

POTENTIATION OF THE D₂ MUTANT MOTOR PHENOTYPE IN MICE LACKING DOPAMINE D₂ AND D₃ RECEPTORS

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Abstract—Within the D₂-class of dopamine receptors, the D₂ and D₃ subtypes share the highest degree of similarity in their primary structure. However, the extent to which these two receptor subtypes have similar or different functional properties is unclear. The present study used gene targeting to generate mice deficient for D₂, D₃, and D₂/D₃ receptors. A comparative analysis of D₂ and D₃ single mutants and D₂/D₃ double mutants revealed that D₂/D₃ double mutants develop motor phenotypes that, although qualitatively similar to those seen in D₂ single mutants, are significantly more severe. Furthermore, increased levels of the dopamine metabolites dihydroxyphenyl acetic acid and homovanillic acid are found in the dorsal striatum of D₂ single mutants. The levels of these metabolites, however, are significantly higher in mice lacking D₂ and D₃ receptors. In addition, results of immunoprecipitation experiments revealed that D₂ single mutants express higher levels of D₃ receptor proteins during later stages of their postnatal development.

These results suggest that D₃ receptors compensate for some of the lacking D₂ receptor functions and that these functional properties of D₃ receptors, detected in mice with a D₂ mutant genetic background, remain masked when the abundant D₂ receptor is expressed. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: gene targeting, homologous recombination, dopamine D₂ receptors, dopamine D₃ receptors, locomotion, dopamine metabolism.

Four major neuronal systems of the brain use dopamine (DA) as the principal neurotransmitter to modulate locomotor behaviour (nigrostriatal system), motivated behaviour (mesolimbic system), learning and memory (mesocortical system), and the release of prolactin (tuberoinfundibular system). Dopamine receptors are expressed in the targets of these pathways as well as in DA synthesizing neurons. They are targets for drugs with antipsychotic potency and they are involved in mediating effects of psychostimulants such as cocaine and

amphetamine. Two functionally distinct classes of DA receptors are known. They are designated as the D₁ class of receptors (D₁, D₅) and the D₂ class of receptors (D₂, D₃, D₄) which couple to stimulatory and inhibitory subsets of heterotrimeric G proteins, respectively.^{7,13} Although some differential distribution of the various subtypes of DA receptors is thought to exist, the anatomic distribution of these receptor subtypes also shows substantial regional overlap. In addition, efforts to identify distinct functions of receptor subtypes that belong to the same functional class, and that show significant similarities in their primary structures, suffer from the lack of antagonists selective for some of the more recently identified receptor subtypes.

In the past few years a number of laboratories has therefore turned to gene targeting as a more precise approach to determining distinct functions of DA receptor subtypes. For example, studies on D₁ and D₂ mutants revealed significant differences with regard to motor and motivated behaviour influenced by these receptor subtypes. Whereas D₁ mutant mice exhibit locomotor hyperactivity and fail to mediate the locomotor stimulant effects of cocaine and

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Abbreviations: DA, dopamine; DOPAC, dihydroxyphenylacetic acid; EDTA, ethylenediaminetetra-acetate; ES, embryonic stem; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; IP, immunoprecipitation; P, postnatal day; RT-PCR, reverse transcriptase-polymerase chain reaction; TH, tyrosine hydroxylase; TMD, transmembrane spanning domain.

amphetamine,^{35,36} D₂ mutant mice show locomotor hypoactivity and postural abnormalities, and they fail to experience the rewarding effects of opioid drugs.^{2,23} Moreover, studies on D₁ and D₂ receptor mutant mice have provided direct evidence for D₁ and D₂ receptor-mediated control of expression of the striatal peptides dynorphin and enkephalin, respectively.^{2,8,35}

Within the D₂ class of dopamine receptors, the D₃ receptor is structurally most closely related to D₂ receptors, suggesting that functional complementarity between these two receptor subtypes may exist in anatomic regions where their expression overlaps.^{7,13} However, the lack of ligands with sufficient D₃ receptor selectivity^{4,6,14,19,20,30} complicates the elucidation of distinct functional properties of D₃ receptors. Studies on mice deficient for the D₃ receptor are therefore of great value, and two recent studies on such mice have already shown that the motor phenotype of these mutants bears no resemblance to the pronounced locomotor hypoactivity seen in D₂ mutants.^{1,37}

In order to investigate in more detail the extent to which the homologous D₂ and D₃ receptors have similar or different functional properties, we used the gene targeting approach to generate mice lacking D₂ and D₃ receptors so that results of studies on these single mutants could be directly compared to results obtained with D₂/D₃ double mutants.

EXPERIMENTAL PROCEDURES

Isolation of D₂ and D₃ receptor genes and targeted gene disruption in mouse embryonic stem cells

A 129Sv mouse genomic library was screened to isolate the genes that code for the D₂ and D₃ receptor. To isolate the D₂ gene, two oligonucleotide probes (5'-TTCGACTCAACAATACAGACCAGAATGAGTGTATCATTGC-3' (sense strand) and 5'-CAGAGTGACGATGAAGGGCACGTAGAATGAGACAATGGAGGAGTAGACCACAAAGGCAGGGTTGGTAC-3' (anti-sense strand) corresponding to sequences that code for partially overlapping regions of the putative third cytoplasmic domain of the rat D₂ receptor³ were used for the initial screening of the library. Two overlapping clones were isolated that together comprise exon sequences coding for the entire D₂ receptor protein. The precise localization of the exons was mapped by endonuclease restriction-site analysis, Southern blot hybridization using cDNA and genomic DNA as probes, and direct nucleotide sequence analysis.

For construction of the D₂ replacement targeting vector

pD22dPgkNeo/polyAless, a 2 kb Bam HI/Hind III restriction fragment of exon 2 of the D₂ gene was replaced with the poly(A⁺)less PGK-neo^r-cassette. The deleted region of the D₂ gene comprises sequences located immediately downstream of the first ATG, encoding the amino terminal extracellular domain, transmembrane spanning domain (TMD) 1, the first intracellular domain, and the majority of TMD 2 of the D₂ receptor (Fig. 1A). The replacement of the majority of exon 2 in this targeting vector by the PGK-neo^r-cassette results in a null-mutation of the D₂ gene due to the lack of a splice-site flanking the neo^r-cassette at the 5' end, and because of the presence of a stop codon in the 5' sequence of the inserted PGK-neo^r-cassette (which is in-frame with the first ATG triplet codon of the D₂ gene).

The isolation and characterization of the D₃ gene has been reported previously.¹¹ For construction of the D₃ replacement targeting vector *pD33iPGKneo/Rev*, a 7 kb Sal I restriction fragment of the D₃ gene comprising 3' sequences of intron 2 and 5' sequences of coding exon 3 was first ligated to a 1.5 kb Xho I-Apa I restriction fragment of the (modified) plasmid pKJ-1²⁸ containing the PGK-neo^r-poly(A⁺) cassette. The 3' end of this ligation product was then ligated to a 0.8 kb Apa I-Xba I restriction fragment that comprises sequences coding for the remaining part of exon 3 and 5' sequences of intron 3. The construct was inserted into the Sal I-Xba I cloning sites of pBluescript SK (+).

The following modifications were then made to enhance the selection of homologously recombinant embryonic stem (ES) cells (see Ref. 32): A 0.4 kb Bam HI restriction fragment of the above construct was released to remove the PGK-poly(A⁺) signal sequence of the neo^r gene. Furthermore, a 0.5 kb Eco RV-Xba I restriction fragment containing the PGK promoter was placed 3' and outside the targeted homology and, finally, a poly(A⁺) signal sequence was placed 5' to the PGK neo^r cassette. The insertion of the PGK-neo^r-poly(A⁺)less cassette into coding-exon 3 of the D₃ gene results in a premature termination of the open reading frame of D₃ mRNA at sequences encoding the putative second intracellular domain of the D₃ receptor (Fig. 2A).

2 × 10⁷ mouse R1 ES cells (kindly provided by Dr Andras Nagy; Samuel Lunenfeld Research Institute, Toronto, Canada) were transfected via electroporation with 55 µg of linearized plasmid DNA (targeting vectors) in 0.8 ml of a buffer described by Thomas and Capecchi,³⁴ using the Bio-Rad Gene Pulser (400 V; 25 µF). Twenty-four hours after transfection, the cell culture medium was supplemented with the selection drug (0.35 mg/ml of G418), and seven to nine days later, G418-resistant stable transfectants were isolated. Restriction fragments of DNA isolated from these clones (D₃: Bam HI; D₂: Eco RV) were analysed by Southern blotting to identify wild-type and mutant alleles. Furthermore, for each of the identified homologous recombinant clones the number of integration events was determined by cutting their DNA 5' to the inserted neo^r-cassette and downstream (in the 3' direction) of the targeted homology. A ³²P-radiolabeled probe that encodes the neomycin-resistance gene was used to identify the size of its restriction fragment on Southern blots. The homologous recombination

Fig. 1. D₂ knockout. (A) Genomic structure of the D₂ clone isolated from a 129/Sv-type mouse genomic library (top), and structure of the replacement targeting vector *pD22dPgkNeo/polyAless* (bottom). Black horizontal bars indicate locations of exon sequences. In the replacement targeting vector, a 1.7 kb fragment represents the 5' portion of the targeted homology and comprises 3' sequences of intron 1 and 5' sequences of exon 2. The 3' end of this DNA fragment was ligated to a PGK-neo^r-poly(A⁺)less cassette which replaces a 2 kb Bam HI/Hind III restriction fragment of the D₂ gene. The 3' portion of the targeted homology is a 5.5 kb Hind III restriction fragment of the D₂ gene that contains exons 3 to 5. (B) Left: Southern blot of Eco RV-digested tail DNA obtained from offspring of heterozygous crosses. The blot was probed with a ³²P-radiolabeled DNA (5'-ext. probe, see Fig. 1A) comprising sequences located upstream of the targeted homology. Middle and right: northern blots of mRNAs extracted from the dorsal striatum of wild-type (+/+) mice, and heterozygous (+/-) and homozygous (-/-) D₂ mutants. Ten micrograms of total RNA was loaded on to each lane. The blot in the middle was probed with a ³²P-UTP-labeled antisense riboprobe complementary to sequences of the entire exon 2 of the D₂ gene. The blot on the right was probed with a ³²P-radiolabeled random-primed 0.52 kb Pst I restriction fragment of the rat enkephalin gene.

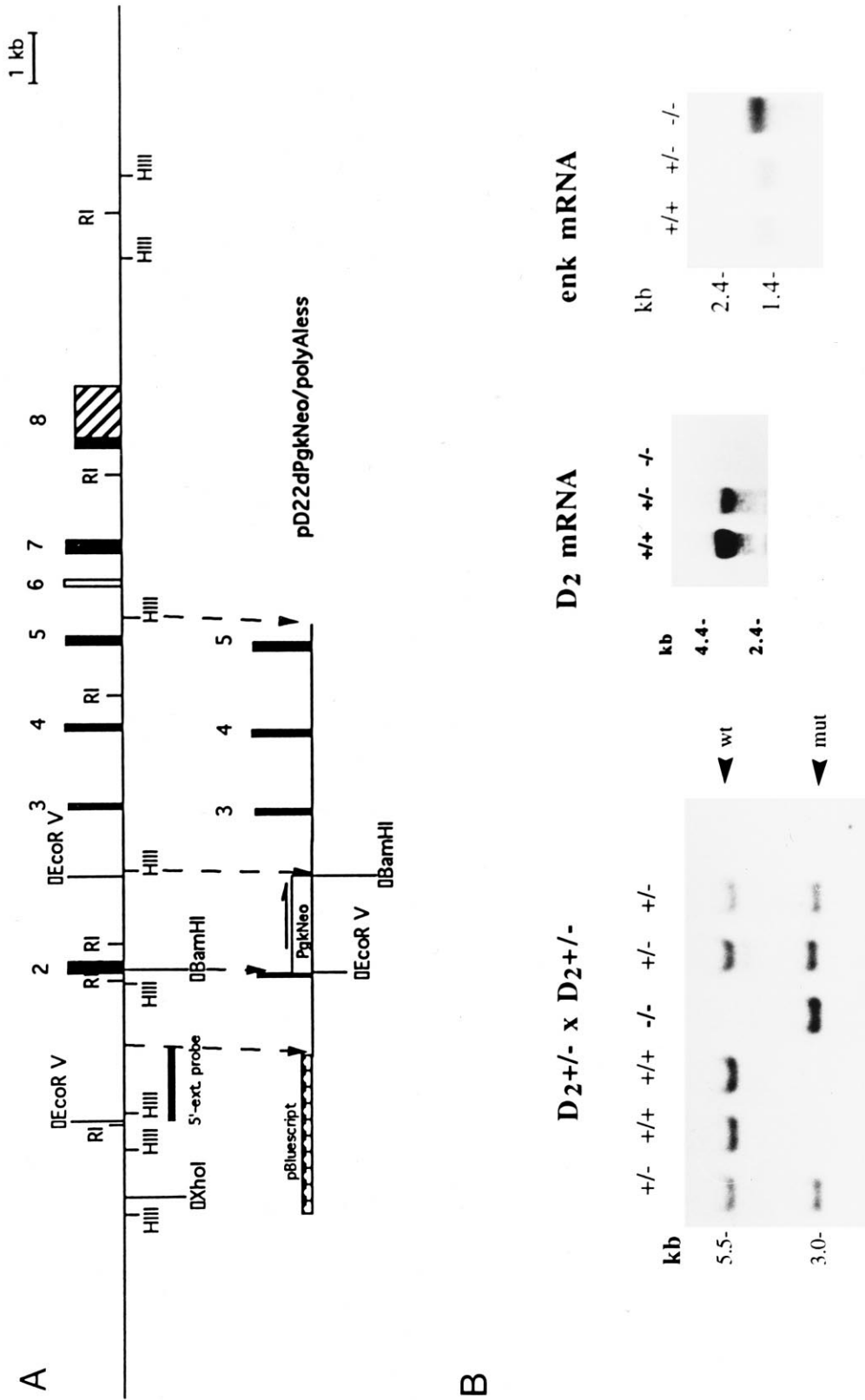


Fig. 1.

frequencies were 1 in 13 for the D₃ gene and 1 in 21 for the D₂ gene. For each homologous recombinant, only a single integration of the replacement targeting vector was detected.

Generation of D₂, D₃ and D₂/D₃ receptor mutant mice

All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to avoid or minimize animal suffering and to reduce the number of animals used.

For each of the two genes targeted, ES cells of four homologous recombinant clones were injected into blastocysts isolated from fertilized C57Bl/6 female mice. Injected blastocysts were implanted into the oviduct of ICR or SW one-day-pseudopregnant mothers. The resulting male chimeric offspring were then bred with C57Bl/6 females, and transmission of the mutant allele to agouti offspring was verified by Southern blotting of tail DNA. For each of the two knockouts, male chimera transmitted the mutant allele to 50% of their offspring. Cross-breeding of these F1 heterozygous mutants produced F2 hybrids in which the D₂ and the D₃ mutant alleles segregated in a Mendelian fashion. Genomic Southern blot analysis of tail DNAs of F1 and F2 offspring confirmed the D₂ (Fig. 1B; left panel) and D₃ receptor gene mutation (Fig. 2B, left panel) in these mice. Homozygous D₂ and D₃ mutants were obtained by cross-breeding the respective heterozygous mutants.

In order to generate D₂/D₃ double mutants, homozygous D₂ males were mated with homozygous D₃ females. Viable, normally developing and fertile offspring were obtained that are heterozygous for both mutations and that are grossly indistinguishable from wild-type mice. Cross-breeding of these heterozygous double mutants resulted in offspring that carried all expected genetic combinations of the D₂ and D₃ mutant alleles (single mutants: D₃^{+/-}, D₃^{-/-}, D₂^{+/-}, D₂^{-/-}; double mutants: D₃(^{+/-})/D₂(^{+/-}), D₃(^{+/-})/D₂(^{-/-}), D₃(^{-/-})/D₂(^{+/-}), and D₃(^{-/-})/D₂(^{-/-}) and, as expected for a Mendelian distribution of these alleles, wild-type and homozygous double mutants occurred with a frequency of 1 in 16.

Locomotor activity

Male mice at postnatal age P70 were housed individually at least 48 h prior to assessment of their locomotor activity. Mice were then placed into locomotor activity boxes (dimensions: 20 × 20 × 15 cm) mounted with photobeam cells (AccuScan Instruments, Columbus, OH, U.S.A.). The horizontal frames of these boxes consist of an 8 × 8 array of beams. For each mouse, a sequential record of the numbers of beam interruptions in the horizontal sensor as well as the total distance traveled (measured in cm) was recorded (Integra System, AccuScan Instruments). Locomotor activity levels were monitored during uninterrupted 20 h sessions to assess activities during light and dark phases. In each session, an equal number of wild-type, D₂ and D₃ single mutants, and D₂/D₃ double mutants was tested. Comparisons between genetic groups of mice were conducted using a two-sample Student's *t*-test for independent samples with unequal variance.

Electrochemical detection of dopamine and metabolites

Tissue levels of dopamine and its metabolites were determined using high-performance liquid chromatography (HPLC)-coupled electrochemical detection [HPLC model CEAS 5500; C-18 column (ESA)] as described by Pothos *et al.*²⁷ In brief, brains were rapidly removed and kept on ice while the dorsal striatum was dissected. Dissected tissues were then homogenized in 0.4 N perchloric acid (1:10; wet weight/vol)/100 μM EDTA and centrifuged for 20 min at 15,000 r.p.m. The supernatants were analysed for DA and

metabolites. Protein concentrations were determined from the remaining tissue pellets using the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.). HPLC-measured transmitter and metabolite levels were corrected for mg of protein and are expressed as mean ± S.D. of measurements obtained from five animals per genetic group.

Antibodies, immunoprecipitations and immunoblotting

Immunoprecipitation (IP) experiments were performed as previously described.²⁵ In brief, the concentration of soluble protein was determined with the BCA Protein Assay (Pierce). Samples containing equal amounts of protein were pre-cleared with protein G agarose (40 μl; Boehringer–Mannheim, Indianapolis, IN, U.S.A.) and subsequently incubated with primary antibody (a mouse monoclonal anti-D₃/IgG antibody²⁵ at 4°C for 15 h. After adding 40 μl of protein G agarose slurry, the incubation was continued for at least 2 h. The protein G–antibody–antigen complexes were boiled for 5 min in 1 × Laemmli buffer (containing 5% β-mercaptoethanol) and the entire IP was separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to membrane (Immobilon PVDF; Millipore, Bedford, MA, U.S.A.) and analysed on western blots that were probed with a mouse monoclonal anti-D₃/IgM antibody²⁵ (dilution: 1:250). Bound antigen was visualized using a peroxidase-conjugated secondary antibody (goat anti-mouse Ig[G + M], Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.), in conjunction with enhanced chemiluminescence (Pierce).

Northern blotting, reverse transcriptase–polymerase chain reaction and exponential reverse transcriptase–polymerase chain reaction

Total cytoplasmic RNA was extracted from dissected mouse brain tissue using the guanidine/cesium chloride ultracentrifugation method.⁵ For northern blotting, RNA was separated on 1.2% formaldehyde/agarose gels, transferred to Zeta Probe blotting membrane (Biorad, Hercules, CA, U.S.A.) and probed with the ³²P-radiolabeled cDNAs specified in the legends to the appropriate figures. For polymerase chain reaction (PCR) experiments, first-strand cDNA was synthesized from 10 μg of total RNA using an oligo-dT₁₅ primer in conjunction with 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (United States Biochemical, Cleveland, OH, U.S.A.). Full-length D₃-encoded cDNA was amplified by PCR using the primer pair D35': ATGGCACCTCTGAGCGAG and D33': GGCTTTGCGGAAGTCTATGT. Amplification of the amino-terminal D₃ cDNA was achieved with the pair of primers D35' and mD313': GATGGCACAGAGGTTTCAG. For exponential reverse transcription (RT)–PCR experiments, the carboxyl terminal half of the mouse D₃ cDNA was amplified by PCR using the primer pair 5'-TAGACTTCGGTGGTCTCTTC/5'-GGCTTTGC-GGAAGTCTATG and equal aliquots of the first strand cDNA reaction. For PCR with templates of each genotype, five PCR tubes containing equal aliquots of one PCR mastermix were taken to different endpoints (10, 15, 20, 25 and 30 cycles of amplification). The respective PCR products were analysed on Southern blots probed with a ³²P-radiolabeled cDNA encoding the mouse D₃ receptor.

RESULTS

Generation of mice lacking D₂, D₃ and D₂/D₃ receptors

Gene targeting via homologous recombination was used to generate mice deficient for D₂ and D₃ receptors (see Experimental Procedures).

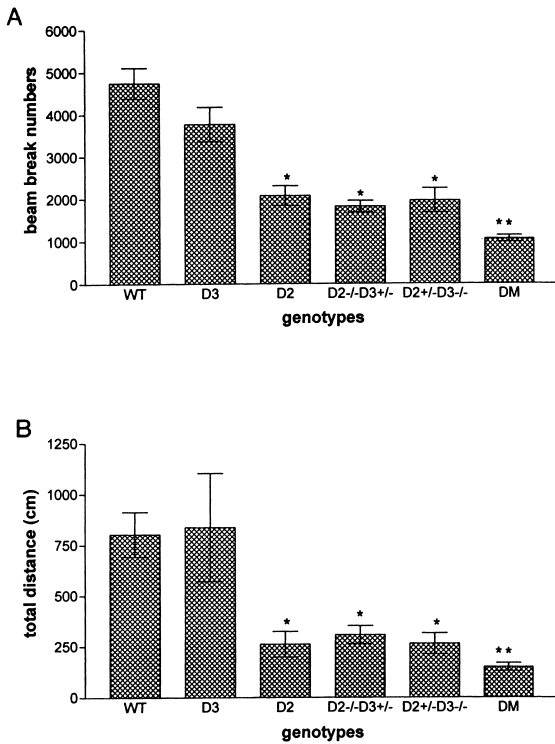


Fig. 3. Locomotor activity of wild-type, D_2 and D_3 single mutants, and D_2/D_3 double mutants. (A) Values represent the mean \pm S.E.M. of total horizontal photobeam cell interruptions recorded during the first 30 min spent in the test chamber. (B) Values represent the mean \pm S.E.M. of the total distance traveled during the same test-period. Seven animals per genetic group were tested. DM, D_2/D_3 homozygous double mutants; WT, wild-type. *Significantly different from WT, **significantly different from D_2 mutants.

The targeted disruption of the D_2 gene results in a null mutation of the D_2 receptor (Fig. 1A). The male and female D_2 homozygous mutants that we generated are fertile and their offspring can be weaned three to four weeks after birth. Northern blot analysis of RNA extracted from the dorsal striatum (the anatomic region with highest density of D_2 receptor expression) of wild-type, and heterozygous and homozygous D_2 mutants showed a \sim 50% reduction in D_2 mRNA in heterozygous mutants and a complete absence of D_2 mRNA in homozygous mutants (Fig. 1B, middle panel). In addition, compared to wild-type, homozygous D_2 mutants express \sim 3-fold higher levels of enkephalin mRNA in the dorsal striatum (Fig. 1B, right panel). This finding was expected (see also Ref. 2) because enkephalin mRNA is co-expressed with D_2 receptors in medium spiny neurons of the striatopallidal pathway in which activation of D_2 receptors inhibits the transcription of the enkephalin gene.¹²

The motor phenotype observed in adult homozygous mutants qualitatively resembles the phenotype previously described for D_2 mutants.² It is characterized by a pronounced bradykinesia (locomotor hypoactivity, delayed initiation of

movements) and postural abnormalities (hunched posture, sprawling of hind legs). However, these motor abnormalities become apparent only after postnatal day 15 (P15), reach their most severe state between P30 and P45 (the mortality at this age is about 20%) and then improve significantly. Mice between P15 and P45 are smaller than their wild-type littermates and their body weight is reduced by \sim 20%. The late postnatal onset of motor abnormalities is consistent with the ontogeny of D_2 receptor expression which is low at birth and increases continuously in the dorsal and ventral striatum to reach adult levels at \sim P21.¹⁵

In order to quantitatively compare the locomotor activity of adult wild-type and homozygous D_2 mutants, male mice at postnatal age P70 were placed into locomotor activity boxes (see Experimental Procedures) to record the total number of horizontal photobeam interruptions and the total distance traveled by the animals. The postnatal age P70 was chosen because, at this age, body weight and size differences between wild-type and mutant animals are no longer apparent. Thus, these parameters cannot account for possible differences in measurements of locomotor activity.

The locomotor hypoactivity of D_2 mutants is most apparent under non-habituating conditions. As shown in Fig. 3, significant differences between wild-type and homozygous D_2 mutant littermates were found for both the horizontal activity recorded over the first 30 min spent in the test chamber during the light phase of the light/dark cycle (4747 ± 361 counts vs 2082 ± 235 counts; $P < 0.01$) and the total distance traveled during this time (804 ± 110 cm vs 262 ± 63 , $P < 0.001$).

Our targeted disruption of the D_3 gene results in a premature termination of D_3 mRNA at sequences encoding the putative second intracellular domain of the D_3 receptor (Fig. 2A). In contrast to the phenotypic appearance of homozygous D_2 mutants, the development and the gross behaviour of heterozygous and homozygous D_3 mutants did not differ from wild-type littermates. In order to prove the absence of full-length D_3 mRNA in homozygous mutants, RT-PCR analysis was performed with a pair of primers that target the full-length open reading frame of D_3 mRNA (see Experimental Procedures). As shown in Fig. 2B (middle panel), only wild-type mice and heterozygous mutants, but not homozygous mutants, express the full-length D_3 mRNA. However, as also shown in Fig. 2B (right panel), the mutant D_3 allele is transcribed. Thus, heterozygous and homozygous mutants express a truncated D_3 mRNA that encodes only the first three transmembrane spanning domains of the receptor.

As shown in Fig. 3, the locomotor activity of male homozygous D_3 mutants at postnatal age P70, assessed with measurements of horizontal photobeam interruptions and total distance traveled as

described above, did not significantly differ from wild-type littermates (horizontal photobeam interruption (wild-type vs D₃ mutants): 4747 ± 361 counts vs 3777 ± 409 counts, $P > 0.2$; total distance traveled: 804 ± 110 cm vs 839 ± 267 cm, $P > 0.5$).

Finally, mice deficient for both D₂ and D₃ receptors were generated by mating homozygous D₂ males with homozygous D₃ females to obtain heterozygous D₂/D₃ double mutants which were then further crossed to obtain homozygous double mutants. All offspring are unremarkable at birth. However, the mortality of homozygous double mutants is ~30% and, compared to single D₂ mutants, death occurs at earlier postnatal ages (i.e. between P4 and P15). Furthermore, between P15 and P60, the body weight of double mutants is ~15% lower than that of homozygous D₂ mutants. Interestingly, the postural abnormalities of homozygous double mutants resemble those seen in homozygous D₂ mutants, but their locomotor activity is more severely impaired. As shown in Fig. 3, the locomotor activity of adult male homozygous double mutants determined at postnatal age P70 is significantly reduced not only when compared to wild-type but also when compared to D₂ mutants. The total number of horizontal photobeam interruptions measured during the first 30 min of the test period is 2082 ± 235 for homozygous D₂ mutants and 1066 ± 78 for homozygous D₂/D₃ double mutants and this difference is statistically significant at $P < 0.01$. Furthermore, significant differences were also found for the total distance traveled during this test period (homozygous D₂ mutants: 262 ± 63 cm vs homozygous double mutants: 150 ± 20 cm; $P < 0.01$).

Furthermore, we tested two additional types of double mutants that are heterozygous for one and homozygous for the other mutant allele (D₃+/-/D₂-/- and D₃-/-/D₂+/-). Similar to D₂ single mutants, the parameters of locomotor activity of D₃+/-/D₂-/- and D₃-/-/D₂+/- double mutants differ significantly from wild-type ($P < 0.001$). They are, however, still significantly different from those of homozygous double mutants (horizontal photobeam interruption: (D₃+/-/D₂-/-): $P < 0.001$ and (D₃-/-/D₂+/-): $P < 0.05$; total distance traveled: (D₃+/-/D₂-/-): $P < 0.01$ and (D₃-/-/D₂+/-): $P < 0.05$).

In conclusion, the motor phenotype of D₂/D₃ double mutants is qualitatively similar to the phenotype observed in homozygous D₂ mutants. However, the absence of both D₂ and D₃ receptors results in a significantly greater severity of these motor abnormalities. Furthermore, as noted above, differences in the motor activity between wild-type and D₂ and D₂/D₃ mutant mice are most apparent during the first 30 min of exposure to locomotor activity boxes (measured between 1.00 p.m. and 2.00 p.m.; light cycle). Records of the locomotor activities during subsequent hours indicate virtually no

locomotor activity of all mice, regardless of their genotype (habituation). Then, during the dark cycle, the locomotor activity of wild-type and D₃ mutants peaks again between 6.00 p.m. and 11.00 p.m. During this time, however, no locomotor activity is recorded for D₂ mutants and D₂/D₃ double mutants (not shown). This suggests that the locomotor hypoactivity of these mutants is, in fact, more severe than the measurements made during the light cycle indicate.

Indices of dopaminergic activity in D₂, D₃ and D₂/D₃ receptor mutant mice

The differences in the severity of locomotor hypoactivity between D₂ and D₂/D₃ mutants suggested a synergistic effect of D₃ receptor inactivation on the development of the D₂ mutant locomotor hypoactivity. We next sought to determine whether alterations in biochemical indices of dopaminergic activity show similar synergistic effects of D₂ and D₃ receptor inactivation. Therefore, levels of DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined for D₂ and D₃ single and D₂/D₃ double mutants using HPLC-coupled electrochemical detection (see Experimental Procedures). For these studies, 10-week old male animals were used and the results are summarized in Table 1. Compared to wild-type, no alteration in the levels of DA and its metabolites DOPAC and HVA are found in the dorsal striatum of heterozygous and homozygous D₃ mutants. Similar results are obtained from double mutants that are heterozygous for both mutant alleles. Surprisingly, although D₂ receptors are thought to be powerful autoreceptors, homozygous D₂ mutants do not show dramatic changes in the levels of DA. However, their levels of DOPAC and HVA are increased by ~50%. Similar results are obtained from D₃(-/-)/D₂(+/-) mice. D₃(+/-)/D₂(-/-) and D₃(-/-)/D₂(-/-) double mutants, however, show a ~100% increase in the levels of DA metabolites. Interestingly, only these latter results mimic precisely the classically known consequence of chronic neuroleptic treatment, namely a two- to three-fold stimulation of striatal DA metabolism due to a lack of DA utilization. Our results therefore indicate that the effects of classical neuroleptics used in the earlier studies^{9,31} are mediated via blockade of these two receptor subtypes. (Note that the measurements summarized in Table 1 of DA and metabolites in homozygous double mutants and heterozygous D₃ mutants were obtained from an experiment that was performed independently from the experiment on the other mutants. The line drawn through the table separates the two experiments from each other and each of the two experiments has their own wild-type control.)

Despite the increased accumulation of DA metabolites the steady-state levels of the neurotransmitter

Table 1. Levels of dopamine and metabolites in the striatum of D₂, D₃, and D₂/D₃ mutant mice

Genotype	DA (ng/mg protein)	DOPAC/DA (%)	HVA/DA (%)
Wild-type	92.7 ± 8.8	3.9 ± 0.3	10.4 ± 0.3
D ₃ ^{-/-}	101.9 ± 15.4	4.2 ± 0.1	12.8 ± 1.8
D ₂ ^{-/-}	94.3 ± 10.7	5.9 ± 0.4 ^a	15.3 ± 1.7 ^b
D ₂ ^{+/-}	92.0 ± 7.1	4.6 ± 0.1	14.4 ± 0.4
D ₂ (^{+/-})/D ₃ (^{+/-})	104.1 ± 19.5	4.4 ± 0.2	13.2 ± 1.2
D ₂ (^{+/-})/D ₃ (^{-/-})	101.4 ± 12.4	5.9 ± 1.2 ^c	16.5 ± 3.1 ^d
D ₂ (^{-/-})/D ₃ (^{+/-})	107.0 ± 11.7	8.1 ± 1.2^e	24.7 ± 3.8^f
Genotype	DA (ng/mg protein)	DOPAC/DA (%)	HVA/DA (%)
Wild-type	100.5 ± 14.7	6.6 ± 0.4	9.2 ± 0.01
D ₂ (^{-/-})/D ₃ (^{-/-})	93.5 ± 14.1	13.0 ± 0.6^g	18.3 ± 2.4^h
D ₃ ^{+/-}	96.0 ± 18.1	6.8 ± 0.8	9.3 ± 0.7

All data are corrected for mg of protein and expressed as mean ± S.D. of measurements obtained from five animals per group. DOPAC and HVA levels of D₂^{-/-} mutants and D₂(^{+/-})/D₃(^{-/-}) and D₂(^{-/-})/D₃(^{+/-}) mutants are significantly higher than the corresponding wild-type levels (^a*P* < 0.001; ^b*P* < 0.01; ^c*P* < 0.05; ^d*P* < 0.05; ^e*P* < 0.05; ^f*P* < 0.01). DOPAC and HVA levels of D₂(^{-/-})/D₃(^{-/-}) mutants differ significantly from corresponding levels of D₂^{-/-} mice at ^g*P* < 0.001 and ^h*P* < 0.05 (Student's *t*-test).

DA itself are indistinguishable in all genotypes, including homozygous double mutants. Furthermore, the expression of the rate-limiting DA-synthesizing enzyme tyrosine hydroxylase (TH), determined with immunoblots using a mouse monoclonal anti-TH antibody (Incstar, Stillwater, MN, U.S.A.), is unaltered in the dorsal striatum of all mutants (not shown). It should be noted, however, that the latter result does not exclude the possibility that the activity of the TH enzyme, modulated by its various phosphorylation states,^{16,17} is altered in brains of these mutants.

In conclusion, the combined inactivation of D₂ and D₃ receptors increases the severity of the D₂ mutant locomotor hypoactivity and results in steady-state levels of DA metabolites in the dorsal striatum that are significantly higher than those found in D₂ single mutants.

Expression of D₃ receptors in brains of D₂ mutant mice

The results summarized above suggest that D₂-like functional properties of D₃ receptors become unmasked in mice with a D₂ mutant genetic background. They further suggest the possibility that compensatory changes in the expression of D₃ receptors in brains of D₂ mutant mice can diminish the severity of a D₂ mutant phenotype. To begin to address this issue we tested whether mice deficient for D₂ receptors show an increased expression of D₃ receptors.

The very low abundance of D₃ mRNA complicates conventional non-PCR-based quantitative determinations of D₃ mRNA levels. Furthermore, radioligand binding studies are less suited to test whether the absence of D₂ receptors results in an altered expression of D₃ receptors because currently available D₃-preferring ligands also have

appreciable affinities to other neurotransmitter/peptide receptors, such as serotonin and sigma receptors^{19,30} (see Discussion). We therefore chose to determine the levels of D₃ protein during the postnatal development of D₂ mutant mice with immunoprecipitation (IP) experiments. For these experiments, the dorsal striatum was dissected and proteins extracted from the brain (-striatum) and striatum were analysed separately. For each genetic group, proteins extracted from at least four animals of different litters were pooled and the protein concentration in each pooled lysate was determined (see Experimental Procedures). Protein (7 mg/ml) were used for IP experiments in which D₃ proteins were immunoprecipitated with our D₃-specific monoclonal antibody IgG/D3.²⁵ The entire protein G-antibody-antigen complex was then analysed on western blots probed with our IgM/D3 antibody²⁵ (see Experimental Procedures). Additional western blots tested whether aliquots of each IP lysate (50 µg of total protein per aliquot) contain equal levels of TH immunoreactivity. A representative example of these control experiments on IP lysates obtained from wild-type and heterozygous and homozygous D₂ mutants at P45 and P60 is shown in Fig. 4A (bottom left panel).

As shown in Fig. 4A, three molecular forms of D₃ protein are found in mouse brain. These molecular forms correspond to the monomeric (~50,000 mol. wt), dimeric (~100,000 mol. wt) and tetrameric (~200,000 mol. wt) D₃ proteins that we have recently reported to be expressed in rat, monkey, and human brain.²⁵ The expression of D₃ proteins in wild-type brains, however, decreases significantly between postnatal ages P15 and P30. A further gradual decline in D₃ protein levels is observed in these mice between postnatal ages P45 and P60, and the lowest levels of D₃ proteins are detected in adult brains (P90). This is best seen for the tetrameric

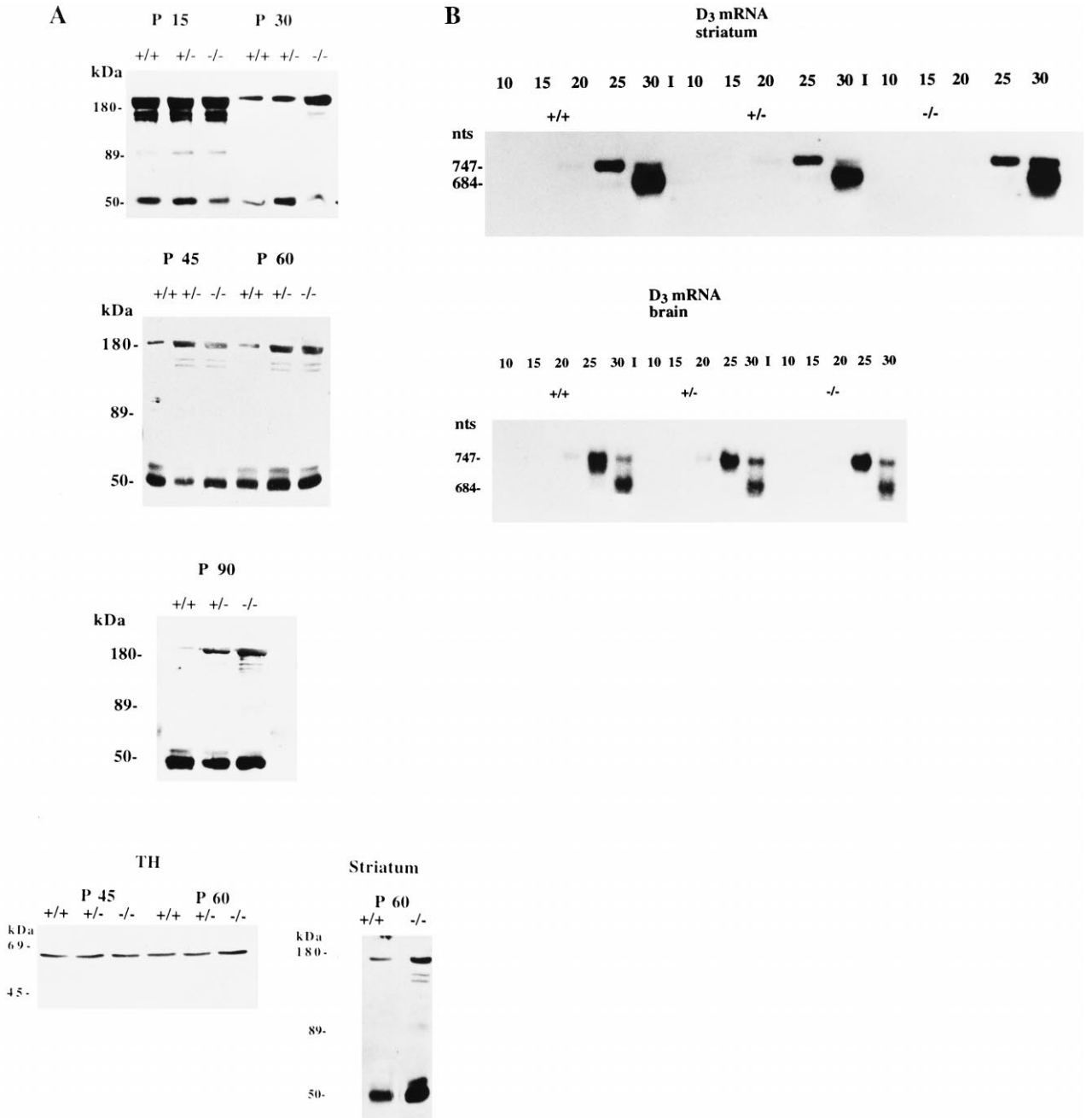


Fig. 4. D₃ Receptor expression during postnatal development of D₂ mutant mice. (A) Western blot of D₃ IPs (IG/D₃) obtained from 7 mg of total protein extracted from brain tissues of mice at postnatal ages P15 to P90. The blots were probed with the monoclonal IgM/D₃ antibody. The IP-antibody (IgG/D₃) band co-migrates with the monomeric D₃ protein of ~50,000 mol. wt. The top three panels show D₃ proteins extracted from brain (-striatum) tissues, the bottom right panel shows D₃ proteins extracted from the striatum. Note the increased expression of the tetrameric D₃ protein of ~200,000 mol. wt in D₂ mutants which becomes apparent at postnatal age P30. The bottom left panel shows the expression of TH immunoreactivity in 50 μg of brain (-striatum) protein lysates used for the P45 and P60 IP experiments. (B) Expression of D₃ mRNA in brains of adult D₂ mutant mice. Southern blots of RT-PCR amplified carboxyl terminal D₃ cDNAs. Equal aliquots of first-strand cDNA reactions were amplified by exponential PCR for 10, 15, 20, 25, and 30 cycles. The blots were probed with ³²P-radiolabeled cDNA encoding mouse D₃. Note that for the PCR analysis of each genotype, five reaction tubes containing equal aliquots of one PCR mastermix were taken to different endpoints of the PCR amplification. A minor D_{3S} cDNA, known to represent an mRNA species expressed in mouse brain,¹⁰ is co-amplified late (25 to 30 cycles) during the PCR amplification. The apparent decrease in D_{3L} cDNA seen after 30 cycles of amplification is likely to be an artifact resulting from D_{3L}/D_{3S} hybrids during the primer-annealing step of the PCR.

form of the D₃ protein which, as we have reported earlier,²⁵ is the most abundant protein species found in D₃ IPs. Furthermore, in mouse brain, dimeric D₃ proteins are most prominent at P15, but they are barely detectable at later postnatal ages. It should also be noted that the determination of the abundance of the monomeric form of the D₃ protein is obscured by the co-migration of the γ -chain of the IP antibody (which is of identical mass and also recognized by the secondary antibody) on sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

It is of interest to note that the above-determined onset of D₃-protein down-regulation coincides with the developmental age at which the expression of D₂ receptors reaches mature levels.¹⁵ In heterozygous and homozygous D₂ mutants, however, such a gradual postnatal down-regulation of D₃ protein expression is not observed. At P15, the expression of D₃ protein in heterozygous and homozygous D₂ mutants is indistinguishable from wild-type. At P30, however, homozygous D₂ mutants continue to express higher levels of D₃ protein. Furthermore, at P45, P60, and P90, the levels of D₃ proteins expressed in brains of heterozygous and homozygous D₂ mutants are substantially higher than the corresponding ones expressed in wild-type. The results shown in the upper three panels of Fig. 4A were obtained from IP experiments on brain (-striatum) proteins. Similar results were obtained with proteins extracted from the dorsal striatum of these brains. One representative result obtained from wild-type and homozygous D₂ mutants at P60 is shown in Fig. 4A (bottom right panel).

To test whether the increased expression of D₃ protein in brains of D₂ mutants results from an increased transcription of the D₃ gene, exponential RT–PCR experiments were performed to allow the amounts of D₃ cDNA, amplified after 10, 15, 20, 25, and 30 cycles of PCR amplification, to be compared. A Southern blot of these amplification products is shown in Fig. 4B. The result of this experiment does not suggest that the increased D₃ protein expression in heterozygous and homozygous D₂ mutants is due to an increased transcription and/or increased stability of D₃ mRNA. Neither the earliest time of the PCR-based detection nor the amount of D₃ cDNA amplified during the exponential phase of the PCR reaction differs for wild-type, and heterozygous and homozygous D₂ mutants. It is therefore likely that an increased translation of D₃ mRNA and/or posttranslational protein-stabilizing modifications of the D₃ protein are responsible for the increased levels of D₃ receptors.

In conclusion, the above data indicate that the lack of D₂ receptors is accompanied by an increased expression of D₃-receptor protein. The increased expression of D₃ protein in brains of D₂ mutants becomes apparent only after postnatal age P15 and appears to result from a lack of down-regulation of

D₃ receptor expression that is characteristically found in wild-type mice.

There is, at present, no evidence for a complementary increase in D₂ receptor expression in brains of D₃ mutants. Results of radioligand binding studies showed that the lack of D₃ receptors does not result in a compensatory increase in D₂ receptor binding sites.^{1,37} Furthermore, results of northern blots of mRNA extracted from the dorsal striatum and from extrastriatal tissues of our mutants show that the expression of D₂ mRNA is not altered in brains of adult D₃ mutants (Fig. 2C). In addition, the expression of striatal enkephalin mRNA, known to be tightly regulated by D₂ receptor activation (see above), is not significantly altered in these mutants (Fig. 2C).

Altered expression of D₃ proteins in brains of heterozygous D₃ mutants

The findings that, compared to homozygous double mutants, D₃(+/-)/D₂(-/-) double mutants differ in the severity of their locomotor hypoactivity (see Fig. 3) but show similar increases in DA metabolism (see Table 1) suggest the possibility that, in heterozygous D₃ mutants, the co-expression of the truncated amino-terminal peptide (encoded by the mutant D₃ allele; see Fig. 2C) and the wild-type receptor results in a dominant negative effect on some, but not all, functions of D₃ receptors. A similar suggestion was made in an earlier study of Accili *et al.*¹ who generated D₃ mutant mice that are genetically almost identical to our D₃ mutants, e.g. these mice express a truncated amino-terminal D₃ peptide derived from the mutant D₃ allele. Interestingly, these authors also showed that their heterozygous D₃ mutants have lost almost all [¹²⁵I]iodosulpiride binding to D₃ receptors. Thus, if the mutant amino-terminal D₃ peptide indeed has dominant negative effects on some of the functions of wild-type D₃ receptors, one possibility is that it alters the expression of the wild-type receptors to render them inaccessible to ligand binding. In this regard it is of interest to note that we have recently shown that some of the D₃ oligomers expressed *in vivo* are heteromeric assemblies of D₃ receptor proteins and a natively expressed, truncated amino-terminal D₃-like protein D_{3nf}.^{25,29} Because these results demonstrated that truncated amino-terminal D₃-like peptides participate in D₃ receptor oligomerization they suggested the possibility that the truncated D₃ protein expressed in our D₃ mutant mice competes with wild-type D₃ and other native D₃-like molecules for D₃ receptor oligomerization. This prompted us to analyse the expression of D₃ receptors in wild-type mice and heterozygous D₃ mutants with IP experiments similar to those shown in Fig. 4.

The results shown in Fig. 5 illustrate that heterozygous D₃ mutants accumulate monomeric forms of the D₃ protein, but they do not express the dimeric

(~100,000 mol. wt) and tetrameric (~200,000 mol. wt) D₃ proteins that are found in wild-type brains. Although small amounts of D₃ proteins of higher molecular weight can also be detected in brains of heterozygous D₃ mutants, the mass of these molecules is less than that of wild-type dimeric D₃ proteins, suggesting that these are heterooligomers of full-length and mutant D₃ proteins. (In contrast to the experiments shown in Fig. 4, larger amounts of total protein were immunoprecipitated in the experiments shown in Fig. 5 in order to be able to clearly visualize dimeric and tetrameric D₃ proteins expressed in adult mouse brains.)

Our data suggest that the expression of the mutant amino-terminal D₃ peptide inhibits the formation of wild-type D₃-receptor oligomers in heterozygous D₃ mutants. The lack of wild-type D₃ oligomers in brains of heterozygous D₃ mutants may be responsible for the dominant-negative effects of the mutant amino-terminal D₃ peptide on some functions of D₃ receptors, namely those mediated by D₃ oligomers.

DISCUSSION

The present study employed gene targeting to generate D₂ and D₃ single and D₂/D₃ double mutant mice. A comparative study on these single and double mutants has identified synergistic effects of inactivation of D₂ and D₃ receptors on the impairment of locomotor activity and on the increased accumulation of dopamine metabolites in the dorsal striatum. Results from immunoprecipitation experiments further indicate an increased expression of D₃ proteins during the postnatal development of D₂ mutant mice.

Does the increased expression of D₃ proteins result in increased expression of functional D₃ receptors that can compensate for some of the functional properties of D₂ receptors? The finding that mice lacking D₃ receptors develop normally, even in the absence of a compensatory increase of the far more abundant D₂ receptor, does not exclude the possibility of a D₃ receptor-mediated compensation for the lack of D₂ receptors, and two observations suggest that the increased expression of D₃ proteins diminishes the severity of D₂ mutant phenotypes.

Firstly, the postnatal development of the motor phenotype of D₂ mutants develops only after postnatal day 15. This relatively late postnatal onset of motor abnormalities is consistent with the postnatal development of D₂ receptor expression, which is low at birth and increases continuously in the dorsal and ventral striatum to reach adult levels at ~P21.¹⁵ However, whereas the severity of motor abnormalities in D₂ single mutants peaks between postnatal age P30 and P45 and then improves substantially, the severity of the motor abnormalities of D₂/D₃ double mutants persists into adulthood. Moreover, during all stages of postnatal development, the locomotor hypoactivity of mice lacking D₂ and D₃

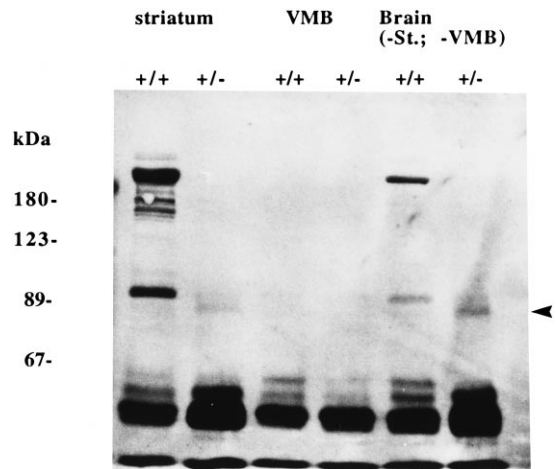


Fig. 5. D₃ IPs of proteins extracted from brains of adult wild-type (+/+) and heterozygous (+/-) D₃ mutants. Proteins were extracted from the striatum, ventral midbrain (VMB), and the remaining brain tissue (-Str.; -VMB). Ten milligrams/milliliter of proteins were immunoprecipitated with the monoclonal IgG/D₃ antibody and the blot was probed with the monoclonal IgM/D₃ antibody. Note that both wild-type and +/- D₃ mutants do not express D₃ oligomers in the VMB. In the other brain regions of +/- D₃ mutants, however, wild-type D₃ oligomers are absent and the position of the band representing abnormal D₃ oligomers is indicated by an arrowhead.

receptors is significantly increased compared to D₂ single mutants, despite the fact that D₃ single mutants do not develop motor abnormalities that resemble those of D₂ mutants. In this regard it is of interest to note that, in contrast to wild-type mice, D₂ mutants do not down-regulate the expression of D₃ proteins between postnatal age P15 and P30, suggesting that D₃ receptors can partially compensate for the loss of D₂ receptors during late postnatal development. Such a potential compensation cannot occur in mice lacking both D₂ and D₃ receptors and, consistent with the suggested D₃ receptor-mediated functional compensation, the motor abnormalities of the double mutants persist into adulthood.

Secondly, the observation that the onset of postnatal down-regulation of D₃ protein expression in wild-type mice coincides with the maturation of D₂ receptor expression¹⁵ further suggests that some functional properties are shared between both receptor subtypes.

Some of the results obtained with studies on our D₂ and D₃ single mutants differ from results published previously. Firstly, whereas we found no differences in the locomotor activity between wild-type and D₃ mutants, Accili *et al.*¹ and Xu *et al.*³⁷ reported a locomotor hyperactivity of their D₃ mutants. This apparent difference is probably due to differences in time intervals of behavioral measurements. Accili *et al.*¹ and Xu *et al.*³⁷ evaluated the locomotor activity of their D₃ mutants during the first 15 min of exposure to the test environment. However, as shown by Xu *et al.*,³⁷

the hyperactivity of D₃ mutants habituates rapidly, thus leading to activity levels of D₃ mutants that, after 15–20 min of exposure to the test chamber, are identical to locomotor activities of wild-type mice. Because our study compared the locomotor activity of wild-type and mutant mice during a 30-min test period it is likely that an initial and rapidly habituating hyperactivity of D₃ mutants was insufficient to result in significantly increased numbers for their locomotor activity measured over a 30-min period.

Secondly, the motor phenotype of our D₂ single mutants is qualitatively and quantitatively similar to that described by Baik *et al.*² In contrast, Kelly *et al.*¹⁸ generated D₂ mutant mice that developed normally and showed no significant motor abnormalities as a consequence of the lack of D₂ receptor expression. In this regard it should be noted that, in contrast to Baik *et al.*² and the present study, Kelly *et al.*¹⁸ did not generate a null mutation of the D₂ gene but rather disrupted the gene at a sequence encoding the third cytoplasmic domain of the D₂ receptor. A potential role of truncated dopamine receptor subtypes (some of which are natively expressed) is only beginning to emerge^{25,29} and it is possible that the targeted disruption of the D₂ gene executed by Kelly *et al.*¹⁸ results in the expression of a relatively long, truncated amino-terminal D₂ protein (which consists of five transmembrane-spanning domains) which can partially mask the functional consequences of a D₂ knockout.

Thirdly, it is noted that two different approaches to determine the expression of D₃ receptors in brains of D₂ mutant mice have given different results. Our IP experiments, performed with D₃-specific monoclonal antibodies,²⁵ detected an increased expression of D₃ protein during the postnatal development of D₂ mutants. In contrast, a previous study of Baik *et al.*² evaluated the anatomic distribution of D₃ receptors by means of visualizing D₃ radioligand binding sites, and these authors failed to detect an increased D₃ receptor expression in brains of adult D₂ mutants. It should be noted, however, that discrepancies also exist with regard to the anatomic distribution of D₃ receptors when results of radioligand binding studies (using D₃-“preferring” ligands)^{22,26} are compared to results of immunocytochemical studies using D₃-specific antibodies.²¹ In addition, the more recently reported widespread D₃ mRNA distribution detected with *in situ* hybridization studies^{24,33} does not correspond to the restricted anatomic distribution of D₃ receptors detected with radioligand binding experiments. It is therefore presently impossible to conclude unequivocally that the anatomic

distribution of D₃ receptors described with radioligand binding studies is indeed most accurately reflecting the expression of this receptor subtype. The development of new D₃-selective ligands, however, will certainly help to clarify this issue.

Finally, our immunoprecipitation studies performed on tissues of heterozygous D₃ mutants demonstrate an absence of wild-type D₃ oligomeric proteins in brain regions where they are normally found. Moreover, we found that not all brain regions of wild-type animals express D₃ oligomers. For example, the ventral midbrain is devoid of oligomers but expresses monomers (see Fig. 5). This suggests the possibility that autoreceptors are expressed exclusively as monomers, and that at least a portion of postsynaptic D₃ receptors are oligomers whose functions would be affected by the expression of the mutant D₃ peptide in brains of heterozygous D₃ mutants. This would further imply that the lack of autoreceptors is largely responsible for the development of the abnormal locomotor phenotype, and it would explain why (in contrast to the results of our study that determined the tissue levels of dopamine metabolites) no dominant-negative effects on locomotor activities were detected in D₃+/-/D₂-/- double mutants.

CONCLUSIONS

A comparative analysis of D₂ and D₃ single mutants and D₂/D₃ double mutants identified distinct functions of D₃ receptors that remain masked in the presence of the abundant D₂ receptor. The absence of both D₂ and D₃ receptors leads to a potentiation of some of the phenotypes that are characteristic for D₂ single mutants, namely locomotor hypoactivity and increased metabolism of striatal dopamine. Furthermore, results of immunoprecipitation studies show that D₂ mutant mice express higher levels of D₃ receptor protein during later stages of their postnatal development. Thus, studies on D₂/D₃ double mutant mice have begun to identify functional properties of D₃ receptors that appear to compensate for some of the functional properties of D₂ receptors.

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