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ORIGINAL ARTICLE

Enhanced Expression of Dopamine D_1 and Glutamate NMDA Receptors in Dopamine D_4 Receptor Knockout Mice

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Abstract

Expression of dopamine ([DA] D_1 and D_2) and glutamate ([Glu], (*N*-methyl-D-aspartic acid [NMDA], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA], and kanaic acid [KA]) receptor types were analyzed autoradiographically in forebrain regions of D_4 receptor knockout mice and their wild-type controls. Selective radioligand binding to D_4 receptors was virtually absent in D_4 receptor knockout mouse brain in contrast to significant specific D_4 binding in forebrain tissue of wild-type controls. Labeling of D_1 receptors was significantly increased in nucleus accumbens (NAc; 39%) and caudate putamen (CPu; 42%) of D_4-knockout mice vs wild-type controls. In addition, NMDA receptor labeling was significantly increased in NAc (31%), CPu (40%), and hippocampal CA_1 (21%) and CA_3 (25%) regions of D_4 knockouts vs wild-type controls. No changes in D_2, AMPA or KA receptors were found. The findings suggest that D_1, D_4, and NMDA receptors might interact functionally and that developmental absence of D_4 receptors might trigger compensatory mechanisms that enhance expression of D_1 receptors in NAc and CPu, and NMDA receptors in NAc, CPu, and hippocampus. The findings also encourage cautious interpretation of results in knockout mice with targeted absence of specific genes, as complex adaptive changes not directly related to the missing gene might contribute to physiological and behavioral responses.

Index Entries: Autoradiography; caudate putamen; dopamine receptors; D_4-receptor; genetic knockout; glutamate receptors; mutant mice.

Introduction

Dopamine (DA) D_4 receptors are members of the D_2-like receptor family, which also includes D_2 and D_3 receptors. Other well-characterized DA receptors (D_1, D_5) constitute the D_1-like family. Members of each family share similar molecular structures and pharmacological profiles but differ greatly in relative abundance and neuroanatomical distribution (Baldessarini and Tarazi, 1996; Neve and Neve, 1997).

Several functional activities have been ascribed to D_4 receptor stimulation, including inhibition of adenylyl cyclase activity, activation of arachidonic acid release, blockade of L-type calcium currents,

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and stimulation of membrane phospholipid methylation in cultured neuroblastoma cell lines (*see* Tarazi and Baldessarini, 1999a). Human D_4 receptors can be transcribed into polymorphic variants that differ in the number of repeats of a 48-amino-acid sequence within the functionally critical third intracellular loop of the peptide. There can be 2–11 repeats, but those with 2, 4, or 7, designated as $D_{4.2}$, $D_{4.4}$, or $D_{4.7}$, are the most prevalent human alleles (Van Tol et al., 1992; Lichter et al., 1993). Clinical genetic studies have associated D_4 receptor polymorphism with specific behavioral traits, including novelty seeking (Benjamin et al., 1996; Ebstein et al., 1996), and with attention deficit-hyperactivity disorder (La Hoste et al., 1996; Swanson et al., 1998). In addition, behavioral neuropharmacological findings link D_4 receptors to hyperactivity in juvenile rats with neonatal lesions of DA neurons (Zhang et al., 2001, 2002).

Immunohistochemical and binding studies indicate that cerebral D_4 receptors are highly expressed in cerebral cortical regions and also in hippocampal formation, including the subiculum (Van Tol et al., 1991; Ariano et al., 1997; Tarazi et al., 1997a). In prefrontal cortex, D_4 receptors are expressed in both inhibitory γ-aminobutyric acid (GABA) interneurons and excitatory glutamate (Glu) pyramidal neurons where they function as inhibitory modulators of Glu neurotransmission (Mrzljak et al., 1996; Rubinstein et al., 2001). Lower, but still significant, levels of D_4 receptors are also expressed in caudate putamen (CPu) and nucleus accumbens (NAc) (Defagot et al., 1997; Tarazi et al., 1997a, 1998).

Mice with targeted gene depletion of D_4 receptors have been generated using recombinant DNA technologies in embryonic stem cells (Rubinstein et al., 1997). These D_4 receptor-lacking mutant mice displayed distinct behaviors compared to wild-type mice, including increased locomotor sensitivity to ethanol, cocaine, and methamphetamine (Rubinstein et al., 1997), reduced exploration of novel stimuli (Dulawa et al., 1999), and enhanced reactivity to unconditioned fear (Falzone et al., 2002). Biochemical and physiological analyses revealed that D_4 knockout mice had higher rates of DA metabolic turnover in dorsal striatum (Rubinstein et al., 1997) and increased excitability of Glu neurons in cerebral cortex (Rubinstein et al., 2001). Therefore, D_4 receptor mutant mice might constitute important neurobiological resources to examine the functions of D_4 receptors and related behaviors. However, these findings should be interpreted carefully, as phenotypic variability, genetic background, and developmental compensatory mechanisms might influence functions and behaviors associated with the absence of specific genes (Bucan and Abel, 2002).

Despite extensive behavioral characterization of D_4 receptor mutant mice (Rubinstein et al., 1997; Dulawa et al., 1999; Falzone et al., 2002), molecular effects of D_4 receptor depletion on concentrations of other DA receptors have not been well defined or compared quantitatively to wild-type mice expressing normal levels of cerebral D_4 receptors. In addition, close functional and often antagonistic interactions between DA and Glu systems (Carlsson and Carlsson, 1990; Carlsson et al., 2001) suggest that knocking out D_4 receptors might trigger changes in Glu receptors. Accordingly, we applied quantitative in vitro receptor autoradiography to assess regulation of DA D_1 and D_2 receptors, as well as the three ionotropic Glu receptors (*N*-methyl-D-aspartic acid [NMDA], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid (KA) types) in forebrain regions of D_4 receptor mutant mice vs wild-type controls.

Materials and Methods

Materials

Radiochemicals from New England Nuclear–Perkin-Elmer (Boston, MA) were DA receptor ligands, [*N*-methyl-^{3}H]R,S-nemonapride (86 Ci/mmol for D_2/D_4 receptors) and [*N*-methyl-^{3}H]SCH-23390 (81 Ci/mmol for D_1 receptors); and Glu receptor ligands, [3-^{3}H](+)-5-methyl-10,11-dihydro-[5H]-dibenzo(a,d)cyclohepten-5,10-imine (MK-801; 24 Ci/mmol for NMDA receptors), [5-^{3}H]AMPA (83 Ci/mmol for AMPA receptors), and [vinylidene-^{3}H]KA (58 Ci/mmol for KA receptors). Tritium autoradiography standards were from Amersham (Arlington Heights, IL). Tritium-sensitive Hyperfilm, as well as D-19 developer and fixative for autoradiography were from Eastman-Kodak (Rochester, NY).

Chemicals and drugs included 6-cyano-7-nitroquinoxaline (CNQX), 1,3-ditolylguanidine (DTG), *cis*-flupenthixol dihydrochloride, KA, ketamine hydrochloride, pindolol, potassium thiocyanate (KSCN), S(–)-raclopride, S(–)-sulpiride, and spermine tetrahydrochloride, all obtained from Sigma–Research Biochemicals International (Sigma–RBI; Natick, MA); ethylene diamine tetra-acetic acid (EDTA) from Fisher Scientific

(Fairlawn, NJ), as well as L-glutamic acid (Glu), L-glycine hydrochloride, and *tris*-(hydroxymethyl)-aminomethane (Tris) hydrochloride from Sigma Chemicals (St. Louis, MO).

D_4 Receptor Knockout Mice

Generation of D_4 receptor knockout mutant mice was detailed previously (Rubinstein et al., 1997). All mice tested were 8- to 10-week-old males F2s derived from the crossing of *Drd4*$^{+/-}$ mice (129/Ola × C57Bl/6J) for more than 10 generations (Rubenstein et al., 1997). Animals were housed in groups of five or six with free access to food and water and a 12/12-h light/dark cycle (lights on at 07.00 h). Age-matched wild-type and D_4 receptor knockout mice were killed by cervical dislocation, and their brains were rapidly removed and chilled on isopentane/dry ice. Brains were stored at –80°C in individual plastic vials until packed in dry ice in a Styrofoam box and mailed from Buenos Aires to Boston. All animal procedures were performed in accordance with the ethical standards set by the Guidelines for the Care and Use of Laboratory Animals of the United States Public Health Service.

Tissue Preparation

Frozen sections (10 μm) were prepared in a cryostat at –20°C, mounted on gelatin-coated, glass microscopic slides, and stored at –80°C until use. Coronal brain sections were taken through NAc septi, CPu, hippocampal regions CA_1 and CA_3, and entorhinal cortex (EC), in which DA and Glu receptor subtypes are well expressed (Tarazi and Baldessarini, 1999b).

Receptor Autoradiography

Brain sections from D_4 receptor knockout mice and wild-type controls were evaluated at the same time in each radioreceptor assay to minimize experimental variability. Sections were first preincubated for 60 min at room temperature (RT) in specified assay buffers to remove endogenous ligands before incubating them with the radioligand.

DA Receptor Autoradiography

D_1 Receptors

Mouse forebrain sections were preincubated for 60 min at RT in 50 m*M* Tris-HCl buffer (pH 7.4) containing 120 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$. Tissue sections were then incubated for 60 min at RT in the same buffer containing 1 n*M* [^{3}H]SCH-23390 with 100 n*M* ketanserin to mask serotonin 5-$HT_{2A/2C}$ receptors. Nonspecific binding was determined with excess (1 μ*M*) *cis*-flupenthixol. After incubation, slides were washed twice for 5 min in ice-cold buffer, dipped in ice-cold water, and dried under a stream of air (Florijn et al., 1997; Tarazi et al., 1997b, 2001). Though the resulting radioligand binding might include some binding to D_5 sites, most of the signal is believed to represent D_1 receptors.

D_2 Receptors

Sections were incubated for 60 min at RT in the same buffer containing 1.0 n*M* [^{3}H]nemonapride with 0.5 μ*M* DTG and 0.1 μ*M* pindolol to mask $\sigma_{1,2}$ and $5HT_{1A}$ sites, respectively. Nonspecific binding was determined with 10 μ*M* S(–)-sulpiride. After incubation, slides were washed twice for 5 min in ice-cold buffer, dipped in ice-cold water, and air-dried (Florijn et al., 1997; Tarazi et al., 1997b, 2001).

D_4 Receptors

Assay methods are modified from those reported to quantify these receptors in rat brain (Tarazi et al., 1997a,b, 2001). Tissue sections were preincubated for 60 min at RT in D_2 assay buffer and then for another 60 min with 1.0 n*M* [^{3}H]nemonapride with 600 n*M* S(–)-raclopride to occupy D_2/D_3 receptors optimally, as well as the masking agents (0.5 μ*M* DTG and 0.1 μ*M* pindolol) used in the D_2 assay. Nonspecific binding was determined with 10 μ*M* S(–)-sulpiride.

Glu Receptor Autoradiography

NMDA Receptors

Sections were preincubated for 60 min at RT in 50 m*M* Tris-HCl buffer (pH 7.4), then for 150 min at RT in fresh buffer containing 10 n*M* [^{3}H]MK-801, with 100 μ*M* L-Glu, 100 μ*M* glycine, 1 m*M* EDTA, and 75 μ*M* spermine to enhance the binding of [^{3}H]MK-801 to its site within the open cation channels associated with NMDA receptors. Nonspecific binding was determined by including 20 μ*M* ketamine. After incubation, slides were washed in ice-cold 50 m*M* Tris-HCl buffer, twice for 20 min, and dried (Tarazi et al., 1996, 1998, 2003).

AMPA Receptors

Tissue sections were incubated for 60 min at RT in 50 m*M* Tris-HCl buffer (pH 7.2) and again for 60 min at RT in fresh buffer containing 30 n*M* [^{3}H]AMPA with 2.5 m*M* $CaCl_2$ and 30 m*M* KSCN. Nonspecific binding was determined by including

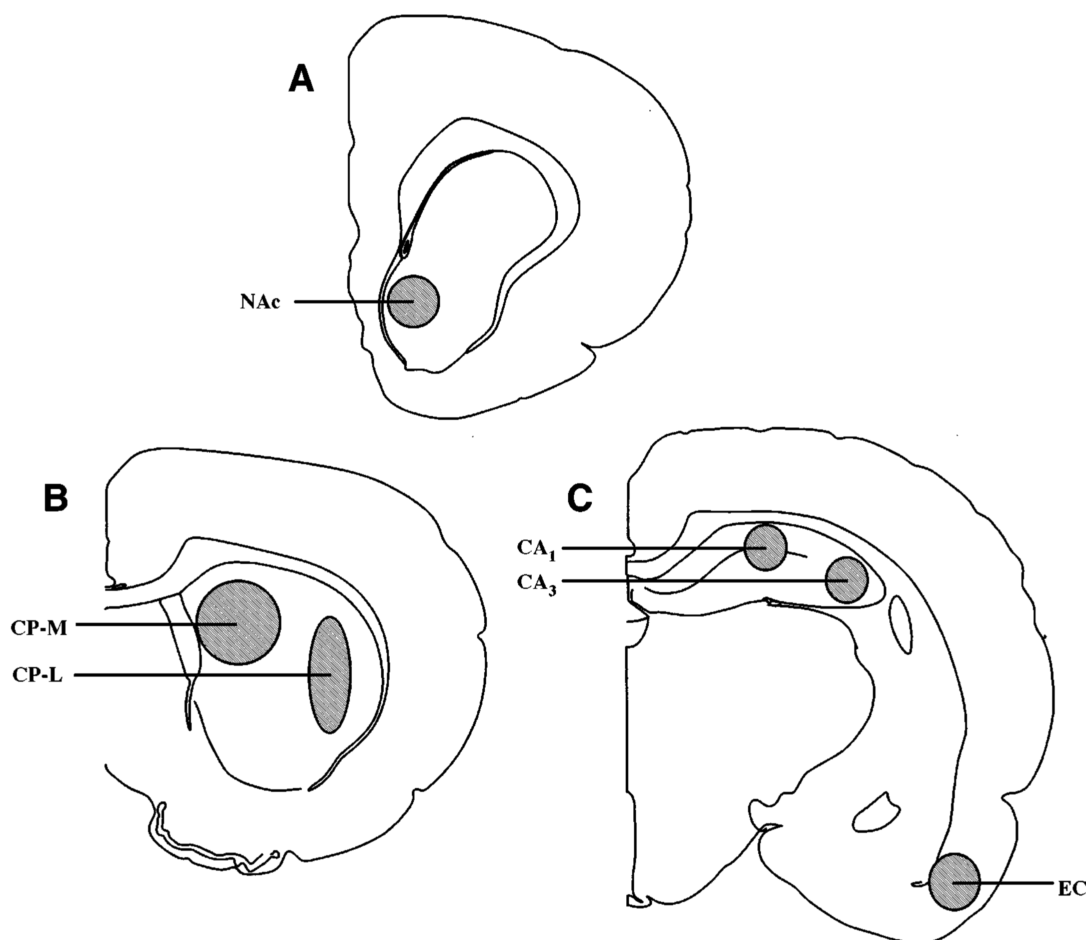

Fig. 1. Sites of autoradiographic analyses of mouse brain regions sampled in 10-µm coronal sections. NAc, nucleus accumbens septi; CPu, caudate putamen (L, lateral; M, medial); EC, entorhinal cortex; CA_1 and CA_3, hippocampal regions.

30 µ*M* unlabeled CNQX. After incubation, slides were washed 3 times in ice-cold Tris buffer for 10 s and dried (Wullner et al., 1994; Tarazi et al., 2003).

KA Receptors

Sections were preincubated for 60 min at 4°C in 50 m*M* Tris-HCl buffer (pH 7.0) and then incubated in this buffer containing 20 n*M* [^{3}H]KA for 60 min at 4°C. Nonspecific binding was determined with 25 µ*M* unlabeled KA. Slides were then washed 3 times in ice-cold 50 m*M* Tris buffer for 10 s and air-dried (Tarazi et al., 1996, 1998, 2003).

Autoradiography and Image Analysis

Radiolabeled slides and calibrated ^{3}H standards (Amersham) were exposed to Hyperfilm (Eastman-Kodak; Rochester, NY) at 4°C for 2–5 wk ([^{3}H]SCH-23390 and [^{3}H]nemonapride), 3 wk ([^{3}H]AMPA and [^{3}H]KA), or 4 wk ([^{3}H]MK-801). Films were developed in Kodak D-19 developer and fixative. Optical density (OD) in brain regions of interest (Fig. 1) was measured with a computerized densitometric image analyzer (MCID-M4, Imaging Research; St. Catharines, Ontario, Canada). Left and right sides of two contiguous sections represented total binding and two additional sections represented nonspecific binding; the four determinations were averaged for each of n = 6 subjects/treatment). Optical density was converted to nCi/mg of tissue using calibrated [^{3}H] standards and specific binding (total minus nonspecific binding) was expressed as fmol/mg tissue.

Data Analysis

Effects of knocking out D_4 receptors on each receptor assay, by anatomical region, were tested by analy-

Table 1
DA D_4 Receptor Binding in Wild-Type and D_4 Receptor Knockout Mice

	Mice	
Brain region	Wild type	D_4 receptor knockout
Nucleus accumbens	38.4 ± 1.0	N.D.
Caudate putamen		
Medial part	35.5 ± 1.7	N.D.
Lateral part	60.3 ± 1.8	N.D.
Hippocampus		
CA_1 region	17.3 ± 1.1	N.D.
CA_3 region	17.5 ± 0.9	N.D.
Entorhinal cortex	11.3 ± 0.8	N.D.

Data are mean ± S.E.M. values for binding (fmol/mg tissue), determined by quantitative autoradiography (n = 6 rats/group; N.D., not detected), as described in Materials and Methods.

Table 2
DA D_1 Receptor Binding in Wild-Type and D_4 Receptor Knockout Mice

	Mice	
Brain region	Wild type	D_4 receptor knockout
Nucleus accumbens	127.9 ± 4.7 (100)	172.7 ± 11.9 **(135)**[a]
Caudate putamen		
Medial part	144.4 ± 16.1 (100)	203.4 ± 12.1 **(141)**[a]
Lateral part	152.1 ± 16.8 (100)	219.3 ± 19.1 **(144)**[a]
Hippocampus		
CA_1 region	31.0 ± 0.9 (100)	30.3 ± 0.7 (98)
CA_3 region	31.0 ± 1.1 (100)	28.5 ± 2.0 (92)
Entorhinal cortex	36.9 ± 0.9 (100)	38.9 ± 1.2 (105)

Data are mean ± S.E.M. values for binding (fmol/mg tissue and percent of control), determined by quantitative autoradiography with significant differences from controls indicated in boldface ([a]$p < 0.05$; n = 6 rats per group), as described in Materials and Methods.

sis of variance (ANOVA), with post hoc Dunnett t-tests used for planned comparisons of mutant vs wild-type mice, and considered significant at two-tailed $p < 0.05$. Data are presented as mean fmol/mg tissue ± S.E.M. binding for n = 6 subjects per condition.

Results

Autoradiographic analysis of tissue receptor levels under D_4-selective binding conditions detected a specific D_4 signal (at 11–60 fmol/mg tissue) in different forebrain regions of wild-type control mice (Table 1). The highly D_4-selective ligands L-745,870 and RBI-257 (Kebabian et al., 1997), both at 1 μM, displaced ≥85% of binding remaining in the presence of raclopride in CPu and NAc tissue from control mice, indicating that most of the raclopride-insensitive binding sites are D_4 receptors. This specific signal was not detected in cerebral tissue sections of D_4 receptor mutant mice, in which levels of total binding were indistinguishable from nonspecific binding in all forebrain regions examined (Table 1).

Distribution of DA D_1 and D_2 receptor labeling in mouse brain was similar to that found in rat brain (Tarazi et al., 1997b, 2001), with higher levels of D_1 and D_2 in NAc and CPu and lower levels in hippocampus and EC (Tables 2 and 3). In tissue from knockout mice vs controls, significant increases in D_1 receptor binding were detected in NAc (by 35%)

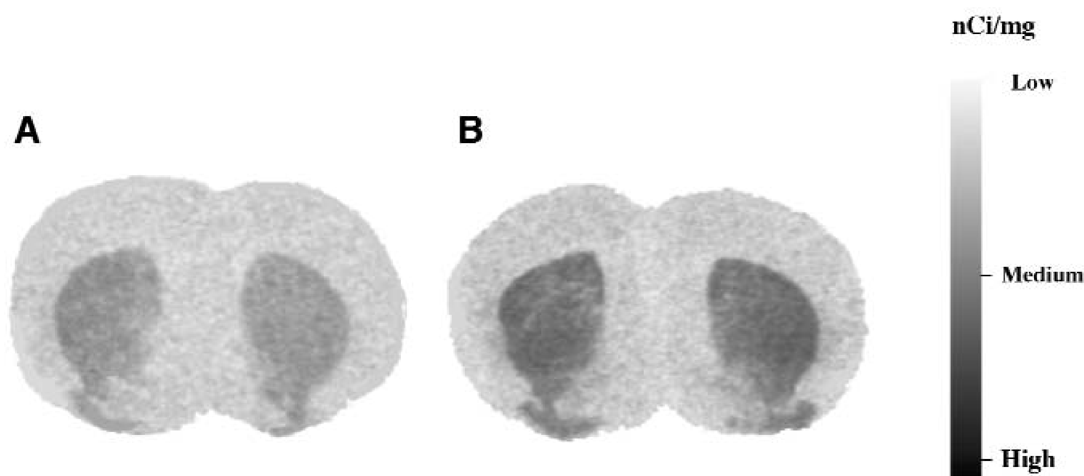

Fig. 2. Autoradiographically determined density (gray scale indicated nCi/mg of [^{3}H]SCH23390 bound per mg tissue) of binding to D_1 receptors in CPu of wild-type mice (**A**) and D_4 receptor knockout mice (**B**). The results indicate significant increases in D_1 receptor binding in medial and lateral CPu of D_4 receptor mutant mice compared to wild-type controls.

and lateral (44%) and medial CPu (41%), but not in hippocampus or EC (Fig. 2; Table 2). In contrast, levels of D_2 receptors did not differ between mutants and controls in any brain region examined (Table 3).

The observed distribution of ionotropic Glu receptors in mouse brain also accorded closely with our previous findings in rat brain (Tarazi et al., 1996, 1998). NMDA and AMPA receptors were highly expressed in hippocampal areas ($CA_1 > CA_3$), followed by NAc and CPu (Tables 4 and 5). In contrast, KA receptors were expressed selectively in the hippocampal CA_3 region and Nac, followed by CPu (Table 6). A significant increase in binding at NMDA receptors also was observed in NAc (31%), lateral (44%) and medial CPu (41%), as well as in hippocampal CA_1 (21%) and CA_3 (25%) regions of D_4 mutants vs controls (Table 4). There were no significant differences in tissue concentrations of AMPA or KA receptors between D_4 mutant mice vs controls (Tables 5 and 6).

Discussion

Autoradiographic quantification of D_4 and D_2 receptors in wild-type mice revealed that D_4 receptors represented a relatively high proportion of $D_2 + D_4$ receptors in mouse hippocampus (52–55%) and EC (40%–44%). In D_2-rich subcortical regions, D_4 receptor levels were only 18% as high as D_2 levels in NAc, and 15–19% in CPu (Tables 1 and 3). The observed proportional distribution of D_4/D_2 receptors in mouse brain accorded closely with another study that quantified D_4 receptor levels in mouse brain (Defagot et al., 2000) and with our previous findings on the proportional distribution of the same DA receptors in rat brain (Tarazi et al., 1997a). Transgenic mice lacking functional D_4 receptor genes showed total quantitative absence of D_4 labeling receptors in comparison to wild-type mice in all brain regions examined (Table 1). In the current absence of D_4-selective radioligands (Kula et al., 1999), these and other findings (Defagot et al., 2000) support our autoradiographic method to quantify D_4 receptors using [^{3}H]nemonapride and optimal concentrations of cold S(–)-raclopride to occupy D_2/D_3 and reveal D_4 sites for selective labeling by [^{3}H]nemonapride that were nearly completely occluded by the highly selective, unlabeled D_4 ligands L-745,870 and RBI-257.

Lack of expression of D_4 receptors in mutant mice was associated with a significant increase in binding levels of DA D_1 receptors in lateral and medial CPu (by 41–44%; Fig. 2; Table 2) and in NAc (35%; Table 2). A substantial proportion of striatal neurons that express D_1 mRNA also coexpress D_4 mRNA (Surmeier et al., 1996). The present findings suggest that D_1 and D_4 receptors might also be functionally associated, perhaps in the same or interacting neurons, so that genetically induced developmental loss of D_4 receptors led to increased expression of D_1 receptors in CPu and NAc (Fig. 2; Table 2). Physiological and behavioral implications of the hypoth-

Table 3
DA D_2 Receptor Binding in Wild-Type and D_4 Receptor Knockout Mice

	Mice	
Brain region	Wild type	D_4 receptor knockout
Nucleus accumbens	213.7 ± 5.6 (100)	200.6 ± 7.3 (94)
Caudate putamen		
Medial part	236.5 ± 11.0 (100)	219.5 ± 8.3 (93)
Lateral part	317.3 ± 8.9 (100)	302.4 ± 16.1 (95)
Hippocampus		
CA_1 region	33.3 ± 2.7 (100)	34.6 ± 1.1 (104)
CA_3 region	31.8 ± 1.5 (100)	28.7 ± 2.4 (90)
Entorhinal cortex	25.6 ± 1.0 (100)	26.0 ± 1.5 (102)

Data are mean ± S.E.M. values for binding (fmol/mg tissue and percent of control), determined by quantitative autoradiography (n = 6 rats per group), as described in Materials and Methods. No difference is statistically significant.

Table 4
NMDA Receptor Binding in Wild-Type and D_4 Receptor Knockout Mice

	Mice	
Brain region	Wild type	D_4 receptor knockout
Nucleus accumbens	315.3 ± 21.8 (100)	413.2 ± 22.0 **(131)**[a]
Caudate putamen		
Medial part	287.7 ± 13.0 (100)	406.1 ± 19.2 **(141)**[a]
Lateral part	330.9 ± 21.1 (100)	463.2 ± 26.7 **(140)**[a]
Hippocampus		
CA_1 region	625.6 ± 22.7 (100)	758.8 ± 20.1 **(121)**[a]
CA_3 region	372.7 ± 31.2 (100)	464.0 ± 21.2 **(125)**[a]
Entorhinal cortex	184.7 ± 18.0 (100)	174.6 ± 16.5 (95)

Data are mean ± S.E.M. values for binding (fmol/mg tissue and percent of control), determined by quantitative autoradiography, with significant differences from controls indicated in boldface ([a]$p < 0.05$; n = 6 rats per group), all as described in Materials and Methods.

esized functional interaction of D_1/D_4 receptors remain to be determined. However, based on the molecular similarities of D_4 and D_2 receptors (Baldessarini and Tarazi, 1996) and evidence of functional synergism between D_2 and D_1 receptors (Hu and White, 1994; Keefe and Gerfen, 1995), it seems plausible that D_4 and D_1 receptors also might interact functionally. In addition, the observed increase in D_1 receptor binding might contribute to the reported supersensitive locomotor responses to psychostimulants in D_4 receptor knockout mice (Rubinstein et al., 1997).

Interestingly, levels of D_2 receptors remained unchanged in forebrain regions of D_4-receptor knockout mice compared to wild-type controls (Table 3). We reported previously that D_1, D_2, and D_4 receptors all follow a similar pattern of postnatal development in several forebrain regions (Tarazi and Baldessarini, 2000). Our present findings suggest further that D_1 and D_4 receptors, but not D_2 receptors, are developmentally linked, in that D_1 expression selectively increased in association with a profound ontogenetic loss of cerebral D_4 receptors. It will be interesting to determine if the D_1/D_4 devel-

Table 5
AMPA Receptor Binding in Wild-Type and D_4 Receptor Knockout Mice

Brain region	Mice	
	Wild type	D_4 receptor knockout
Nucleus accumbens	364.4 ± 11.9 (100)	372.2 ± 16.7 (102)
Caudate putamen		
Medial part	235.0 ± 15.2 (100)	237.7 ± 15.8 (101)
Lateral part	251.9 ± 16.2 (100)	252.6 ± 17.9 (100)
Hippocampus		
CA_1 region	485.3 ± 17.9 (100)	476.5 ± 10.8 (98)
CA_3 region	368.9 ± 17.5 (100)	388.5 ± 15.2 (105)
Entorhinal cortex	346.7 ± 17.4 (100)	367.7 ± 11.2 (106)

Data are mean ± S.E.M. values for binding (fmol/mg tissue and percent of control), determined by quantitative autoradiography (n = 6 rats per group), as described in Materials and Methods. No difference is statistically significant.

Table 6
KA Receptor Binding in Wild-Type and D_4 Receptor Knockout Mice

Brain region	Mice	
	Wild type	D_4 receptor knockout
Nucleus accumbens	192.3 ± 11.4 (100)	196.0 ± 7.8 (102)
Caudate putamen		
Medial part	133.4 ± 3.4 (100)	125.5 ± 3.7 (94)
Lateral part	152.8 ± 4.8 (100)	147.0 ± 5.1 (96)
Hippocampus		
CA_1 region	84.4 ± 2.7 (100)	88.9 ± 2.9 (105)
CA_3 region	202.7 ± 7.9 (100)	211.5 ± 8.6 (104)
Entorhinal cortex	163.4 ± 6.4 (100)	154.5 ± 5.9 (95)

Data are mean ± S.E.M. values for binding (fmol/mg tissue and percent of control), determined by quantitative autoradiography (n = 6 rats per group), as described in Materials and Methods. No difference is statistically significant.

opmental link is reciprocal and if genetic loss of D_1 receptors leads to increased expression of D_4 receptors.

Effects of genetically halting the expression of D_4 receptors included significant increases in Glu NMDA receptors in NAc and CPu of D_4 receptor mutants compared to wild-type controls (Table 4). We previously reported that D_4 heteroceptors seem to co-localize with presynaptic NMDA autoreceptors on the same or parallel corticostriatal fibers innervating CPu and NAc, suggesting that D_4 (inhibitory) and NMDA (excitatory) receptors might play opposite roles in controlling release of Glu in these subcortical brain regions (Tarazi et al., 1998, Tarazi and Baldessarini, 1999b). The observed increases in NMDA receptors in CPu and NAc might reflect neuronal adaptation to compensate for the loss of D_4 receptors in mutant mice. In agreement with the reported increased excitability of glutamatergic neurons in cerebral cortex of D_4 receptor-deficient mice (Rubinstein et al., 2001), the domination of NMDA receptors on terminals of corticostriatal projections might also increase excitability of glutamatergic neurons innervating CPu and NAc.

Functional interaction and perhaps colocalization of D_4 and NMDA receptors are not limited to CPu and NAc but evidently also extend to the hippocampal formation, as significant increases in NMDA receptor binding were detected in hippocampal CA_1 and CA_3 regions of D_4 receptor-deficient mice vs. controls (Table 4). A recent study provides evidence for D_4/NMDA receptor functional interactions in the hippocampus, in that stimulation

of D_4 receptors inhibited NMDA-evoked currents in hippocampal CA_1 neurons, reportedly mediated by transactivation of receptor tyrosine kinases (Kotecha et al., 2002). These observations suggest that D_4 receptors act as inhibitory modulators of excitatory neuronal activity in the hippocampus. Therefore, the absence of D_4 receptors, together with the observed increase in hippocampal NMDA receptors in D_4 receptor mutant mice, suggests that a state of hyperglutamatergic excitability might occur in hippocampal neurons of these genetically altered mice. This hypothesis remains to be tested experimentally. AMPA and KA receptors are less likely to interact developmentally or functionally with D_4 receptors, as tissue levels of both of these ionotropic Glu receptor subtypes did not differ significantly between D_4 knockouts and their controls (Tables 5 and 6).

Altogether, the present findings suggest that novel functional interactions occur between a trio of DA and Glu receptors—D_1, D_4, and NMDA—particularly in the corpus striatum. A functional interaction has been reported for D_1 and NMDA receptors. For example, NMDA receptor activation reversed D_1/cAMP-stimulated phosphorylation of DARPP-32 (dopamine and cAMP-regulated phosphoprotein of molecular mass 32K) in striatal brain slices (Halpain et al., 1990), whereas stimulation of D_1 receptors enhanced NMDA-evoked responses (Flores-Hernandez et al., 2002). Similarly, administration of the noncompetitive NMDA receptor antagonist MK-801 limited increases of striatal acetylcholine produced by D_1 receptor agonists (Damsma et al., 1991) and prevented D_1-mediated increases in rat striatal neurotensin levels following methamphetamine treatment (Singh et al., 1990). It is tempting to speculate that D_4 receptors play a pivotal regulatory role in mediating the functional interactions between D_1 and NMDA receptors. Therefore, genetic manipulations that knock out expression of D_4 receptors are hypothesized also to disrupt D_1/NMDA interactions, lead to an increased expression of striatal D_1 and NMDA receptors, and subsequently alter signal transduction mechanisms and cellular responses linked to D_1 and NMDA receptors.

In conclusion, generation of D_4 receptor mutant mice has advanced knowledge of functions and behaviors presumably mediated by D_4 receptors. However, molecular mechanisms and neural pathways that underlie behavioral changes reported in D_4 receptor knockout mice might not be influenced solely by their lack of D_4 receptors, as apparent secondary changes were also found in these mutant mice, including increased expression of DA D_1 receptors in NAc and CPu, and NMDA receptors in NAc, CPu, and hippocampus. These presumably reactive or compensatory developmental changes in D_1 and NMDA receptors might also contribute to the altered functions and behaviors observed in D_4 receptor knockout mice. In general, our findings indicate the need for cautious interpretation of targeted knockout of specific receptor genes in mice, as complex adaptive changes involving other receptor subtypes and/or neurotransmitter systems directly or indirectly related to the missing gene might contribute to physiological and behavioral responses of such mutants.

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