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Dopamine receptor functions: lessons from knockout mice

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Abstract

In the past few years, a number of laboratories have used gene targeting via homologous recombination to generate mice deficient for key molecules involved in dopaminergic (DAergic) transmission. This tremendous effort has resulted in the successful generation and characterization of mice deficient for the neurotransmitter DA, the main terminator of DAergic neurotransmission (the DA transporter), and all five subtypes of DA receptors. This review summarizes the results from studies of the various DA receptor knockout mice and of mice deficient in proteins that mediate DA receptor signaling. It focuses on a comparison of the locomotor phenotypes and responses to drugs of abuse (psychostimulants), and reviews the results of anatomic studies examining the morphological and neurochemical differentiation of the striatum in these mutants. Moreover, an overview of recently published results highlighting the physiological relevance of the interaction between different DA receptors and between DA receptors and other neurotransmitter receptors in the modulation of behavioral and molecular responses to DAergic stimulation is presented. Finally, in view of the recently discovered heteroligomeric assemblies of neurotransmitter receptors that involve DA receptor subtypes, the potential value of knockout mice as a tool for testing the *in vivo* significance of these heteroligomeric receptors is discussed. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Gene targeting; Dopamine; Dopamine receptors; Striatum; Immediate early genes; Heteroligomers

Abbreviations: AChE, acetylcholinesterase; cAMP, cyclic AMP; CB, calbindin; ChAT, choline acetyltransferase; DA, dopamine; DARPP-32, dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32; DAT, dopamine transporter; DOPAC, dihydroxyphenylacetic acid; DYN, dynorphin; ENK, enkephalin; ES, embryonic stem; GABA, γ -aminobutyric acid; GAD, glutamic acid decarboxylase; GPCR, G-protein-coupled receptor; HVA, homovanillic acid; IEG, immediate early gene; NMDA, *N*-methyl-D-aspartate; NT, neurotensin; 6-OHDA, 6-hydroxydopamine; ORF, open reading frame; PARV, parvalbumin; PKA, cyclic AMP-dependent protein kinase A; PPI, prepulse inhibition; SP, Substance P; SST, somatostatin; TH, tyrosine hydroxylase; TMD, transmembrane-spanning domain.

Contents

1.	Introduction	64
2.	Dopamine receptor mutants: spontaneous locomotor phenotypes and responses to drugs of abuse	65
2.1.	D ₁ receptor mutants	65
2.2.	D ₂ receptor mutants	65
2.3.	D ₃ receptor mutants	68
2.4.	D ₄ receptor mutants	68
2.5.	D ₅ receptor mutants	69
2.6.	Autoreceptor functions of D ₂ -like dopamine receptors	69
3.	Neuroanatomical and neurochemical correlates of locomotor abnormalities in dopamine receptor mutants	69
3.1.	Hallmarks of the wild-type striatum and nucleus accumbens	70
3.2.	Neurochemistry of the striatum and nucleus accumbens in dopamine-deficient and dopamine receptor mutant mice	70

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3.3.	Implications of the role of dopamine in brain development, gene expression, and function	73
4.	Other knockouts affecting dopaminergic neurotransmission	74
4.1.	G_{olf}	74
4.2.	Dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32.	74
5.	Interactions among dopamine receptors	75
5.1.	Interactions among D_1 , D_2 , and D_3 receptors in the expression of behavioral responses to pharmacological stimuli	75
5.2.	Dopamine receptor agonist- and psychostimulant-induced <i>c-fos</i> responses in dopamine receptor knockout mice	75
6.	Interactions between dopamine receptors and other neurotransmitter receptors	77
6.1.	Heterologous receptor assemblies involving dopamine receptor subtypes—a new challenge for studies on knockout mice	77
6.2.	Adenosine A_2 receptor functions in D_2 mutant mice	78
7.	Concluding remarks	79
	Acknowledgements	80
	References	80

1. Introduction

Several medical conditions are known to involve alterations in dopaminergic (DAergic) neurotransmission. They include neurological disorders such as Parkinson's disease, which is caused by a selective degeneration of midbrain nigrostriatal DAergic neurons, and Huntington's disease, which is due to a deterioration of dopaminergic projection neurons in the neostriatum. Moreover, drugs that alter DAergic neurotransmission are successfully employed in the management of certain psychiatric disorders. Neuroleptic drugs that predominantly block the D_2 class of DA receptors have powerful antipsychotic potencies, and drugs that regulate the tone of DAergic neurotransmission alleviate some of the symptoms that characterize attention deficit hyperactivity disorders. Finally, psychostimulants, such as cocaine, amphetamine, and opioid drugs, as well as nicotine and alcohol, have addictive properties that are thought to be (at least partially) due to their ability to alter DAergic neurotransmission.

The central actions of DA are mediated by five distinct receptors that are expressed in DA synthesizing neurons of the substantia nigra, ventral tegmental area, and the hypothalamus and/or in the targets of the DAergic pathways known as the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular pathways that are implicated, respectively, in the modulation of locomotor behavior, motivated behavior, learning and memory, and the regulation of prolactin release. DA receptors are members of the large class of neurotransmitter receptors whose actions are mediated through the activation of heterotrimeric guanine nucleotide regulatory proteins (G-proteins). The five DA receptor subtypes identified to date by molecular cloning differ in their primary structure and show distinct affinities for DA receptor agonists and antagonists. Their current classification is based on the functional properties of these receptors. The D_1 class of receptors is composed of the

receptor subtypes D_1 and D_5 , which couple to the stimulatory G-proteins G_s and G_{olf} and activate adenylyl cyclase to increase cytosolic cyclic AMP (cAMP) levels. The D_2 class of receptors is composed of the subtypes D_2 , D_3 , and D_4 , which couple to the inhibitory G-proteins G_i and G_o in order to modulate ion channel activity and/or depress adenylyl cyclase activity. The anatomical distribution and functional properties of the individual receptor subtypes have been reviewed extensively (Missale et al., 1998; Levant, 1997).

In the past few years, several laboratories have used gene targeting via homologous recombination to generate mice deficient for DA, the DA transporter (DAT), or individual members of the DA receptor family (Zhou & Palmiter, 1995; Giros et al., 1996; for reviews on DA-receptor knockouts, see Sibley, 1999; Schmauss, 2000a). The most dramatic phenotypes were observed in mice deficient for DA and the DAT. Although mice unable to synthesize DA survive embryogenesis and early postnatal life and have normal midbrain DAergic neurons and telencephalic DA terminals, after postnatal age P15, these mutants become severely hypoactive and fail to feed. This phenotype is lethal, unless the mutants are rescued with a continuous L-DOPA treatment (Zhou & Palmiter, 1995). In contrast to the locomotor hypoactivity of DA-deficient mice, the inactivation of the DAT leads to an extreme locomotor hyperactivity in knockout mice that can result in death-promoting exhaustion, and studies on these mice have unequivocally demonstrated that the DAT is the major terminator of the action of exocytotically released DA (Giros et al., 1996).

Compared with mice deficient for DA or the DAT, knockout mice deficient for each of the five DA receptor subtypes have less dramatic phenotypes. The DA receptors that mediate the central effects of DA are widely expressed throughout the neuroaxis, where they can be found in distinct cell populations of the forebrain, cerebellum, brainstem, spinal cord, and retina (for a review, see Missale et al., 1998). In general, whereas the D_1 class of receptors is

thought to be expressed postsynaptically to most DA nerve terminals, D₂-like receptors are located both on DA nerve terminals as well as postsynaptically to sites of DA release. Although each receptor subtype is expressed in a unique pattern, the expression of several receptor subtypes also overlaps and, in some instances, the receptor subtypes are co-expressed within individual cells. This situation often precluded the unique assignment of one individual receptor subtype to a particular neuronal system. Moreover, studies on the distinct functional properties of DA receptor subtypes expressed *in vivo* are limited by the lack of agonists and antagonists with selectivity for individual receptor subtypes that belong to the same functional class. Numerous recent studies on several different DA receptor knock-out mice, however, have aided efforts of biochemical and pharmacological studies in the identification of distinct functional properties of the individual members of DA receptors, and some of the main findings of these studies are summarized below.

2. Dopamine receptor mutants: spontaneous locomotor phenotypes and responses to drugs of abuse

2.1. D₁ receptor mutants

The era of studies on DA receptor mutants began with the successful generation of D₁ mutants. Xu et al. (1994b) generated a genetic null mutation of the D₁ gene by removing the majority of the intronless region of the gene that comprises the entire open reading frame (ORF) of the D₁-encoded mRNA, and Drago et al. (1994) disrupted the D₁ gene at sequences encoding transmembrane-spanning domain (TMD) V of the receptor protein. Both gene-targeting strategies resulted in homozygous D₁ mutants that are viable, but that show a number of abnormalities. First, they are smaller than their wild-type littermates (by 30%). Second, their postnatal development is retarded, but adult mutants are generally healthy. Third, they show alterations in basal and psychostimulant-induced locomotor activity. The genetic null mutants of Xu et al. (1994b) show an increased basal locomotor activity and an increased locomotor response to a novel environment. Although the D₁ deletion mutants of Drago et al. (1994) are less active than their wild-type littermates when tested in the open field, they also display an increased locomotor activity when first placed into this novel environment (Miner et al., 1995). One study on these mutants also detected an impairment in the initiation of spontaneous locomotion and/or responses to external stimuli (Smith et al., 1998), and another study detected an impairment of the sequential integrity of the serially ordered pattern of grooming movements (Cromwell et al., 1998).

Both the genetic null mutants and the D₁ deletion mutants fail to show an increased locomotor behavior in response to psychostimulant administration (Xu et al., 1994a; Miner

et al., 1995; Drago et al., 1996). This finding demonstrated clearly that the D₁ receptor is essential for the expression of the locomotor-activating effects of psychostimulants. In contrast, however, as shown by Miner et al. (1995), the lack of D₁ receptor expression does not affect the rewarding and reinforcing effects (assessed by conditioned place preference experiments) of the psychostimulant cocaine.

Although cortical D₁ receptors have been implicated in the control of spatial learning and memory, only a few studies have assessed cognitive functions of D₁ mutants. El-Ghundi et al. (1999) utilized place navigation tests, performed in the Morris water maze, that are thought to be particularly sensitive to hippocampal dysfunction. In this study, the performance of homozygous D₁ mutants differed significantly from wild-type littermates, i.e., D₁ mutants exhibited longer escape latencies and an absence of a spatial bias. Prefrontal cortical D₁ receptors have also been implicated in the control of working memory (Goldman-Rakic, 1999). El-Ghundi et al. (1999) have tested D₁ mutant mice in a spontaneous alternation task performed in a Y-maze, but found no significant differences between wild-type and mutants. However, in view of the increasing evidence for a critical role of D₁ receptors in working memory (see Goldman-Rakic, 1999), additional tests that detect more sensitively deficits in spatial working memory associated with prefrontal cortical dysfunction (for example, delayed alternation tests) are needed to rigorously assess the working memory of D₁ mutants.

Finally, it has long been postulated that there are as yet uncloned D₁-like receptors that bind the D₁/D₅ antagonist [³H]SCH23390 with high affinity, but that are not linked to adenylyl cyclase, and are enriched in distinct anatomic regions of the brain, most prominently in the amygdala (Mailman et al., 1986a, 1986b). Moreover, Friedman et al. (1997) found functional effects of D₁ agonists on phosphoinositide hydrolysis (Undie et al., 1994) in D₁ knockout mice that are indistinguishable from wild-type. However, a recent study utilized quantitative autoradiography and homogenate saturation binding to determine the residual binding of [³H]SCH23390 in D₁ knockout brains, and found that it is delimited to D₅ receptors of the hippocampus. Furthermore, the authors demonstrate that the D₁ knockout eliminates detectable binding in the striatum, as well as in other regions (e.g., the amygdala), where non-cyclase-coupled D₁ receptors were thought to be expressed (Montague et al., 2001). These results strongly suggest that the non-cyclase-coupled D₁-like receptors are also encoded by the (cloned) D₁ gene (Table 1).

2.2. D₂ receptor mutants

Shortly after the first reports on D₁ mutant mice, Baik et al. (1995) successfully generated genetic D₂-null mutant mice. Two other D₂ knockouts have been generated subsequently, one resulting from a genetic null mutation of the D₂ gene (Jung et al., 1999) and the other resulting from the

Table 1
Locomotor phenotypes of mice deficient for DA receptors

Receptor knockout	Genetic null mutants ¹	Deletion mutants ²
D ₁	Increased basal locomotor activity and increased locomotor response to a novel environment Absent locomotor responses to psychostimulants	Decreased basal locomotion, but increased locomotor response to a novel environment Absent locomotor responses to psychostimulants
D ₂	Postural abnormalities, bradykinesia with delayed initiations of movements, impairments in the coordination of movements, prolonged periods of immobility Increased stereotypic responses to D ₁ agonists	Normal posture, decreased locomotor activity with delayed initiations of movements
D ₃	Rapidly habituating increased locomotor activity in a novel environment Increased locomotor activity in response to co-stimulation of D ₁ and D ₂ receptors	Rapidly habituating increased locomotor activity in a novel environment
D ₄	?	Reduced spontaneous locomotor activity in a novel and familiar environment, enhanced motor coordination, locomotor supersensitivity to ethanol and psychostimulants
D ₅	?	Increased horizontal and rearing activity, enhanced motor coordination

¹ Deletion of sequences constituting the N-terminus of the ORF or the entire ORF.

² Deletion of sequences located in the 3' half of the ORF.

removal of a C-terminal region of the D₂ gene comprising the 3' portion of the third cytoplasmic domain and TMDs VI and VII (Kelly et al., 1997). Homozygous genetic null mutants are viable, but their body weight is 15% less compared with wild-type littermates, their postnatal growth is delayed, and their fertility is greatly reduced (Baik et al., 1995; Jung et al., 1999). Another very characteristic feature of the two genetic null mutants is pronounced postural abnormalities that include a hunched posture, paw flattening, and sprawling of hind legs (Baik et al., 1995; Jung et al., 1999). These postural abnormalities are not observed in the D₂ deletion mutants (Kelly et al., 1997, 1998). It should also be noted that the locomotor abnormalities of genetic D₂-null mutants are most pronounced when demands are placed on motor function, but they are only subtle when these mutants are observed in a more naturalistic environment resembling the home cage of the animal. In fact, the evaluation of the spontaneous unconditioned psychomotor behavior by Clifford et al. (2000) revealed only subtle topographic shifts between individual elements of behavior, and the motor deficits appeared less prominent under these less stressful and less demanding conditions.

A cardinal phenotype of both the genetic null mutants and the D₂ deletion mutants is a delayed initiation of movement (Baik et al., 1995; Kelly et al., 1998; Jung et al., 1999). The genetic null mutants, however, show a more severe reduction in spontaneous locomotor activity (Baik et al., 1995; Jung et al., 1999); they have pronounced impairments in their ability to coordinate movements, and they spend more time in immobility (Baik et al., 1995). These phenotypes may relate to imbalances between the direct (striatonigral) and indirect (striatopallidal) pathways that are thought to underlie both the hypokinetic syndromes seen in Parkinson's disease and the hyperkinetic syndromes of Huntington's disease, and the release of movements is thought to be achieved by a balance between these two striatal output pathways (Albin et al., 1989).

Recent data indicate that the relative activities of the striatal striosome and matrix compartments determine the degree of repetitive, stereotypic behavior elicited by DA agonists or psychostimulants, i.e., a relatively higher activity in striosomes correlates with a higher degree of stereotypy (Canales & Graybiel, 2000). Thus, it is of interest that D₂ mutants exhibit more stereotypy in response to D₁ agonist stimulation than wild-type or D₃ mutants. This is particularly apparent when such mice are treated with the D₁ agonist SKF-82958, which, compared with other agonists of the SKF group, has been shown to be the most efficacious inducer of stereotypic behavior (Meyer & Shults, 1993). In mice, the systemic application of SKF-82958 (1 mg/kg) elicits oral stereotypies (licking and nibbling behaviors) within 10 min that end 45 min later. Our laboratory used locomotor activity boxes equipped with photobeams to obtain a quantitative estimate of the number of these stereotypic movements that were exhibited during this time period by wild-type, D₂ and D₃ single mutants, and D₂/D₃ double mutants (the D₃ and D₂/D₃ mutants are described in Section 2.3). Fig. 1 summarizes the results of these experiments, which assessed the total horizontal locomotor activity (number of beam break interruptions in the horizontal sensor), stereotypy counts (the number of beam breaks that occur during a period of uninterrupted stereotypic activity), the number of stereotypies (which corresponds to the number of times the monitor observes stereotypic behavior), and the stereotypy time [the total amount of time (in sec) that stereotypic behavior is exhibited]. In Fig. 1, the respective values are expressed as the ratio of SKF versus saline treatment for all four genotypes. In wild-type, as well as in all three types of mutants, SKF treatment resulted in a significant increase in all four parameters measured when compared with the corresponding numbers obtained after saline treatment. However, whereas the SKF-induced increase in stereotypic locomotor activity does not differ between wild-type and D₃ single mutants, mice lacking D₂

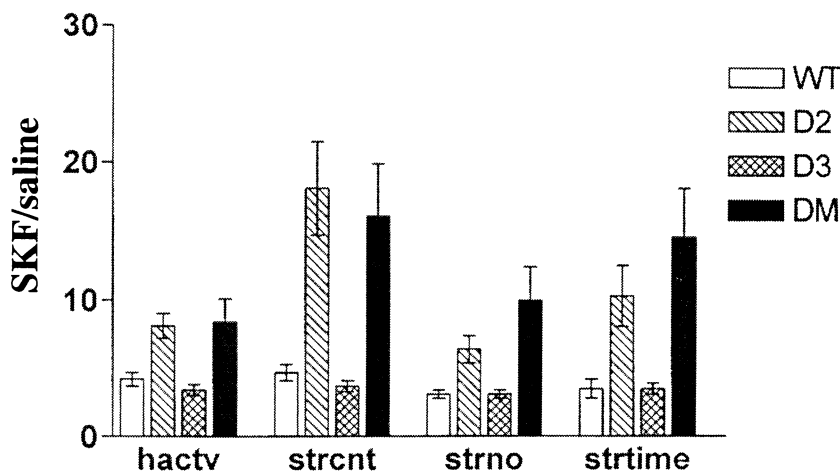


Fig. 1. Locomotor activity/stereotypies following SKF-82958 (1 mg/kg) administration to wild-type and D_2 , D_3 , and D_2/D_3 mutant mice at postnatal age P70. Values represent the mean \pm S.D. of determinations made in 7–10 animals per genetic group. To assess quantitatively stereotypic behavioral responses to SKF-82958 treatment, locomotor activity boxes were used. This automatic system allows assessments of locomotor behavior (including stereotypies) that highly correlate with observer ratings (Sanberg et al., 1987). When animals displaying repetitive behavior are placed in locomotor activity boxes, they break the same beam (or set of beams) repeatedly and the monitor considers that the animal is exhibiting stereotypies. In this experiment, male mice were allowed to habituate to the test boxes for 60 min. Saline was then injected, followed 30 min later by an injection of SKF-82958 (1 mg/kg i.p.). For each mouse, at any given sample period, a sequential record of the number of beam interruptions in the horizontal sensor (total horizontal locomotor activity), the stereotypy counts, the stereotypy number, and the stereotypy time was obtained (see text). These parameters were monitored during uninterrupted 30-min sessions, following saline and drug injections during the light phase (2–5 p.m.) of the light/dark phases. In each session, an equal number of wild-type, D_2 , and D_3 single mutants, and D_2/D_3 double mutants was tested. Comparisons of ratios of drug/saline values between genetic groups were conducted using a two-sample Student's *t*-test for independent samples with unequal variance (*wild-type* vs. D_2 ; horizontal activity (hactv): $P < 0.001$, stereotypy counts (strcnt): $P < 0.01$, stereotypy number (strno): $P < 0.05$, stereotypy time (strtime): $P < 0.05$; *wild-type* vs. D_2/D_3 ; hactv: $P < 0.05$, strcnt: $P < 0.02$, strno: $P < 0.02$, strtime: $P < 0.05$).

and D_2/D_3 receptors show a significant (2- to 4-fold) increase in stereotypic behavior (see Fig. 1). Given the results of Canales and Graybiel (2000), the data shown in Fig. 1 may indicate a larger increase in the activity of the striosomal compartment in D_1 agonist-treated D_2 mutants compared with wild-type or D_3 single mutants.

D_2 mutant mice have also been used to study the role of D_2 receptors in the modulation of prepulse inhibition (PPI), a measure of sensorimotor gating in which the magnitude of a startle response is diminished when the startling stimulus is preceded by a low-intensity prepulse. PPI is disrupted by direct and indirect DA agonists, an effect attributed to the activation of the D_2 class of DA receptors (Mansbach et al., 1988). The precise role of the individual members of the D_2 class of DA receptors in the disruption of PPI, however, has been elusive. Thus, Ralph et al. (1999) tested mice deficient for D_2 , D_3 , and D_4 receptors (the D_4 mutants are described in Section 2.4) and found that all genotypes displayed robust PPI and acoustic startle. However, the indirect DA agonist amphetamine failed to disrupt PPI only in D_2 mutants, suggesting that the D_2 receptor plays a critical role in mediating the PPI-disruptive effects of indirect DA agonists.

Several other studies addressed the role of D_2 receptors in mediating the locomotor and rewarding effects of various drugs of abuse (for reviews, see Sibley, 1999; Schmauss, 2000a). Most interestingly, whereas D_1 receptors have been shown to be essential for mediating locomotor, but not the rewarding, effects of drugs of abuse (see Section 2.1), the

study of Maldonado et al. (1997) revealed that the locomotor hyperactivity exhibited in response to acutely administered morphine is preserved in the D_2 mutants. However, in place preference tests, these mutants show no preference for the drug-associated compartment, suggesting that responses to the rewarding properties of opioid drugs require the expression of D_2 receptors.

Finally, whereas the studies discussed above analyzed phenotypes of mice deficient for both isoforms of the D_2 receptor, D_{2L} and D_{2S} , two laboratories recently have generated mice lacking only the D_{2L} receptor isoform (Wang et al., 2000; Usiello et al., 2000). This approach was straightforward because D_{2L} - and D_{2S} -encoded mRNAs are derived from the same primary transcript that can be spliced alternatively to either include (D_{2L}) or exclude (D_{2S}) an exonic sequence of 87 nucleotides of the D_2 gene that encodes 29 amino acid residues of the putative third cytoplasmic loop of the receptor protein (see Missale et al., 1998). Thus, the deletion of the alternative exon 6 of the D_2 gene (which encodes the 29 amino acids) resulted in homologously recombinant mice expressing only D_{2S} receptors.

The generation of isoform-specific D_2 mutants is of particular importance for the study of distinct functional properties of both isoforms, which, in wild-type animals, is impossible not only because both isoforms are co-expressed in many brain regions, but mainly because isoform-selective pharmacological agents are not available. Thus, the generation of D_{2L} mutants enabled for

the first time a precise delineation of the specific functions mediated by the D_{2L} receptor. Wang et al. (2000) analyzed mice deficient for D_{2L} receptors (both on a hybrid, as well as congenic, C57Bl/6 background) and found an increased expression of D_{2S}, particularly in the striatum where the normal expression of D_{2L} receptors greatly exceeds that of D_{2S} receptors. Several striatal functions are altered in these mutants. In open-field tests, D_{2L} mutants exhibit reduced locomotion and rearing behavior, as well as a slightly (but not significantly) increased time spent in immobility. Haloperidol-induced catalepsy, however, is significantly reduced in D_{2L} mutants. In addition, other functions were unaffected, including motor coordination, as measured with the rotarod test, which was only impaired on the first day of testing, but not on subsequent test days. A key finding of this study, however, is that D₂ autoreceptor functions are well preserved in these mutants. For example, the initial suppression of locomotor activity in response to low doses of a D₂ agonist (thought to be due to stimulation of release-modulating D₂ autoreceptors), as well as the DA-mediated inhibition of the spontaneous firing rate of substantia nigra neurons (thought to be mediated by impulse-regulating D₂ autoreceptors), are unaffected in the mutants.

The study of Usiello et al. (2000) came to similar conclusions. The authors also found increased expression of the D_{2S} receptor and an absence of haloperidol-induced catalepsy in mice lacking D_{2L}. Low doses of haloperidol led to a wild-type-like increase in extracellular DA levels, and low doses of the D₂-like agonist quinpirole induced a suppression of motor activity. Thus, both studies found a preservation of D₂ autoreceptor functions in mice lacking D_{2L} receptors. They further showed that the neuroleptic-induced catalepsy is mediated mainly by D_{2L} receptors. In contrast to the study of Wang et al. (2000), however, Usiello et al. (2000) found that the spontaneous locomotor activity of D_{2L} mutants was normal. In addition, these authors found that, in contrast to wild-type mice that show increased locomotor activity in response to either D₁ or D₁/D₂ agonist stimulation, mice lacking D_{2L} receptors do not respond to this pharmacological manipulation. This finding suggests that the cooperative effect of postsynaptic D₁ and D₂ receptors in producing locomotor activation involves D_{2L}, but not D_{2S}, receptors.

2.3. D₃ receptor mutants

To generate mice deficient for D₃ receptors, Accili et al. (1996) used a targeting vector that, upon homologous recombination, disrupted the D₃ gene at sequences coding for the second intracellular loop of the receptor. Later, Xu et al. (1997) reported the generation of genetic D₃-null mutants, and Jung et al. (1999), using a targeting strategy similar to that of Accili et al. (1996), disrupted the D₃ gene at sequences coding for the second intracellular loop of the receptor protein.

Despite the similarity of the primary peptide sequences of the three members of the D₂ class of DA receptors (which is highest between D₂ and D₃ receptors), the locomotor phenotype of D₃ mutants does not resemble that of D₂ mutants. In fact, all D₃ mutants generated to date develop normally, are fertile, and at most, show a transient and rapidly habituating locomotor hyperactivity in a novel environment (Accili et al., 1996; Xu et al., 1997; Jung et al., 1999). Studies on the mice generated by Accili et al. (1996) revealed a reduced thigmotaxis in the open field and behavioral abnormalities in the elevated maze that suggest the possibility that reduced anxiety of D₃ mutants underlies the brief locomotor hyperactivity in a novel environment (Steiner et al., 1998). Studies on the genetic D₃-null mutants, however, found no indication for a reduced anxiety (Xu et al., 1997).

Measurements of the basal and D₁ agonist- or psychostimulant-induced locomotor activity of D₃ single mutants (Xu et al., 1997) suggest that D₃ receptors play no major role in the expression of locomotor phenotypes. However, this is only true as long as either higher doses of psychostimulants are tested (see Section 5) or as long as the expression of other DA receptor subtypes is unaffected. For example, when double-mutant mice deficient for D₂/D₃ receptors or D₁/D₃ receptors are examined for their locomotor activity, the lack of D₃ receptors alters locomotor phenotypes that are characteristic for either D₁ or D₂ single mutants. This was first reported by Jung et al. (1999), who showed that the postural and locomotor phenotypes of D₂/D₃ double mutants are significantly more severe compared with D₂ single mutants (although they are qualitatively similar). This result suggested that in D₂ single mutants, D₃ receptors can compensate for some (but not all) of the functional consequences of the D₂ receptor knockout. Indeed, immunoprecipitation experiments revealed an increased expression of the D₃ protein during later stages of the postnatal development of D₂ single mutants (Jung et al., 1999).

Karasinska et al. (2000) used the D₁ mutants generated by Drago et al. (1994) and D₃ mutants of Accili et al. (1996) to generate homozygous D₁/D₃ double mutants. Their studies showed that the decreased exploratory activity of D₁ mutants is further decreased in D₁/D₃ double mutants, suggesting that D₃ receptors exert an inhibitory effect on the magnitude of the decreased exploratory activity resulting from D₁ receptor blockade. The decreased anxiety of D₃ single mutants reported by Steiner et al. (1998), however, is absent in D₁/D₃ double mutants. Moreover, the impaired performance of D₁ single mutants in motor coordination (rotarod performance) and spatial learning tests (Morris water maze task) are indistinguishable from those of D₁/D₃ double mutants.

2.4. D₄ receptor mutants

At present, only one D₄ knockout has been reported that resulted from a deletion of exon II of the D₄ gene and an earlier

termination of the ORF downstream thereof (Rubinstein et al., 1997). Genetic null mutants remain to be generated.

D₄ mutants develop normally. Their spontaneous locomotor and rearing activity (determined in the open field), however, is reduced compared with wild-type mice in both novel and familiar environments (Rubinstein et al., 1997). However, for reasons not well understood, D₄ mutants show a superior performance in rotarod tests, and they display locomotor supersensitivity to ethanol and psychostimulants (Rubinstein et al., 1997).

A recent study on these mutants tested the hypothesis that D₄ receptor activity modulates behavioral responses to novelty, a hypothesis that arose from earlier observations of an association between polymorphism of the human D₄ gene and novelty-seeking behavior (Ebstein et al., 1996; Benjamin et al., 1996). Indeed, Dulawa et al. (1999) found a reduced behavioral response of D₄ mutants to novelty that was largely accounted for by a reduction in approach (exploratory) behavior. Thus, a prominent role for D₄ receptors in the expression of novelty-seeking behavior remains a strong possibility.

2.5. D₅ receptor mutants

At present, only one D₅ receptor knockout has been reported (see Sibley, 1999). These mutants were generated by inserting a premature stop codon within sequences coding for the second intracellular loop of the receptor protein. D₅ mutants are viable, develop normally, and are fertile. In the open field, however, D₅ mutants exhibit a significant increase in horizontal and rearing activity, and this apparent increase in exploratory behavior is not due to a reduced level of anxiety. Like D₄ mutants, D₅ mutants also outperformed their wild-type littermates in the rotarod test, suggesting that these receptors have the capacity to depress or inhibit locomotor activity.

2.6. Autoreceptor functions of D₂-like dopamine receptors

The observation that both D₂ and (to a lesser extent) D₃ receptors are expressed in the substantia nigra and ventral tegmental area has led to the hypothesis that both receptors can act as autoreceptors. However, results of studies on D₂ and D₃ receptor mutants suggest that D₂-like autoreceptor functions are exclusively mediated by the D₂ receptor subtype. For example, in wild-type slice preparations, the spontaneous firing of midbrain DAergic neurons is inhibited upon application of DA or the D₂/D₃ agonist quinpirole. This inhibition, however, is completely absent in corresponding slice preparations obtained from D₂ mutants, suggesting that D₂ autoreceptors alone are responsible for mediating the effect of DA on the inhibition of spontaneous firing of midbrain DA neurons (impulse-modulating DA receptors) (Mercuri et al., 1997). Moreover, stimulation of D₂-like autoreceptors is also known to reduce both the synthesis and the release of DA. Studies on [³H]DA-loaded

purified striatal synaptosomes, obtained from wild-type and D₂ mutant mice, confirmed that D₂ (but not D₃) receptors are involved in the autoreceptor-mediated inhibition of evoked DA release (release-modulating DA receptors) (L'hirondel et al., 1998).

Corresponding studies on the D₃ mutant mice generated by Xu et al. (1997) confirmed that D₃ receptors do not mediate impulse- or synthesis-regulating autoreceptor functions (Koeltzow et al., 1998). Moreover, these studies suggest that potential effects of D₃ receptor activation on DA release are mediated by mechanisms that are distinct from a release-modulating autoreceptor function.

In summary, studies on D₂ and D₃ knockout mice provided strong evidence for an exclusive role of D₂ receptors in autoreceptor-mediated functions. Moreover, as outlined above, the recent studies of Wang et al. (2000) and Usiello et al. (2000) showed that D_{2S} receptors have the full capability to mediate these functions. Because D₂ knockout mice have also been shown to exhibit a decreased clearance of locally applied DA (Dickinson et al., 1999), further studies on D_{2L} knockout mice can now test more directly whether D₂ autoreceptors or postsynaptic D₂ receptors (or both) are involved in modulating the DAT activity.

3. Neuroanatomical and neurochemical correlates of locomotor abnormalities in dopamine receptor mutants

Some of the locomotor phenotypes of the DA receptor mutants described in Section 2 could result either from alterations in the tissue content of the neurotransmitter DA and/or from abnormalities in DA-innervated brain regions that are critically involved in the modulation of locomotor behavior. However, despite the different locomotor phenotypes described for D₂, D₃, D₄, and D₂/D₃ double mutants, their tissue levels of striatal DA are unaltered (Rubinstein et al., 1997; Kelly et al., 1998; Jung et al., 1999). Differences that were detected in these studies are delimited to DA metabolites. For example, in D₂ and D₂/D₃ double mutants, the levels of dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are increased more than 2-fold (Jung et al., 1999) and levels of DOPAC are 2-fold higher in D₄ mutants compared with wild-type (Rubinstein et al., 1997). Such increases in DOPAC and HVA levels have been described previously for rats treated chronically with the D₂-like antagonist haloperidol (Essig & Kilpatrick, 1991), and they are thought to reflect a higher metabolism of apparently non-utilized DA. In any case, striatal DA levels are normal in mice lacking D₂-like receptors. This raises the possibility that the various locomotor phenotypes detected in the DA receptor knockouts are rooted in structural or neurochemical alterations within anatomic structures receiving DA input. At present, the most extensively studied structures of DA-receptor knockout mice are the striatum and the nucleus accumbens, regions with dense DAergic innervation and comparably robust expression of DA receptors. Importantly,

these structures are critically involved in modulating locomotor and stereotypic behavior. The main findings of these studies, which have focused mainly on light-microscopic analyses of immunostained material, are summarized here following a brief discussion of the normal (wild-type) striatal neuroanatomy and neurochemistry.

3.1. Hallmarks of the wild-type striatum and nucleus accumbens

The rodent neostriatum and the ventromedially apposed nucleus accumbens share many important neurochemical and morphological similarities, despite being functionally distinguishable (the former is predominantly affiliated with locomotor behavior, while the latter is associated with motivation, reward, and attention systems). Both structures are composed primarily (90–95%) of γ -aminobutyric acid (GABA)ergic medium spiny projection neurons (Chang et al., 1982; Chang & Kitai, 1985). Additionally, both regions receive dense DAergic innervation, as well as excitatory input from the cortex, and they abundantly express DA receptors and several neuropeptides known to modulate striatal cell firing rate, including Substance P (SP), dynorphin (DYN), enkephalin (ENK), and neurotensin (NT) (Le Moine et al., 1991; Le Moine & Bloch, 1996).

Significant differences in the structures can be found in the topographic source of excitatory and DAergic input and, importantly, in expression of the D₁, D₂, and D₃ receptor subtypes (Le Moine et al., 1991; Gerfen, 1992; Le Moine & Bloch, 1996; Groenewegen et al., 1999). Whereas the neostriatum is innervated by the substantia nigra pars compacta, the nucleus accumbens receives DAergic projections primarily from the more medially located ventral tegmental area. Furthermore, the nucleus accumbens receives excitatory input from the limbic cortex, including the prefrontal cortex, hippocampus, and amygdala, while the dorsal striatum receives excitatory glutamatergic innervation that arises mainly from other regions of cortex and the intralaminar thalamus. Perhaps most importantly for the scope of this review, these structures differ in the expression of DA receptor subtypes (Le Moine et al., 1991; Le Moine & Bloch, 1996). The neostriatum and nucleus accumbens abundantly express D₁ and D₂ receptors with only little cellular co-expression. Moreover, the nucleus accumbens expresses the (generally low-abundant) D₃ receptor at highest levels.

Functionally, the projection fields of neuronal populations expressing either the D₁ or D₂ receptor segregate (Gerfen & Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990). In the neostriatum, neurons expressing the D₁ receptor project directly to the substantia nigra pars reticulata (direct pathway), while D₂-expressing neurons project to the pallidum and the subthalamic nucleus, ultimately reaching the substantia nigra indirectly (indirect pathway). In both the neostriatum and nucleus accumbens, D₁-expressing neurons co-localize with the neuropeptides SP and

DYN, while neurons expressing the D₂ receptor co-express ENK and NT. This highly correlated expression of particular neuropeptides with the two DA receptor subtypes, together with the observation that neuropeptide expression levels are modified by DA depletion or specifically by D₁ or D₂ antagonism, indicate that neuropeptide expression is regulated by DA signaling (Anderson & Reiner, 1990; Gerfen et al., 1990). In addition, three major classes of interneurons have been characterized in each structure, and these include large cholinergic neurons (which stain with choline acetyl transferase), smaller interneurons that co-express somatostatin (SST) and nitric oxide synthase (NADPH-diaphorase), or parvalbumin (PARV) and GABA.

Independent of their expression of either the D₁ or D₂ receptor, neostriatal medium spiny neurons are further compartmentalized into matrix and striosomal compartments. Whereas the matrix can be identified by its expression of the striosome-sparing Ca²⁺-binding protein calbindin (CB) and relatively low expression levels of DYN, striosomes distinctly label positively for the μ -opioid receptor and DYN (Graybiel et al., 1981; Gerfen et al., 1983; Graybiel, 1984, 1990; Liu & Graybiel, 1992a, 1992b). The functional role of this neurochemical organization is not completely understood. However, as outlined in Section 2.2, the relative activities of matrix and striosome compartments is thought to play a role in the expression of stereotypic behavioral patterns (Canales & Graybiel, 2000).

3.2. Neurochemistry of the striatum and nucleus accumbens in dopamine-deficient and dopamine receptor mutant mice

Tables 2 and 3 outline the comparative expression of the distinct neurochemical markers described in the previous section in the neostriatum and nucleus accumbens of wild-type and DA receptor mutants. The main findings are summarized below.

Mice lacking DA exhibit no obvious structural abnormalities in the brain (Zhou & Palmiter, 1995). Despite the absence of immunoreactivity for the rate-limiting enzyme of DA synthesis, tyrosine hydroxylase (TH), similar levels of aromatic amino acid decarboxylase, the enzyme that converts L-DOPA to DA, are expressed in DA-deficient mice, indicating the preservation of normal midbrain DAergic projections (Zhou & Palmiter, 1995). Analyses of ligand-binding autoradiography for D₁- and D₂-like receptors and D₁- and D₂-encoded mRNA levels by *in situ* hybridization revealed no changes in the expression of either receptor compared with wild-type. These data, therefore, illustrated for the first time that the expression of DA receptors in the striatum is not dependent upon developmentally regulated DA signaling (Kim et al., 2000). In addition, the unaltered immunohistochemical staining for dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein (DARPP)-32, a protein whose phosphorylation is dependent upon DA receptor stimulation (see Greengard et al., 1999),

Table 2
DA signaling regulates striatal and accumbens neuropeptide expression

Anatomical region	Molecular marker	Type of changes	DA ^{1,2}	D ₁ ^{3,4,5,6,7}	D ₂ ^{8,9}	D ₃ ^{9,10,11}	D ₂ /D ₃ ⁹
Striatum: medium spiny neurons	D ₁ receptors (SP, DYN)	None	D ₁ and D ₂ (LB and IS)	DARRP-32 (IC) ENK(HC, IS)	DYN (IS) CB ¹³ (IC)		
	D ₂ receptors (ENK, NT)	Reduced	ENK and DARPP-32 (IC)	SP and DYN (IS, IC)	SP (IS)	CB (IC)	CB ¹³ (IC)
	D ₃ receptors		SP and DYN (IC)	NMDA-R1 (IC)			
	GABA DARPP-32 CB	Increased		Glu-R1 (IC) ENK (IS)	ENK (IS)		
Striatum: non-projecting interneurons	ChAT SST/ NADPH-diaphorase	None		NADPH-diaphorase ChAT, PARV/ GABA (HC)			
	PARV/GABA	Increased		GABA (HC)	GAD (IS)		
Striatum and accumbens: nerve terminals	AADC, TH, DAT	None	AADC (IC) DAT (LB)	TH (IC) DAT (LB)	TH (IC, IS)	TH (IC) DAT (LB)	TH (IC)
Nucleus accumbens	D ₁ receptors (SP, DYN)	None	D ₁ and D ₂ (LB and IS)				
	D ₂ receptors (ENK)	Reduced	ENK (IC)	SP and DYN (IS)		CB (IC)	CB (IC)
	D ₃ receptors CB		DYN (IC)	DAT ¹² (LB)			

Table of neurochemical markers examined in mutant mice. Consistent with previous pharmacological studies on intact and 6-OHDA-lesioned animals, experiments in mutant mice have shown that D₁ and D₂ receptors are critically involved in the modulation of SP/dynorphin- or enkephalin-expression levels, respectively. AADC, aromatic acid decarboxylase; IC, immunocytochemistry; IS, in situ hybridization; LB, ligand-binding autoradiography.

¹ Zhou and Palmiter (1995).

² Kim et al. (2000).

³ Xu et al. (1994b).

⁴ Drago et al. (1994).

⁵ Drago et al. (1996).

⁶ Moratalla et al. (1996).

⁷ Ariano et al. (1998).

⁸ Baik et al. (1995).

⁹ Jung et al. (2000).

¹⁰ Xu et al. (1997).

¹¹ Accili et al. (1996).

¹² Change is very subtle.

¹³ Altered cellular distribution.

suggests a normal capacity for DA receptor function in these mutants (Zhou & Palmiter, 1995).

Consistent with results of previous studies on rodents that showed reductions in SP and DYN expression levels in response to both 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway and antagonists of D₂-like receptors (Hanson et al., 1981; Tang et al., 1983; Gerfen et al., 1990, 1991; Le Moine et al., 1990; Gerfen, 1995), the DA-deficient mutants express less SP and DYN immunoreactivities (Zhou & Palmiter, 1995). Thus, the transcription of these neuropeptides is tonically modulated by DA. However, whereas the expression of ENK increases in response to either denervation or D₂-like receptor blockade (Tang et al., 1983; Gerfen et al., 1990, 1991; Le Moine et al., 1990; Gerfen, 1995), there was no detectable change in ENK immunoreactivity in the DA knockout mouse. It should be noted, however, that this result does not necessarily indicate that DA plays no role in modulating striatal ENK expression in D₂ receptor-expressing neurons. These studies were performed in young mice at postnatal age P16, an age at

which the expression of D₂ receptors (which critically modulate ENK expression) have not reached mature expression levels (Guennon & Bloch, 1991).

D₁ mutants are presently the only DA receptor mutants reported to have a reduced brain size. Nevertheless, the brains of D₁ knockout mice appear anatomically well formed. Their midbrain DAergic projections appear intact, mutants display wild-type levels of TH immunoreactivity, and the expression of DAT ligand-binding sites is generally unaltered (Xu et al., 1994b; Moratalla et al., 1996). Similar to the DA-deficient mutants, no changes were found for the expression of DARPP-32 immunoreactivity (although striatal D₁ receptors co-localize with DARPP-32), suggesting an intact DA-signaling system, despite the absence of D₁ receptors during development (Xu et al., 1994b).

The examination of striatal interneuronal populations revealed no apparent abnormalities. No qualitative differences were found in tissues processed immunohistochemically for the interneuronal markers NADPH-diaphorase, choline acetyltransferase (ChAT), PARV, and GABA. In medium-spiny neurons, however, neurochemical changes

Table 3
Neurochemistry of the striatal matrix and striosome compartments

	Wild-type		D ₁ ¹	D ₂ ^{2,3}	D ₃ ³	D ₂ /D ₃ ³
Matrix	CB	Same	ENK (IS)	AChE (HC)	AChE (HC)	AChE (HC)
	ENK	Reduced	DYN (IS)		CB (ventromedial)	CB(ventromedial)
	AChE	Increased		ENK (IS)		
		Different	Blurred CB, poor zones (IC), ENK ⁵ poor striosomes (IC)	CB ¹ (IC)		CB ⁴ (IC)
Striosome	DYN clusters	Same		DYN (IS)		
	μ-opioid receptor patches	Reduced	DYN (IC)			

Table of all neurochemical markers used to examine the striosomal and matrix compartments of wild-type and DA receptor mutant mice. Although the expression of some markers varied, both compartments were identifiable and, thus, their development does not require DA signaling. HC, histochemistry; IC, immunocytochemistry; IS, in situ hybridization.

¹ Xu et al. (1994b).

² Baik et al. (1995).

³ Jung et al. (2000).

⁴ Altered cellular distribution.

⁵ Change is very subtle.

are evident. Whereas ENK mRNA and protein levels have been reported to be either unchanged (Xu et al., 1994b; Drago et al., 1994) or slightly elevated (Drago et al., 1996) compared with wild-type, D₁ mutants show a reduced expression of DYN and SP mRNA and protein in the striatum and nucleus accumbens (Drago et al., 1994, 1996; Xu et al., 1994b; Moratalla et al., 1996) (see Fig. 2). Reductions in SP immunoreactivity were also observed in axonal projections in striatal output targets (Xu et al., 1994b). The reduction of SP and DYN expression in striata of D₁ mutants, together with the observation that the expression of ENK is not significantly altered in this structure, is in good agreement with the results of phar-

macological studies showing that SP and DYN levels are decreased in rodents after 6-OHDA lesions (with or without subsequent treatment with D₂-like agonists) (Gerfen, 1995). Thus, these findings confirmed that DA signaling increases the expression of DYN and SP via activation of D₁ receptors (see Table 2).

Despite the lack of the D₁ receptor, the matrix and striosomal compartments develop normally. This is indicated by matrix localization of CB and ENK and the labeling of striosomal opiate μ-receptor and DYN clusters of cells, although subtle changes in expression patterns made the striosomal compartments less obviously defined relative to wild-type (Xu et al., 1994b) (see Table 3).

Finally, the expression of excitatory amino acid receptors, essential for the reception of excitatory afferents from the cortex and thalamus, was found to be altered in D₁ mutants. Ariano et al. (1998) reported a reduction in immunohistochemical labeling for the *N*-methyl-D-aspartate (NMDA)-R1 receptor and an increased expression of the glutamate receptor subtype GluR1, suggesting a functional interaction between either receptor and the D₁ receptor.

Altogether, the characterization performed to date supports the conclusion that the D₁ receptor is not required for proper development of the striatum. However, these studies have clearly illustrated that striatal D₁ receptors play a major role in regulating the expression of the neuropeptides DYN and SP, and some presumed compensatory changes in the expression of excitatory amino acid receptors have been found.

Intact midbrain DAergic projections are also found in D₂ mutants whose tissue levels of striatal DA are normal, and no alterations in the expression of TH-encoded mRNA or protein were detected (Baik et al., 1995; Jung et al., 2000). Whereas mRNA levels for the D₁ receptor-associated neuropeptide DYN are unchanged, the levels of mRNA encoding SP are reduced (Baik et al., 1995). Importantly, consistent

Prodynorphin mRNA levels in wild type and D1 mutants

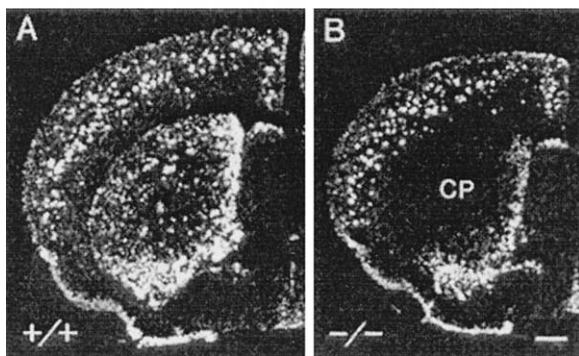


Fig. 2. Down-regulation of prodynorphin mRNA expression in the striatum of mice deficient for DA D₁ receptors. In situ darkfield autoradiographic images of wild-type (+/+) (A) and homozygous D₁ mutant (-/-) (B) mice. CP, caudoputamen. Scale bar (in B): 0.5 mm. Reproduced from Moratalla et al. (1996), with permission of the copyright holder, National Academy of Sciences.

with the results of earlier studies showing increased expression of striatal ENK in response to either 6-OHDA lesions or D₂-like receptor antagonism (Gerfen, 1995), ENK mRNA levels are significantly increased in mice lacking the D₂ receptor (Baik et al., 1995; Jung et al., 1999). Moreover, the mRNA levels of glutamic acid decarboxylase (GAD), an enzyme essential for the synthesis of GABA, are elevated. As this elevation was delimited to the striatum and the cortex, the authors interpreted these results as a compensatory change that occurs in the striatal circuitry following a disinhibition of the subthalamic neurons secondary to removal of the D₂-associated indirect pathway (Baik et al., 1995) (see Table 2).

D₂ deletion mutants (Kelly et al., 1998) exhibit some, but not all, of the neurochemical phenotypes described for striata of D₂-null mutants (Murer et al., 2000). Whereas the expression of striatal ENK mRNA is increased and the expression of SP mRNA is decreased, no changes were found for the expression of GAD-67 mRNA in the striatum, but this mRNA was found to be reduced in the globus pallidus. Whether this difference between the genetic null mutants and the deletion mutants contributes to the differences in distinct behavioral phenotypes of both types of mutants remains to be examined further.

The striatal matrix and striosomal compartmentalization of D₂ mutants were examined with histochemical staining for acetylcholinesterase (AChE) and immunocytochemical staining for CB. Although no obvious differences in the distribution of either AChE or CB were found, D₂ mutants show a very characteristic change in the cellular distribution of CB immunoreactivity. In wild-type neurons, CB is homogeneously distributed throughout the cell, including the nucleus. In contrast, CB-expressing neurons of D₂ mutants are devoid of nuclear CB expression and the majority of CB clusters in close proximity to the plasma membrane (Jung et al., 2000) (see Table 3). Although the reasons for this altered cellular distribution of CB remain to be identified, it is compelling to hypothesize that these changes reflect a compensatory response to cellular stress that is secondary to some excitotoxic process related to the lack of D₂ receptors. In this regard, it is of interest to note that Bozzi et al. (2000) recently have shown that D₂ mutants have a greater susceptibility to kainate-induced hippocampal excitotoxicity, i.e., D₂ mutants exhibit seizures when administered doses of kainic acid that are not epileptogenic in wild-type mice, and these seizures result in extensive hippocampal apoptotic cell death. Thus, the distinct cellular distribution of CB in neurons of D₂ mutants could increase the buffering capacity for Ca²⁺ entering either through voltage-sensitive Ca²⁺ or NMDA channels to protect cells that have lost D₂ receptors against the damaging effects of excessive Ca²⁺ influx during prolonged periods of excitation. The altered cellular distribution of CB could also affect the ability of the cell to modulate rising intracellular Ca²⁺ signals that are critical for the establishment of synaptic plasticity. Indeed, as

shown by Calabresi et al. (1997), at corticostriatal synapses, for example, the lack of D₂ receptors induces alterations in synaptic plasticity. Finally, it is also possible that the changes in striatal CB expression detected in D₂ mutants result in a general reduction of activation of the striatal matrix in response to DA stimulation and thus, underlie the increased D₁ agonist-induced stereotopies of D₂ mutants described in Fig. 1.

Similar to the other mutants discussed above, the normal expression of TH immunoreactivity and the unaltered expression of DAT ligand-binding sites in striata of D₃ mutants indicate a non-compromised capability for endogenous DA synthesis and re-uptake (Xu et al., 1997; Jung et al., 2000). Also, the lack of histochemical changes in AChE expression indicates a normally formed matrix and an unaltered capability for the degradation of acetylcholine. Deletion of the D₃ receptor does not alter the expression of D₁ or D₂ class receptor ligand-binding sites (assessed with autoradiography), indicating that there are no compensatory changes in DA receptor expression per se (Accili et al., 1996; Xu et al., 1997) (see Tables 2 and 3). In contrast to D₂ mutants, the cellular expression pattern of CB immunoreactivity is unaltered in D₃ mutants. Surprisingly, however, CB labeling is notably decreased in the nucleus accumbens of D₃ mutants, a region in which D₃ receptors are normally expressed at highest levels (Jung et al., 2000) (Table 3). This unexpected finding raises the possibility that D₃ receptors either promote the expression of CB in neurons, where both proteins are co-expressed, or play a significant role in the development of the ventral striatal CB-expressing system. Further studies are needed to address this issue.

To date, D₂/D₃ double mutants are the only double mutants that have been subjected to anatomical studies (Jung et al., 2000). Results of histochemical labeling for AChE and immunocytochemical labeling of TH are similar to results obtained from either single mutant. Thus, the double mutants also appear to have intact DAergic projections, and no differences were found in the distribution of AChE or the demarcation of the striatal matrix. As one would expect, however, the labeling for CB revealed a combination of the phenotypes detected in D₂ and D₃ single mutants. The double mutants display both a notable decrease in CB expression in the nucleus accumbens (as detected in D₃ single mutants) and the altered cellular distribution of CB characteristic of D₂ single mutants.

3.3. Implications of the role of dopamine in brain development, gene expression, and function

At this time, light and confocal microscopy on the brains of DA receptor mutants have not provided a structural basis for the various phenotypes detected in the mutants. However, these studies have documented a few noteworthy neurochemical alterations for each mutant. They include

reduced expression of DYN and SP in the striatum and nucleus accumbens of D₁ mutants, increased expression of ENK and GAD, as well as an altered cellular distribution of CB in striata of D₂ mutants, and reduced expression of CB in the nucleus accumbens of D₃ mutants. Thus, these studies have identified neurochemical changes that are likely to contribute to the locomotor phenotypes detected in the mutants. Importantly, these studies have also shown that DA signaling is not a prerequisite for the normal development of dopaminergic structures.

Although the gross anatomy of the structures examined in the above studies is normal in DA receptor mutants, more rigorous comparisons are required to confirm that the absence of DA signaling leads only to neurochemical alterations (as opposed to changes in morphology) and to elucidate the nature of these neurochemical abnormalities and their relationship to the respective phenotypes.

DA also plays an important role in other systems, including those that are well characterized, such as the pituitary, retina, and olfactory bulb. To date, three studies have used D₂ mutant mice to study the role of DA in the control of pituitary function (Saiardi et al., 1997; Kelly et al., 1997; Asa et al., 1999). D₂ receptors are highly expressed in lactotrophs and melanotrophs of the anterior and intermediate pituitary lobes, respectively. Studies on D₂ mutant mice demonstrated that DA has antiproliferative effects on the developing pituitary gland and a negative control over prolactin synthesis. The absence of D₂ receptors leads to a progressive increase in the number of lactotrophs and a chronic elevation of prolactin levels (Saiardi et al., 1997; Kelly et al., 1997). As first shown by Saiardi et al. (1997) and later confirmed by Asa et al. (1999), this phenotype is associated with the development of tumors in the aging animal. Thus, despite the suggested presence of D₃ and D₄ receptors in the pituitary, these effects are mediated solely via D₂ receptors. It is presently controversial whether the absence of D₂ receptors also affects the intermediate pituitary lobe. Saiardi et al. (1997) found that genetic D₂-null mutants also exhibit an intermediate lobe hyperplasia. Such a morphological change, however, was not found in the deletion mutants (Kelly et al., 1997; Asa et al., 1999). Whether these discrepant results are due to genetic background differences between the two different mutants remains to be tested in both congenic C57Bl/6 and 129Sv mice.

4. Other knockouts affecting dopaminergic neurotransmission

Since DA exerts its effect via receptor-mediated activation of a variety of signaling cascades, the inactivation of individual components of DA receptor signaling cascades must have consequences for normal DA signaling. Indeed, it has now been shown that inactivation of one member of the family of inhibitory G-proteins, G_{oif}, abolishes D₁ receptor

signaling in the striatum, and that inactivation of DARPP-32 affects a large variety of DA-mediated striatal functions. Results of these studies are summarized below.

4.1. G_{oif}

Beside its role in odorant signal transduction, G_{oif} interacts with other neurotransmitter receptors. In the rat brain, G_{oif} is highly enriched in the striatum, where it appears to be preferentially localized in the perikarya and dendrites of striatonigral and striatopallidal neurons. Its expression in nerve terminals in the substantia nigra, however, is much lower compared with the striatum (Hervé et al., 1993). Although the expression of G_{oif} is not restricted to DA target neurons that express D₁ receptors, the abundant expression of G_{oif} in striatonigral neurons (and the minor role that G_{os} plays in striatal neurons) has led to the suggestion that striatal D₁ receptors couple to G_{oif} (Hervé et al., 1993). Indeed, mice lacking G_{oif} are not only anosmic, but, similar to the D₁ knockout mice described by Xu et al. (1994a, 1994b), they also display a locomotor hyperactivity when tested in the open field (Belluscio et al., 1998). Moreover, G_{oif} mutant mice do not show wild-type-like D₁ agonist-stimulated locomotor activity and rearing, and neither cocaine-induced stereotypic behavior nor induction of striatal *c-fos* expression is detected in these mutants (Zhuang et al., 2000). In short, in G_{oif} knockout mice, striatal D₁ receptors have a decreased affinity for DA because they are uncoupled from their G-protein (Zhuang et al., 2000).

4.2. Dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32

DARPP-32 is expressed at high levels in nearly all striatal medium spiny neurons, where it is thought to play a central role in mediating the effects of DA under physiological conditions. DARPP-32 appears to mediate (the opposing) cross-talk between D₁- and D₂-like receptors. On one hand, stimulation of the D₁ class of DA receptors leads to activation of the adenylyl cyclase/cAMP-dependent protein kinase A (PKA) pathway that results in phosphorylation of DARPP-32 at a single threonine residue (Thr34). This phosphorylated form (but not the dephosphorylated form) is a very potent inhibitor of the serine/threonine protein phosphatase-1, which regulates the phosphorylation states of many downstream effectors (neurotransmitter receptors, ion channels, ion pumps, and transcription factors). On the other hand, activation of D₂-like receptors decreases the D₁ agonist-stimulated phosphorylation of DARPP-32 via mechanisms involving inhibition of PKA and activation of Ca²⁺/calmodulin-dependent protein phosphatase (PP-2B, calcineurin) (for a review, see Greengard et al., 1999).

Thus, it is not surprising that DARPP-32 knockout mice (Fienberg et al., 1998) show a variety of alterations

in DA receptor-mediated responses. They include the abolition of DA-mediated phosphorylation of the NMDA-R1 receptor subunit and the D₁ receptor-mediated reduction in the activity of [Na⁺/K⁺]ATPase, a decreased modulation of Ca²⁺ currents by D₁ receptors, and an attenuated inhibition of glutamate-evoked currents in response to D₁ agonists.

Psychostimulants such as cocaine and amphetamine (acting as indirect D₁ receptor agonists), as well as D₂-like antagonists, increase DARPP-32 phosphorylation. In DARPP-32 knockout mice, however, amphetamine stimulation leads to less striatal DA and DA-induced GABA release when compared with wild-type. Striatal *c-fos* responses to low doses of amphetamine are also reduced, and the chronic administration of the drug does not lead to the expression of the 35- and 37-kDa ΔFosB proteins that normally accumulate during chronic exposure to psychostimulants. Finally, the locomotor stimulant effects of cocaine and amphetamine are significantly attenuated in the mutants, and the cataleptic effects of low doses of D₂-like antagonists are also significantly lower (Fienberg et al., 1998). Thus, the DARPP-32/protein phosphatase-1 cascade, which in medium spiny neurons appears to enable the integration of different striatal inputs to modulate proper physiological responses, plays an important role in mediating the effects of DA acting upon striatal DA receptors.

5. Interactions among dopamine receptors

Investigations on the functional properties of non-mutated DA receptors in DA receptor mutant mice have begun to provide novel insights into the role of individual DA receptors in the modulation of behavioral responses to DAergic stimulation and the induction of expression of immediate early genes (IEGs). Results of these studies revealed complex interactions among D₁, D₂, and D₃ receptors in vivo. Because there is no strict cellular co-localization of all three receptors, the interactions described in the following sections are unlikely to be mediated solely via cellular mechanisms, but they are also likely to reflect complex interactions at the level of neuronal circuits.

5.1. Interactions among D₁, D₂, and D₃ receptors in the expression of behavioral responses to pharmacological stimuli

Xu et al. (1997) found that D₃ mutants exhibit differences in their locomotor activity when both D₁ and D₂ receptors are co-stimulated, either directly with D₁- and D₂-like receptor agonists or indirectly with low doses of the DA re-uptake blocker cocaine. The combined stimulation of D₁ and D₂ receptors elicits more locomotor activity in D₃ mutants than in wild-type. Since

this effect was also observed in DA-depleted mice, these findings point to a previously unrecognized role for postsynaptic D₃ receptors in dampening the magnitude of cooperative postsynaptic effects of D₁ and D₂ receptor co-stimulation. Similarly, in the conditioned cue preference paradigm, D₃ mutants show an abnormally strong conditioning effect at low doses of amphetamine that in wild-type mice do not lead to the expression of conditioned cue preference. This further suggests that D₃ receptors also inhibit the expression of conditioned cue preference to lower doses of psychostimulants.

5.2. Dopamine receptor agonist- and psychostimulant-induced *c-fos* responses in dopamine receptor knockout mice

Induction of expression of the IEG *c-fos*, a gene with low baseline expression levels, is an important mechanism in the control of neurotransmitter-regulated gene expression (Harlan & Garcia, 1998). The administration of D₁ receptor agonists and psychostimulants (cocaine, amphetamine) leads to a robust induction of striatal *c-fos* expression, which, in striatonigral neurons that express D₁ receptors, is paralleled by an increase in striatal proDYN mRNA expression. Moratalla et al. (1996) observed, however, that cocaine administration fails to increase proDYN mRNA levels in the neostriatum of D₁ mutant mice, a result that provided an elegant proof for the widely proposed concept that an increase in cocaine-stimulated DYN gene transcription requires activation of D₁ receptors. Moreover, both cocaine and amphetamine fail to induce the expression of *c-fos* and JunB in the striatum and the cerebral cortex in these mutants (Moratalla et al., 1996). Drago et al. (1996) confirmed, with studies on their D₁ mutants, that cocaine treatment does not induce striatal *c-fos* and *zif/268* expression.

It is now clear that expression of D₁ receptors is the ultimate requirement for the induction of expression of IEGs and the neuropeptide DYN. However, there is also a role for the D₂ class of DA receptors in modulating the expression levels of IEGs. In fact, the D₂-like antagonist haloperidol induces *c-fos* and JunB expression in the striatum of wild-type, as well as D₁ mutant mice. Thus, this effect is entirely mediated by D₂ receptors and interestingly, in the absence of a D₁/D₂ synergism, it is augmented in D₁ mutant mice (Moratalla et al., 1996). In contrast, mice deficient for D₂ and D₃ receptors show blunted forebrain *c-fos* mRNA and protein responses to D₁ agonist stimulation (Jung & Schmauss, 1999; Schmauss, 2000b). The greatest decrease of D₁ agonist-stimulated induction of *c-fos* immunoreactivity is detected in both striatal and extrastriatal tissues of D₃ mutant mice. Moreover, pretreatment of D₃ mutants with the D₂/D₃ antagonist eticlopride further reduces *c-fos* protein expression. Eticlopride also reduces D₁ agonist-stimulated *c-fos* responses of wild-type mice, suggesting that the expression of both

D₂ and D₃ receptors is required for maximum *c-fos* responses to D₁ receptor stimulation (Jung & Schmauss, 1999). Indeed, studies on *c-fos* mRNA expressed in response to D₁ agonist stimulation revealed clearly that

both D₂ and D₃ mutants show blunted forebrain *c-fos* responses compared with their wild-type littermates, although basal *c-fos* mRNA levels are substantially higher in both mutants (see Fig. 3). However, the forebrain *c-fos*

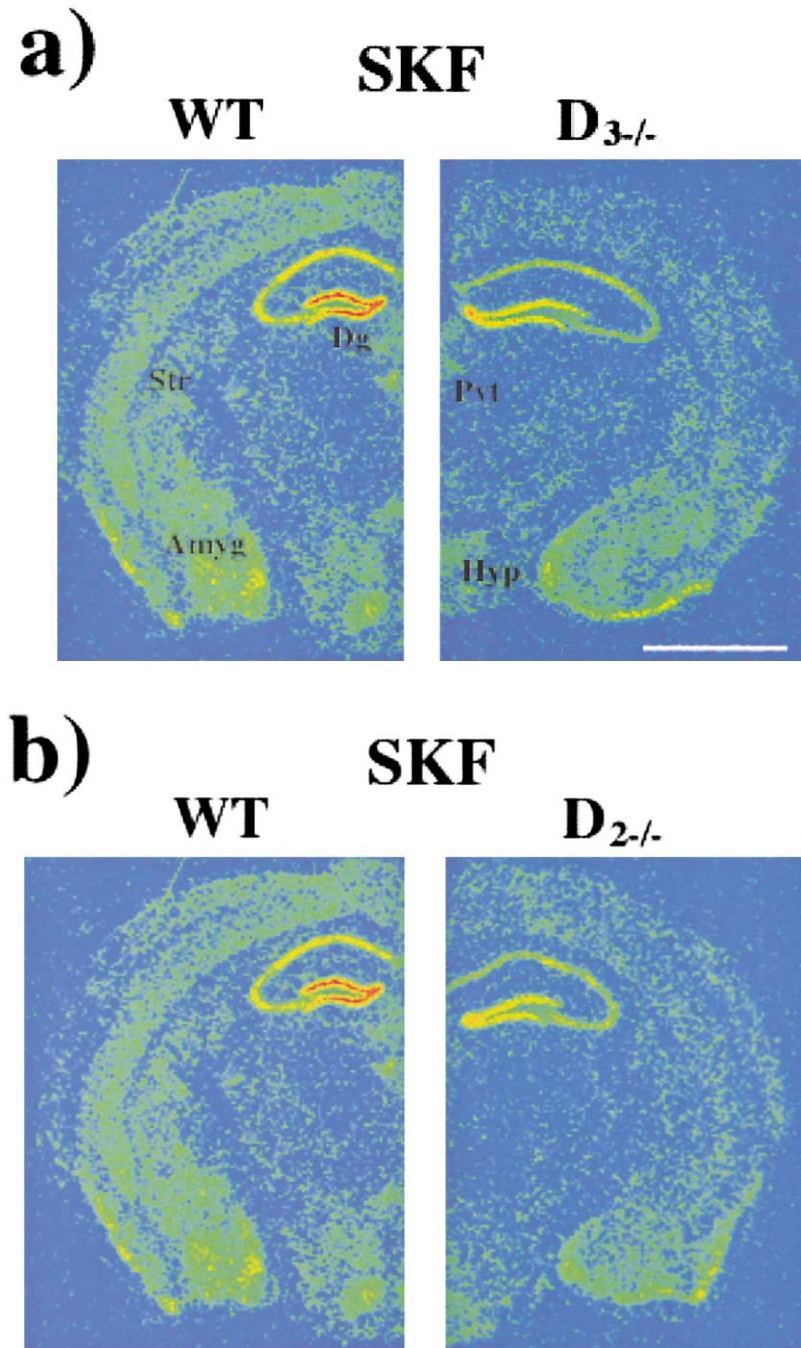


Fig. 3. Blunted SKF-induced *c-fos* mRNA expression in D₂ and D₃ receptor knockout mice. Adult wild-type and mutants were sacrificed by decapitation 1 hr following the application of SKF-82958 (i.p. 1 mg/kg) or vehicle (saline). To detect *c-fos* mRNA, tissue sections (16 μ m) were exposed for 16 hr at 55°C to a [³⁵S]labeled, antisense riboprobe comprising 540 nucleotides of the mouse *c-fos* mRNA (see Schmauss, 2000b). Images on film were digitized using the MCID image analysis system (St. Catharines, Ontario) and colorized uniformly to highlight *c-fos* signal intensities. Representative sections of wild-type (WT) and D₃ mutants (a) and wild-type and D₂ mutants (b) are taken at 2 mm rostral to the interaural line. In all genotypes, the application of SKF elicits an increase in *c-fos* mRNA relative to the basal expression evident in mice injected only with saline [not shown, but see Schmauss (2000b) for Northern blot analysis]. Note the reduced *c-fos* mRNA response of both D₂ and D₃ mutants, which is most apparent in cortical (amygdala, dentate gyrus, dorsal and lateral cortices), posterior striatal, and midline thalamic and hypothalamic structures. Amyg, amygdala; Dg, dentate gyrus; Hyp, hypothalamic nuclei; Pvt, paraventricular nucleus of the thalamus; Str, striatum. Scale bar = 2 mm.

mRNA responses to methamphetamine (which reach < 20% of the corresponding levels expressed in response to D₁ agonist stimulation and which are completely blocked by pretreatment with a D₁ antagonist) are indistinguishable between wild-type and D₂/D₃ mutants (Schmauss, 2000b).

The mechanisms underlying the blunted *c-fos* mRNA and protein responses of D₂ and D₃ mutants to direct D₁ agonist stimulation remain to be elucidated. It is, however, of interest to note that a single, low-dose of methamphetamine (5 mg/kg) leads to a long-term reversal of these blunted *c-fos* responses, an effect that lasts as much as 2 weeks after methamphetamine injection (Schmauss, 2000b). Moreover, the reversal of the blunted *c-fos* responses to D₁ agonist stimulation by methamphetamine is most pronounced in the neocortex, a region in which methamphetamine normally elicits the largest *c-fos* responses. It is further noted that a similar long-term reversal of the blunted *c-fos* responses to D₁ agonist stimulation is also achieved with a single dose of a full D₁ agonist. In contrast to methamphetamine, however, the effects of the D₁ agonist pretreatment are not delimited to the neocortex, but are found throughout the forebrain (Schmauss, 2000b). These data indicate that despite the unaltered expression of D₁ receptor ligand-binding sites in the brains of D₂ (Baik et al., 1995) and D₃ mutants (Xu et al., 1997), the chronic inactivation of either D₂ or D₃ receptors leads to a decreased responsiveness of D₁ receptors to agonist stimulation, and this decreased responsiveness can be reversed by a single administration of either methamphetamine or a D₁ agonist.

6. Interactions between dopamine receptors and other neurotransmitter receptors

6.1. Heterologomeric receptor assemblies involving dopamine receptor subtypes—a new challenge for studies on knockout mice

During the past few years, evidence has accumulated that suggests that G-protein-coupled receptor (GPCR) exist not exclusively as monomers. In fact, a large number of studies have provided evidence for the expression of homodimeric, -trimeric, and -tetrameric forms of DA receptors (for a

review, see Schmauss, 2000a). Recent evidence also points to the existence of heterologomers formed between various subtypes of DA receptors and other neurotransmitter receptors (Table 4). For example, in hippocampal neurons that co-express D₅ and GABA_A receptors, the carboxyl terminus of the D₅ receptor (a protein sequence thought to confer D₅ receptor-specific functions) directly interacts with the second intracellular loop of the γ 2GABA_A receptor subunit (Liu et al., 2000). This is the first example of a heterologomeric assembly formed between a GPCR and a multimeric ligand-gated neurotransmitter channel. In contrast to homologomeric DA receptors, which are pre-formed (see Schmauss, 2000a), the formation of D₅/ γ 2GABA_A heterologomers requires agonist stimulation, and pretreatment with either a D₁/D₅ or a GABA_A receptor antagonist blocks the formation of the protein complex. The heterologomers are functionally antagonistic: GABA decreases the maximal D₅ receptor-mediated stimulation of adenylyl cyclase and DA decreases GABA_A receptor-mediated functions. This suggests a specific role for D₅ receptor activation in modulating inhibitory synaptic inputs in neurons that co-express D₅ receptors and the γ 2GABA_A subunit (hippocampal and neocortical neurons). Such a modulation should be absent in mice deficient for D₅ receptors. Thus, D₅ mutants are potentially a valuable tool for testing the significance of a D₅/ γ 2GABA_A interaction *in vivo*.

Recently, the D_{2L} receptor and a subtype of the SST receptor family (SSTR5) have been found to form functional heterologomers (Rocheville et al., 2000a). Both receptors are known to form homologomers (D₂ homologomers are pre-formed; SSTR5 oligomers form upon agonist stimulation), and the SSTR5 receptor also forms heterologomers with other members of the SSTR family (Rocheville et al., 2000b). D₂ receptors and SSTR5 are co-localized in striatal medium spiny and neocortical pyramidal neurons, and both receptors couple to inhibitory subsets of G-protein (G_i). The D₂/SSTR5 protein complex is a functionally synergistic heterologomer, i.e., ligand binding to one of the two receptor molecules increases ligand affinity for the other partner of the heteromer and thus, leads to a greater activation of downstream effectors.

Direct evidence for the physical interaction between D₂ and SSTR5 receptors *in vivo* is still elusive. However, with suitable antibodies in hand, co-immunoprecipitation studies

Table 4
Heterologomeric assemblies involving DA receptors

DA receptor subtype	Heterologomer partner	Regions of co-localization	Requirement for heterologomer formation	Functional consequence of heterologomer formation
D ₅	γ 2-subunit of GABA _A receptor	Hippocampus	Co-stimulation of D ₅ and GABA _A receptors	DA decreases GABA _A currents; GABA decreases DA-stimulated activation of adenylyl cyclase
D _{2L}	SSTR5	Striatum, neocortex	SSTR5 or D ₂ receptor agonists	Synergistic interaction (ligand binding and adenylyl cyclase activity)
D ₁	Adenosine A ₁ receptor	Striatum	A ₁ agonists	Uncoupling of D ₁ receptors from G-protein

and comparative fluorescence resonance energy transfer studies on wild-type and mutant mice are now possible. Such studies on striatal or cortical tissues and slice preparations obtained from genetic D₂-null mutants (Baik et al., 1995; Jung et al., 1999), D₂-deletion mutants (Kelly et al., 1997), D_{2L} mutants (Wang et al., 2000; Usiello et al., 2000), and wild-type mice could potentially provide valuable insight into the functional significance of these heterologomers.

D₁ and adenosine A₁ receptors also form functionally interacting heteromeric complexes (Ginés et al., 2000). This finding was perhaps the least surprising since antagonistic interactions between specific subtypes of striatal adenosine and DA receptors that are co-expressed in GABAergic striatopallidal neurons (A₂ and D₂ receptors) and in GABAergic striatonigral and striatoentopeduncular neurons (A₁ and D₁ receptors) have been widely reported and, thus, suggested to directly interact with each other (for a review, see Ferré et al., 1997). Ginés et al. (2000) found that a combined treatment of cells expressing D₁/A₁ heterologomers with a D₁ and an A₁ agonist results in a significant reduction of D₁ agonist-induced cAMP formation. These results led the authors to suggest that co-activation of A₁ and D₁ receptors in the heteromeric receptor complex leads to an uncoupling of D₁ receptors from G-protein (G_s) and that this mechanism contributes to the functional A₁/D₁ antagonism observed in behavioral and biochemical studies. Despite the widely observed antagonistic interaction between A₁ and D₁ receptors, further investigation on the functional consequences of A₁ receptor inactivation in the absence of D₁ receptors, i.e., in D₁ knockout mice, is necessary to provide insight into the extent to which A₁ receptors that are tonically activated by endogenous adenosine function independently of the DAergic system.

The latter question is also of interest for adenosine A₂ receptors, which, in striatopallidal neurons, oppose the action of D₂ receptors (Ferré et al., 1997). Although published evidence for the (highly probable) heterologomeric assembly of A₂/D₂ receptors is still elusive, the role of A₂ receptors in brains of D₂ mutant mice already has been extensively investigated, and results of these studies are summarized in the next section.

6.2. Adenosine A₂ receptor functions in D₂ mutant mice

Opposing interactions between A₂ and D₂ receptors have been observed both in biochemical and behavioral experiments, and they are summarized in a comprehensive review by Ferré et al. (1997). Briefly, stimulation of A₂ receptors decreases the affinity of agonists (but not antagonists) for the D₂ receptor in a manner that is independent of G-protein coupling. A₂ receptor stimulation also counteracts D₂ receptor-mediated Ca²⁺ influx. Moreover, blockade of D₂ receptors leads to an increase in the expression of the IEG *c-fos* in striatopallidal neurons, an effect that can be partially counteracted by A₂ antagonists. Conversely, *c-fos* expression in response to A₂ receptor agonists is partially

counteracted by D₂ receptor agonists. In addition, stimulation of A₂ receptors reduces D₂ receptor-mediated locomotor activity and antagonizes the D₂ agonist-induced reduction in GABA release from neurons of the globus pallidus. Other phenomena, however, such as the effect of the adenosine receptor antagonist caffeine on the expression of those IEGs that have high basal expression levels, were not counteracted by D₂ antagonists, suggesting a function of tonically active A₂ receptors that is independent of D₂ receptors. In this regard, it is also possible that the motor effects mediated by adenosine are not entirely attributable to the modulation of DA-mediated signaling and, in fact, may involve a D₂ receptor-independent role of A₂ receptors in modulating GABA release and recurrent feedback inhibition of striatal neurons (see Ferré et al., 1997). This question has been addressed in two recent studies on D₂ and A₂ mutant mice (Aoyama et al., 2000; Chen et al., 2001). Both studies found a normal expression of A₂ receptor-encoded mRNA and ligand-binding sites in the striatum of D₂ mutants. Interestingly, in D₂ mutants, blockade of A₂ receptors reverses the abnormal striatal neuropeptide expression caused by the D₂ receptor knockout. Hence, the abnormally increased expression of ENK and the abnormally decreased expression of SP revert to normal wild-type levels (Aoyama et al., 2000). Consistent with this finding, Chen et al. (2001) found that the inactivation of A₂ receptors in A₂/D₂ double mutants partially reverses the increase in ENK mRNA expression that is due to the inactivation of D₂ receptors. Because ENK is co-expressed with D₂ and A₂ receptors in striatopallidal neurons and SP is co-expressed with D₁ receptors in striatonigral neurons, Aoyama et al. (2000) concluded that blockade of A₂ receptors normalizes the unbalanced neuronal activities in both (indirect and direct) striatal output pathways in D₂ mutants. Indeed, the authors found that blockade of A₂ receptors rescues the locomotor hypoactivity of D₂ mutants. Chen et al. (2001) further found that A₂ agonists reduce both spontaneous and amphetamine-stimulated locomotion in D₂ mutant mice. Thus, adenosine mediates its proper physiological effect through A₂ receptors, and does so partially independent from its antagonistic interaction with DA-mediated signaling, a finding that has important implications for novel treatment strategies for Parkinson's disease.

At present, it is difficult to reconcile the findings of Aoyama et al. (2000) and Chen et al. (2001) with results of another study suggesting that A₂ receptors expressed in D₂ mutants are "functionally uncoupled" (i.e., agonist stimulation does not lead to G-protein activation) (Zahniser et al., 2000). The authors found that an A₂ agonist (CGS 21680) significantly increased the stimulation-evoked striatal GABA release in striatal/pallidal slice preparations from wild-type tissues, but that the agonist had no effect when applied to corresponding slices obtained from the D₂ mutants (a result that could not be replicated with wild-type slices that were treated with the A₂ agonist and the D₂-like antagonist raclopride). Similarly, the same A₂ receptor agonist significantly increased cAMP levels in wild-type slices, but failed to

stimulate adenylyl cyclase in slices obtained from D₂ mutants. Finally, in contrast to the results of Aoyama et al. (2000) and Chen et al. (2001), the locomotor stimulant caffeine, an A₁/A₂ receptor antagonist, did not lead to a sustained locomotor activation in D₂ mutants. In fact, 1 hr after caffeine injection, a locomotor depressant rather than activating effect is observed in D₂ mutants. This phenomenon is similar to the observation that caffeine, administered to A₂ receptor knockout mice, has locomotor depressant rather than activating effects (Ledent et al., 1997). Thus, the expression of an apparently non-functional A₂ receptor in the D₂ knockout mice studied by Zahniser et al. (2000) cannot explain the functional rescue of the neurochemical and behavioral phenotype of D₂-null mutants by A₂-specific receptor blockade or the A₂ knockout, and further studies are needed to clarify whether the A₂ receptor-stimulated GABA release is strictly dependent upon D₂ receptors.

7. Concluding remarks

In the past few years, studies on DA receptor knockout mice have provided a wealth of information about behavioral and biochemical/molecular phenotypes associated with the inactivation of the individual subtypes of DA receptors. To a great extent, these studies have built upon (rather than overturned) results of pharmacological and biochemical studies on intact (wild-type) animals, and they have also provided novel information about those receptor subtypes (D₃, D₄, and D₅), for which highly selective agonists and antagonists are not available yet. It is clear that the mutants will help tremendously in the *in vivo* testing of already marketed receptor-selective ligands, as well as in the receptor-subtype selectivity screening of newly developed ligands. Studies on D₃ mutant mice, for example, already have addressed the *in vivo* selectivity of a variety of compounds originally thought to represent D₃ receptor “selective” ligands, and the results of these studies suggest the putative selectivity of these ligands should be viewed cautiously (Boulay et al., 1999; Xu et al., 1999).

It is apparent that, in some cases, studies on genetic null mutants and deletion mutants of the same receptor have given different or even discrepant results. This is particularly evident for D₂ deletion mutants, which differ from genetic null mutants not only in the severity of the locomotor and postural impairments, but that have also given different results in anatomical and biochemical studies. Such discrepancies include the absence of a hyperplasia of the intermediate lobe of the pituitary (Kelly et al., 1997; but see Saiardi et al., 1997; Asa et al., 1999) and the lack of detectable changes in the expression of glutamate decarboxylase-67 mRNA expression in the striatum of deletion mutants (but not in the genetic null mutants) (Murer et al., 2000; but see Baik et al., 1995). Reasons for these discrepancies remain to be identified. Kelly et al. (1998) proposed that differences in the genetic backgrounds of the D₂

mutants generated by Baik et al. (1995) and Kelly et al. (1997) account for the differences in the locomotor phenotypes described by both laboratories. Indeed, the effect of background genes (derived from the parental strains that are either linked to the mutation or located at other chromosomal loci) on the expression of complex behavioral phenotypes is widely recognized as a confounding problem in the interpretation of such phenotypes (Crawley et al., 1997; Silva et al., 1997). Most targeting experiments utilized embryonic stem (ES) cells derived from 129Sv mice, i.e., genetically complex ES cells of a collection of substrains with various backgrounds (see Simpson et al., 1997). Therefore, to facilitate the comparison of results between experiments and among different laboratories, it is strongly suggested that congenic mutants be generated by backcrossing the F1 mutants to standard inbred mice. With regard to D₂ mutants, however, it should also be noted that while two laboratories that have generated genetic D₂-null mutants with different sources of ES cells (Baik et al., 1995; Jung et al., 1999), both lines of homozygous mutants have similar postural abnormalities, bradykinesia, and a significantly reduced fertility. Thus, if the expression of these phenotypes depends solely on the genetic backgrounds of these (independently generated) mutants, as proposed by Kelly et al. (1998), the severity of these phenotypes should be alleviated in congenic C57Bl/6 mutants. Our laboratory, therefore, has initiated backcrossings of our mutants to the C57Bl/6 inbred strain, and contrary to the above prediction, we find that the phenotypes of homozygous D₂ mutants, resulting from heterozygous crosses of the 5th backcross generation, are pronounced and, in fact, more severe compared with our mutants with a mixed (129Sv/C57Bl/6) genetic background. Thus, further rigorous studies on higher generations (12 generations or more) of congenic C57Bl/6 D₂-null mutants ultimately will show whether (and to what extent) the 129Sv genetic background, rather than the D₂ mutation, contributed to the phenotypes of these mice.

Another unresolved issue is whether different targeting strategies (genetic null mutations versus deletion mutants) have effects on the phenotype. There is precedence in the literature that the expression of a truncated D₃ receptor resulting from a deletion of targeted sequences that are located downstream of the 5' end of the ORF alters both the expression and function of the wild-type receptor (Accili et al., 1996; Jung et al., 1999). Moreover, amino-terminal peptides of GPCR that contain 2-5 TMDs have been shown to be independent folding units that are targeted to the plasma membrane, where they can interact with carboxyl-terminal folding units to form functional protein complexes (Schöneberg et al., 1995) that could involve heterooligomeric assemblies with other neurotransmitter receptors or ion channels, a possibility that, in view of the increasing evidence for heterooligomeric assemblies involving not only full-length, but also truncated, GPCR molecules receptors (Schmauss, 2000a; this review), remains to be tested for deletion mutants. Relevant findings made in *in vivo*, such as the

heterologous assembly of the D₃ receptor and its truncated version D_{3nf} in rodent and primate brain (Schmauss, 1996; Nimchinsky et al., 1997), and the lack of wild-type oligomeric forms of the D₃ receptor protein in brains of heterozygous D₃ mutants that co-express the wild-type D₃ receptor and an amino terminal truncated form thereof (Jung et al., 1999) indeed suggest that the expression of truncated GPCRs in brains of mutant mice has significant physiological consequences. Nevertheless, the *in vivo* significance of the recently reported heterologous remains to be demonstrated, and genetic null mutants incapable of forming such heterologous protein complexes potentially could provide a powerful tool for the identification of functions mediated by these heterologous. In addition, a further rigorous comparison of genetic null versus deletion mutants could also open new avenues for studies on the functional consequences of GPCR truncations expressed *in vivo*.

In addition to genetic factors, complex behavioral traits are also influenced by environmental factors, and differences in the environmental and technical details of behavioral test procedures can lead to inter-laboratory differences in the results. For example, the results of Crabbe et al. (1999) illustrate that measurements of behaviors with smaller genetic effects, i.e., those that often result from single gene knockouts, can be significantly affected by environmental conditions that are specific to individual laboratories and, thus, do not necessarily reflect an effect of the targeted gene deletion. The authors, therefore, suggest that genotypes should be tested in multiple laboratories and evaluated with multiple tests of a single complex behavior (such as anxiety/aggression or reward-related behavior). Moreover, standardization of behavioral tests (although perhaps difficult to achieve for most commonly employed tests of this nature) would enhance replicability of results across laboratories. Indeed, as recently demonstrated by Crestani et al. (2000), in some cases, inter-laboratory differences can be rapidly resolved if tests are repeated under identical conditions. In addition, maintaining knockouts on fully inbred strains (by continuous back-crossing) will prevent phenotypic drifts that ultimately can result in the loss of a phenotype in independently maintained breeding populations (Phillips et al., 1999).

Finally, it is widely understood that the phenotypes of the constitutive knockouts may not entirely relate to the inactivation of the receptors *per se*, as they may also result from complex adaptations to the loss of the receptor during the development of the mutant. For this reason, comparative studies on tissue-specific and inducible knockout mice are clearly needed, and they will define the future directions of the knockout work.

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