (26, 27), these studies support our conclusion that hyperactivity in D_3 receptor mutant mice is the result of ablation of D_3 receptors rather than the effect of compensatory changes. Taken together, these findings indicate that D_3 receptors play an inhibitory role in behavior.

D_3-deficient mice differ considerably from D_1- and D_2-deficient mice. D_1 receptor-deficient mice are growth-retarded with reduced body and brain size and weights (refs. 16 and 28; J.D., C.R.G., H.W., and H.S., unpublished data). In exploratory tests, they show drastically reduced rearing behavior, whereas locomotor activity is minimally affected (ref. 16; J.D., C.R.G., H.W., and H.S., unpublished data). Inactivation of the D_2 dopamine receptor results in Parkinsonian-like locomotor impairment and reduced fertility (29). In contrast, as demonstrated here, D_3 receptor-deficient mice do not show gross developmental deficits and are hyperactive. Thus, our findings suggest that D_3 receptors contribute to dopamine control of behavior, though in a qualitatively different manner from D_1 and D_2 receptors. The development of mouse strains with mutations in multiple dopamine receptors will further our understanding of the complex dopamine system.

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